

Cytokine resistance in melanoma

Von der Fakultät für Mathematik, Informatik und Naturwissenschaften
der Rheinisch-Westfälischen Technischen Hochschule Aachen
zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften
genehmigte Dissertation

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Tag der mündlichen Prüfung: 30. November 2007

Diese Dissertation ist auf den Internetseiten der Hochschulbibliothek online verfügbar.

Publications from this work

1. **Komyod, W., Böhm, M., Metze, D., Heinrich, P.C. & Behrmann, I. (2007)**
Constitutive suppressor of cytokine signaling 3 expression confers a growth advantage to a human melanoma cell line.
Mol Cancer Res 5, 271-281.
2. **Komyod, W., Bauer, U.M., Heinrich, P.C., Haan, S. & Behrmann, I. (2005)**
Are STATs arginine-methylated?
J Biol Chem 280, 21700-21705.

Further publications:

3. **Behrmann, I., Wallner, S., Komyod, W., Heinrich, P.C., Schuierer, M., Buettner, R. & Bosserhoff, A.K. (2003)**
Characterization of methylthioadenosin phosphorylase (MTAP) expression in malignant melanoma.
Am J Pathol 163, 683-690.
4. **Behrmann, I., Smyczek, T., Heinrich, P.C., Schmitz-Van de Leur, H., Komyod, W., Giese, B., Müller-Newen, G., Haan, S. & Haan, C. (2004)**
Janus kinase (Jak) subcellular localization revisited: the exclusive membrane localization of endogenous Janus kinase 1 by cytokine receptor interaction uncovers the Jak/receptor complex to be equivalent to a receptor tyrosine kinase.
J Biol Chem 279, 35486-35493.
5. **Kortylewski, M., Komyod, W., Kauffmann, M.E., Bosserhoff, A., Heinrich, P.C. & Behrmann, I. (2004)**
Interferon-gamma-mediated growth regulation of melanoma cells: involvement of STAT1-dependent and STAT1-independent signals.
J Invest Dermatol 122, 414-422.

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Abbreviations

Ab	antibody
AdoMet	S-adenosylmethionine
AdoHcy	S-adenosylhomocysteine
α 2M	α 2-macroglobulin
Amp ^R	ampicillin resistance
APS	ammonium peroxodisulfate
ATP	adenosine triphosphate
β -Gal	β -galactosidase
bp	base pairs
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CIS	cytokine inducible SH2-containing protein
CLC	cardiotrophin-like cytokine
CNTF	ciliary neurotrophic factor
CT-1	cardiotrophin-1
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EBI3	Epstein-Barr virus induced gene 3
<i>E. coli</i>	<i>Escherichia coli</i>
ECL	enhanced chemoluminescence
EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
Erk	extracellular signal-regulated kinase
ESS	extended SH2 subdomain

Fab	fragment antigen binding
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAS	γ -activated sequence
gp	glycoprotein
Grb	growth factor receptor-bound protein
GST	glutathione S-transferase
h	hour
HA	haemagglutinin
HEPES	N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid
HRP	horseradish peroxidase
IFN	interferon
IFNAR	IFN- α receptor
Ig	immunoglobulin
IHC	immunohistochemistry
IL	interleukin
IP	immunoprecipitation
IRF	interferon regulatory factor
ISGF3	interferon-stimulated gene factor 3
ISRE	interferon-stimulated response element
Jak	Janus kinase
JNK	c-jun N-terminal kinase
JH	Jak homology
kDa	kilo Dalton
KIR	kinase inhibitory region
LB	Luria-Bertani Medium
LIF	leukemia inhibitory factor
luc	luciferase
MAPK	mitogen-activated protein kinase
MAPKAP	MAP kinase activated protein kinase
MDA	N-methyl-2-deoxyadenosine
MEK	MAPK/Erk kinase
MEKK	MEK kinase

MHC	major histocompatibility complex
min	minute
mRNA	messenger RNA
MTA	5'-methylthioadenosine
MTAP	methylthioadenosine phosphorylase
Neo ^R	Neomycin resistance
NF- κ B	nuclear factor κ B
NHEM	normal human epidermal melanocytes
NP	neuropoietin
OD	optical density
ONPG	<i>o</i> -Nitrophenyl- β -D-galactopyranoside
OSM	oncostatin M
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PI3K	phosphoinositide 3-kinase
PIAS	protein inhibitor of activated STAT
PMSF	phenylmethylsulfonylfluoride
PRMT	protein arginine methyltransferase
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
PVDF	polyvinylidene difluoride
pY	phosphotyrosine
R	receptor
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
RT-PCR	reverse transcriptase PCR
SAPK	stress-activated protein kinase
SDS	sodium dodecyl sulfate
sec	second
SH	src homology
Shc	SH2-containing collagen-related protein

SHP	SH2-domain-containing tyrosine phosphatase
SIE	sis-inducible element
siRNA	short interference RNA
sIL-6R	soluble IL-6 receptor
SOCS	suppressor of cytokine signalling
SOS	son of sevenless guanine nucleotide exchange factor
STAT	signal transducer and activator of transcription
SUMO	small ubiquitin-related modifier
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TBS	Tris-buffered saline
TEMED	N, N, N', N' - Tetramethylethylenediamine
tk	thymidine kinase
TNF	tumor necrosis factor
TNS	trypsin neutralization solution
Tris	Tris (hydroxymethyl) aminomethane
U	unit
UV	ultraviolet
WB	Western blot
WSX-1	WSXWS type I cytokine (IL-27 receptor α)
WT	wild-type

1 Introduction

1.1 Progression of cutaneous melanoma

Melanoma is a skin cancer that develops from the malignant transformation of melanocytes. Melanocytes are pigment cells that are found predominantly in the skin, where they produce melanins, the pigments responsible for skin colour. In the skin, melanocytes are localized in the basal layer of the epidermis (Fig. 1), and their homeostasis is regulated by epidermal keratinocytes. In response to ultraviolet (UV) radiation, keratinocytes produce factors that regulate melanocyte survival, differentiation, proliferation and motility, stimulating melanocytes to produce melanin, causing the skin to tan, or darken. Melanocytes can proliferate and spread, leading to formation of a naevus or common mole (Fig. 1).

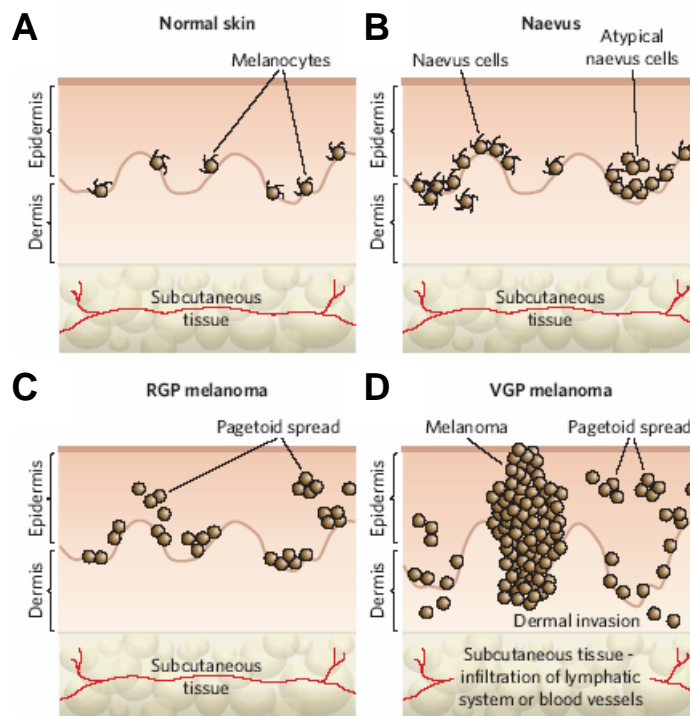


Figure 1. Progression of melanocyte transformation (taken from [1]).

There are various stages of melanocytic lesion, each of which is marked by a new clone of cells with growth advantages over the surrounding tissues. **A.** Normal skin. This shows an even distribution of dendritic melanocytes within the basal layer of the epidermis. **B.** Naevus. In the early stages, benign melanocytic naevi occur with increased numbers of dendritic melanocytes. According to their localization, naevi are termed either junctional (restricted to the epidermis), dermal (restricted to the dermis), or compound. Some naevi are dysplastic, with morphologically atypical melanocytes. **C.** Radial-growth-phase (RGP) melanoma. This is considered to be the primary malignant stage. **D.** Vertical-growth-phase (VGP) melanoma. This is the first stage that is considered to have malignant potential and leads directly to metastatic malignant melanoma, the most deadly stage, by infiltration of the vascular and lymphatic systems. Pagetoid spread describes the upward migration or vertical stacking of melanocytes that is a histological characteristic of melanoma.

Melanoma develops from precursor lesions, i.e. benign naevi or from normal melanocytes to become so-called dysplastic lesions that are capable of malignant transformation. These premalignant lesions can progress to *in situ* melanoma with melanocytic cells strictly confined to the epidermis, and subsequently to the radial-growth phase (RGP) melanoma, which has the same features as *in situ* melanoma except that it can involve some local microinvasion into the dermis. RGP melanoma becomes invasive by undergoing a vertical-growth phase (VGP) in which the cells have metastatic potential, with nodules or nests of cells invading the dermis (Fig. 1). Not all melanomas pass through each of these individual phases — RGP or VGP can both develop directly from isolated melanocytes or naevi, and both can progress directly to metastatic malignant melanoma [1].

Cutaneous melanoma can be subdivided into four main clinical subtypes due to a combination of clinical and pathological features (see Table 1):

- **Superficial spreading melanoma (SSM)**

This is the most common type, and it can cause tumors on any part of the body. This cancer spreads on the surface of the skin before it invades deeper tissues.

- **Nodular melanomas (NM)**

This type of melanoma invades the deeper tissues, making it one of the more dangerous forms of melanoma.

- **Acral lentiginous melanoma (ALM)**

This type of melanoma is found most commonly in dark-skinned people, usually on the palms, soles and nail beds. This is the most serious form of melanoma.

- **Lentigo maligna melanoma (LMM)**

This is the slowest-growing form of melanoma. It usually occurs in elderly people on sun-damaged skin (usually the head or neck). A precancerous skin spot called lentigo maligna sometimes develops before the cancer.

Table 1: Clinical classification of melanoma [2]

Subtype	Frequency	Common site	Key distinguishing features
SSM	70%	Trunk of men Legs of women	RGP, 1-5 years
NM	10-25%	Trunk of men Legs of women	RGP, 6-18 months
ALM	5%	Palm, soles, nails	Not related to sun damage
LMM	<1%	Head and neck of elderly	Associated with chronic sun exposure RGP, 3-15 years

The American Joint Committee on Cancer (AJCC) has defined a staging system for melanoma (TNM system) that describes the extent of local tumor involvement (the T stage) and the presence or absence of spread to lymph nodes (the N stage) or other distant metastasis (the M stage) [3].

The level of invasion as defined by Dr. Wallace Clark (called the Clark level) is one of the staging system used for microscopic staging of primary melanoma [4]. The Clark level of a melanoma uses a scale of I to V to describe the thickness of a melanoma in relation to its penetration into the skin.

- Clark level I: the cancer stays in the epidermis (*in situ* melanoma)
- Clark level II: the cancer has begun to penetrate to the upper dermis
- Clark level III: the cancer involves most of the upper dermis
- Clark level IV: the cancer has penetrated to the lower dermis
- Clark level V: the cancer has penetrated very deeply into the subcutaneous tissue

Melanoma incidence is increasing at faster rates than any other cancer. It is estimated that 130,000 malignant melanomas occur globally each year (World Health Organization). A large number of studies indicate that the risk of malignant melanoma correlates with genetic and personal characteristics, and a person's UV exposure behaviour. Although melanoma accounts for only about 4% of all skin cancers, it causes most skin cancer deaths. Treatment of melanoma in its early stages provides the best opportunity for cure.

Therapies for malignant melanoma include surgical excision, chemotherapy, radiation therapy, vaccine therapy and immunotherapy using interferons (IFN) or interleukin-2 (IL-2). There are several approved postoperative adjuvant therapies for malignant melanoma. IFN- α is the most commonly used adjuvant therapy for patients with malignant melanoma but has only a modest success rate [5]. High-dose IL-2 has also been approved, but response rates are low and toxicity is a problem.

Cytokine resistance is of major clinical importance for immunotherapy of melanoma. Thus, it is necessary to identify molecular mechanisms that determine cytokine resistance in order to optimize immunotherapy of patients with advanced melanoma.

1.2 IL-6-type cytokines

Cytokines are protein mediators playing an important role in the regulation of immune responses, inflammation, proliferation, differentiation and apoptosis. They exert their effects by binding to specific receptors on the plasma membrane of target cells. Distinct responses of target cells to cytokines are determined by signal transduction events which differ between various cell types, or even between separate phases of cell development.

Interleukin-6 (IL-6) and oncostatin M (OSM) belong to the family of IL-6-type cytokines which to date comprises nine members: IL-6, IL-11, IL-27, ciliary neurotrophic factor (CNTF), cardiotrophin-like cytokine (CLC), neuropoietin (NP), cardiotrophin-1 (CT-1), leukaemia inhibitory factor (LIF) and OSM [6-8]. The IL-6-type cytokines are characterized by a four long-chain α -helix-bundle structure and by the shared usage of the signal transducing receptor chain glycoprotein (gp) 130 [6]. Depending on the second participating signal transducing receptor chain the family can be further subdivided. IL-6 and IL-11 use a homodimer of two chains of gp130; IL-27 signals via a heterodimer of gp130 and WSX-1 receptor; CNTF, CLC, NP, CT-1 and LIF signal via a heterodimer of gp130 and LIFR [8,9]. Human OSM has the exceptional capability to signal via two receptor complexes: the type I receptor complex composed of gp130/LIFR and the type II receptor complex consisting of gp130/OSMR [10,11]. The IL-6-type cytokine receptors belong to the cytokine receptor class I family and are defined by the presence of at least one cytokine-binding module (CBM) consisting of two fibronectin-type-III-like domains of which the N-terminal domain contains a set of four conserved cysteine residues and the C-terminal domain a WSXWS motif [12].

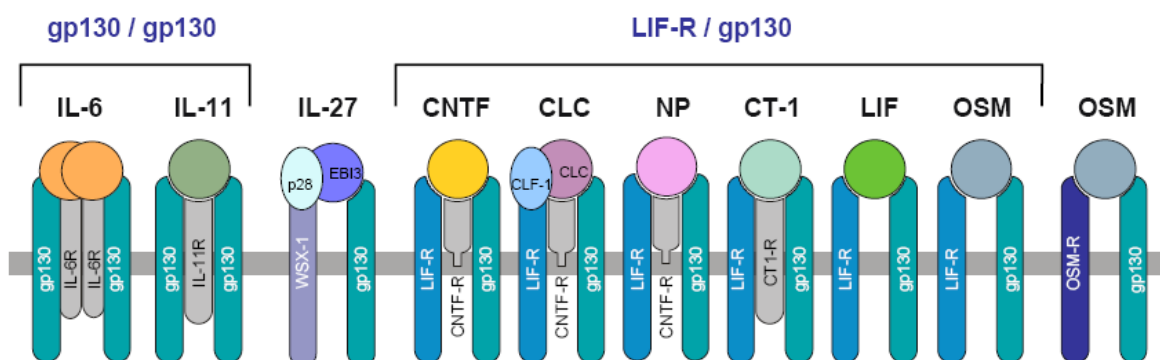


Figure 2. IL-6-type cytokine receptor complexes.

IL-6-type cytokine receptor complexes signal through different combinations of the signaling receptor subunits gp130, WSX-1, LIFR and OSMR, with gp130 being used by all the family members. IL-6, IL-11, CNTF, CLC, NP and CT-1 require their respective α -receptor subunits, which are shown in light grey, to efficiently recruit the corresponding signal transducing receptor components.

1.2.1 Interleukin 6 (IL-6)

IL-6 is a pleiotropic cytokine with a wide spectrum of biological functions involved in hematopoiesis, immune response, inflammation, the acute phase response and oncogenesis. It was first described as B-cell stimulatory factor-2 (BSF-2) that induced the final maturation of B cells into immunoglobulins-producing cells [13,14]. IL-6 is a 26 kDa glycoprotein produced by a variety of cells including T cells, B cells, monocytes, macrophages, fibroblasts, keratinocytes, endothelial cells, mesangial cells, and several tumor cells [15]. IL-6 expression is also induced by stimulation with pro-inflammatory cytokines such as IL-1 and tumor necrosis factor- α (TNF- α) [16,17]. IL-6 is a potent growth factor for human myeloma and plasmacytoma cells [18-20], on the other hand, it mediates growth inhibition and differentiation of mouse M1 myeloid leukemia, melanoma, breast and lung cancer cells [21-24]. Transfer of the IL-6 and IL-6R α gene into melanoma cells led to a lower rate of tumor growth in mice and enhanced survival [25].

IL-6 signals through two different receptor proteins. It first binds to an α -receptor subunit (IL-6R α). The low affinity complex of IL-6 and IL-6R α engages the signal transducing receptor subunit gp130 leading to a high affinity receptor complex and the initiation of the cytoplasmic signaling cascades. Recently, the structure of the soluble hexameric IL-6 receptor complex consisting of two molecules of each IL-6, sIL-6R α , and sgp130 has been solved by x-ray crystallography [26].

1.2.2 Oncostatin M (OSM)

Oncostatin M (OSM) is a 28 kDa glycoprotein and was originally identified by its ability to inhibit the growth of human A375 melanoma cells [27,28]. OSM is produced mainly by activated T lymphocytes, monocytes, macrophages and neutrophils [29,30]. Like the other IL-6-type cytokines, OSM is involved in various cellular responses such as regulation of the acute phase reaction, hematopoiesis, bone remodeling, and homeostasis of the extracellular matrix. OSMR knock-out mice display defects in hematopoiesis and liver regeneration [31,32]. Furthermore, OSM can act as a mediator for both the proliferation and the growth arrest of various cell lines [33]. OSM stimulates the proliferation of Kaposi's sarcoma, muscle cells and endothelial cells [34-36] while it inhibits the growth of melanoma, breast carcinoma and lung carcinoma [27,28,37,38].

1.3 Signaling pathways activated by IL-6-type cytokines

1.3.1 Jak/STAT signaling pathway

Like other cytokine receptors, the signal transducing receptor chains of IL-6-type cytokines do not contain an intrinsic tyrosine kinase activity and therefore signaling events are mediated by members of the Janus kinase (Jak) family of protein tyrosine kinases, which are constitutively associated with cytokine receptors. Jaks are non-receptor tyrosine kinases with molecular masses of 120-140 kDa. Four members are known in mammalian cells: Jak1, Jak2 and Tyk2, which are widely expressed, and Jak3, which is primarily expressed in hematopoietic cells. The Jak kinases are characterized by the presence of seven regions of sequence similarity found between Jaks and designated as Jak homology (JH) domains. The general structure of Jaks is shown in Fig. 3. The C-terminal tyrosine kinase domain (JH1), is preceded by a kinase-like domain (JH2), which itself lacks catalytic activity, but regulates the activity of the kinase domain [39,40]. The N-terminal half of the Jaks, domains JH3–JH7, comprises a potential Src homology 2 (SH2) domain [41] and a postulated FERM (four-point-one, ezrin, radixin, moesin) domain which is crucial for cytokine receptor binding [42-44]. Tyr1022/1023 of Jak1, Tyr1007/1008 of Jak2 and Tyr1054/1055 of Tyk2 in the kinase activation loop are important for the regulation of kinase activity [45,46].

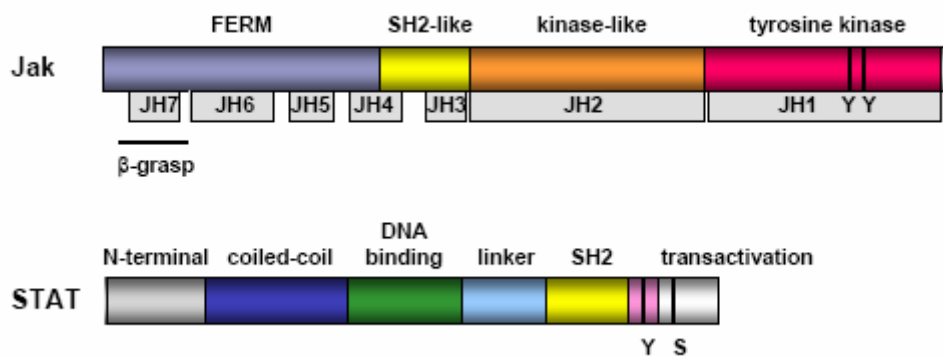


Figure 3. Domain structures of Jaks and STATs.

Although activation of the Jak1, Jak2 and Tyk2 has been reported for IL-6-type cytokines [6], studies in Jak1-deficient cells have indicated that Jak1 plays a crucial role in signal transduction of this family of cytokines [47,48]. Apart from their role in signal transduction, Jaks are important for the regulation of the surface expression of several cytokine receptors. Co-expression of Jak1, Jak2 and Tyk2 substantially enhanced the surface expression of human OSMR β [49].

Upon ligand binding and receptor clustering, tyrosine kinases of the Jak family associating with the cytoplasmic region of cytokine receptors become activated. They subsequently phosphorylate tyrosine residues of the receptor, which recruits other signaling proteins with matching SH2 domains such as STATs to the receptor. Subsequent to receptor binding, the STAT proteins are phosphorylated on a single tyrosine residue (Tyr701 in STAT1 and Tyr705 in STAT3). The phosphorylated STATs dissociate from the receptors, form active dimers through interactions of their SH2 domains, and translocate to the nucleus to induce transcription of target genes [6]. In addition to tyrosine phosphorylation, STATs 1, 3, and 5 also require phosphorylation on a serine residue in the C-terminal region to achieve maximal transcriptional activity [50-52].

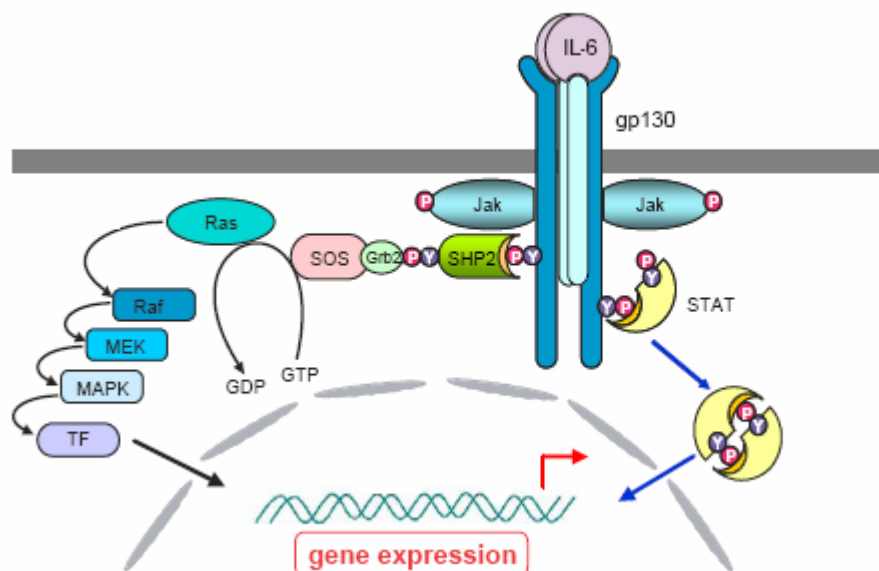


Figure 4. IL-6 activates both Jak/STAT and MAPK cascades.

Representation of the two major pathways activated by IL-6-type cytokines. TF, transcription factor.

The STAT family of transcription factors consists of seven mammalian members: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. The domain structure of STAT proteins comprises from N- to C-terminus an oligomerization domain, a coiled-coil domain, a DNA-binding domain, a linker domain, an SH2 domain and a transactivation domain (Fig. 3).

STAT1 is a critical mediator of IFN signaling. STAT1^{-/-} mice were found to develop normally but had extreme susceptibility to viral and some bacterial infections [53]. STAT3 is the major mediator of IL-6 signaling and gene targeting of STAT3 leads to early embryonic lethality [54]. In contrast to STAT1, STAT3 is often associated with oncogenesis [55]. STAT3 is

constitutively activated in various types of human cancer, including hepatocellular carcinoma, head and neck squamous cell carcinoma, breast cancer, and prostate cancer. STAT3 has been shown to prevent programmed cell death and to promote cell proliferation through regulating genes involved in cell growth and apoptosis, including Bcl-x_L, Mcl-1, c-Myc, p21^{WAF1/CIP1} and cyclin D1 [55]. Furthermore, constitutive STAT3 activation has been shown to up-regulate VEGF expression and to promote tumor angiogenesis [56]. Inhibition of STAT3 signaling in tumor cells induced apoptosis and/or cell cycle arrest *in vitro* and *in vivo* [55]. However, the role of STAT3 in melanoma seems to be complex because STAT3 activation in association with either inhibition or promotion of growth has been described [57,58].

1.3.2 Activation of MAPK cascades

Besides activating the Jak/STAT pathway, IL-6-type cytokines can also induce the activation of the mitogen-activated protein kinase (MAPK) cascades (Fig. 4). MAPKs are a family of serine/threonine protein kinases widely conserved among eukaryotes and are involved in many cellular programs such as cell proliferation, cell differentiation and cell death [59,60]. Three main MAPK cascades have been identified: extracellular signal-regulated kinase Erk1 and Erk2, p38, and c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPKs) [59]. IL-6 induced activation of the MAPK cascade is mediated through recruitment of the SH2 domain-containing protein tyrosine phosphatase-2 (SHP2) to the receptor via Tyr759 in the cytoplasmic region of gp130. SHP2 is itself activated by Jak1 and Jak2 and upon phosphorylation acts as a docking target for the adaptor protein Grb2, which is constitutively associated with the GTP-exchange factor SOS. The SHP2-Grb2-SOS route thereby leads to the activation of the Ras protein, and thus results in the activation of the Erk MAPK cascade [6]. In contrast to gp130, the OSMR does not recruit SHP2. Nevertheless, activation of the Ras/Raf/MAPK pathway by OSM can be mediated through recruitment of the adapter protein Shc (SH2-containing collagen-related protein) to the OSMR via Tyr861 [61]. Moreover, OSM has recently also been shown to activate p38 and JNK [62].

1.4 Interferon signaling pathway

Interferons (IFNs), initially identified as antiviral agents, are implicated in a variety of biologic functions including antiviral and antiproliferative responses, induction of cell differentiation, and modulation of the immune response. They are divided into two classes according to their ability to bind common receptor types. Type I IFNs comprising IFN- α ,

IFN- β , IFN- ω and IFN- τ bind to a type I IFN receptor. The only type II IFN is IFN- γ , as it binds to a distinct type II receptor. Both types of IFN have strong anti-proliferative and immunomodulatory effects on melanoma cells [28,63-65].

IFN- α is a 19.2 kDa protein produced in most cell types in response to viral and other microbial infections. IFN- α exerts its pleiotropic effects via a receptor complex consisting of two chains, the ligand-specific IFNAR1 (IFN- α receptor 1) and IFNAR2, expressed on nearly all cell types [66]. Binding of IFN- α leads to the oligomerization of the receptor subunits and initiates signal transduction (Fig. 5). Tyk2 and Jak1 protein tyrosine kinases that are constitutively associated with IFNAR1 and IFNAR2 receptor subunits, respectively, become activated, leading to phosphorylation of tyrosine residue 466 of the IFNAR1, which serves as a docking site for STAT2. The activated kinase subsequently phosphorylates STAT2 and STAT1 on Tyr690 and Tyr701, respectively. Phosphorylated STAT1 and STAT2 then dissociate from the receptor, form heterodimers and translocate to the nucleus, where they bind IFN regulatory factor 9 (IRF-9) and form the ISGF3 (interferon-stimulated gene factor 3) transcription factor complex that binds ISRE (interferon-stimulated response element) to initiate transcription of a wide variety of genes, including several antiviral genes. In addition, phosphorylated STAT1 also forms homodimers, which translocate to the nucleus and bind γ -activated sequence (GAS) elements to induce transcription of target genes [66].

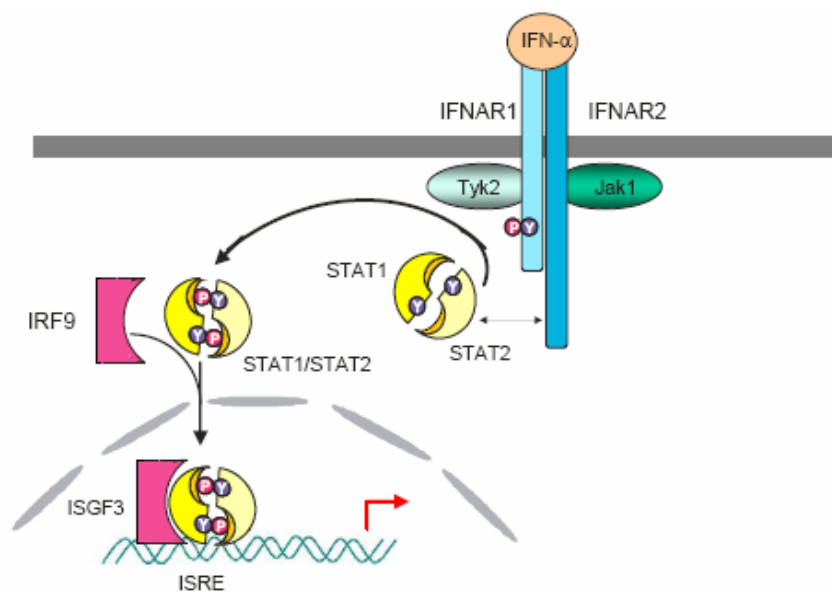


Figure 5. Schematic representation of IFN- α signaling pathway.

1.5 Negative regulation of the Jak/STAT signaling pathway

The Jak/STAT signaling pathway is negatively regulated by a number of proteins including protein tyrosine phosphatases (PTPs), such as SHP2 and T-cell PTP (TcPTP), protein inhibitors of activated STAT (PIAS) and suppressors of cytokine signaling (SOCS) [6,67].

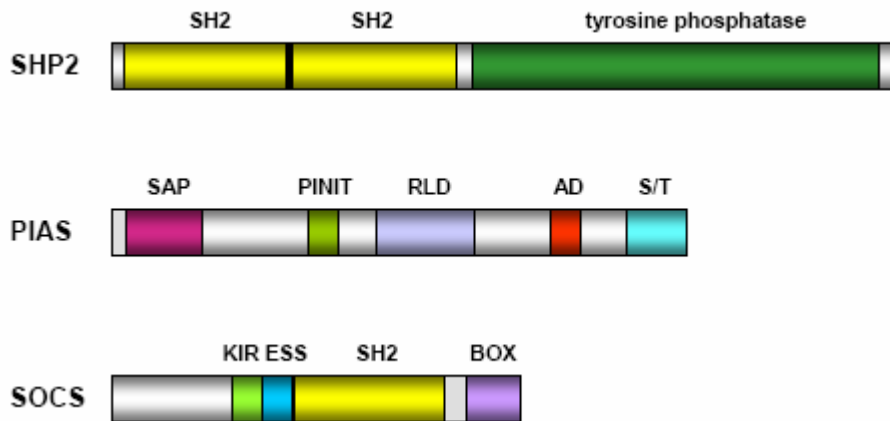


Figure 6. Domain structures of SHP2, PIAS and SOCS proteins.

SAP, scaffold attachment factor (SAF)-A/B, acinus and PIAS domain; RLD, RING-finger-like zinc-binding domain; AD, acidic domain; S/T, serine/threonine rich region; KIR, kinase inhibitory region; ESS, extended SH2-subdomain.

1.5.1 Protein tyrosine phosphatases (PTPs)

SHP2 is a ubiquitously expressed cytoplasmic PTP containing two N-terminal SH2 domains and a catalytic phosphatase domain in the C-terminal half (Fig. 6). It participates in the negative regulation by dephosphorylating the activated Jaks, STATs or cytokine receptors [67]. The crystal structure of SHP2 suggests that, in the absence of a tyrosine-phosphorylated binding partner, the N-terminal SH2 domain interacts with the PTP domain and thereby inhibits the enzymatic activity [68]. Binding of the SH2 domains to phosphotyrosine motifs of receptors or adapters leads to an unfolding of the protein and an activation of the enzyme [69-71]. SHP2 can also be activated by the phosphorylation of tyrosine residues 542 and 580 within the C-terminal part of the enzyme. Subsequently, these phosphotyrosines interact with the N- and C-terminal SH2 domain, respectively, relieving the PTP domain from the N-terminal SH2 domain-mediated inhibition [72].

Besides SHP2, further phosphatases are suggested to affect Jak/STAT signaling. The nuclear isoform of TcPTP was recently identified as the nuclear tyrosine phosphatase of STAT1 and STAT3 [73,74]. TcPTP is widely expressed in different tissues, but particularly abundant in

lymphoid and hematopoietic cell lineages, suggesting that TcPTP plays an important role in both hematopoiesis and immune function [75-77]. TcPTP-deficient mice showed impaired bone marrow microenvironment and immune function [78]. Several other PTPs, such as PTP1B, CD45 and SHP1, have also been reported to be involved in Jak/STAT signaling [79-81].

1.5.2 Protein inhibitor of activated STAT (PIAS)

The PIAS family consists of four mammalian members: PIAS1, PIAS3, PIASx (also known as PIAS2) and PIASy (also known as PIAS4). Except for PIAS1, two isoforms of each PIAS protein have been identified [82]. PIAS proteins contain several conserved domains (Fig. 6): the N-terminal SAP (Saf-A/B, acinus and PIAS) domain, which contains a conserved LXXLL motif; the PINIT domain, which is essential for nuclear retention [83]; the RING-finger-like zinc-binding domain (RLD), which is required for SUMO (small ubiquitin-related modifier) ligase activity [84]; the highly acidic domain (AD) and a C-terminal serine/threonine-rich region, which is not present in PIASy. Each member of the PIAS family has been shown to inhibit STAT-mediated gene activation through distinct mechanisms. PIAS1, a known inhibitor of STAT1, exerts its inhibition by binding to the N-terminal region of activated STAT1 and thereby preventing STAT1 from binding to DNA [85,86]. Similarly, PIAS3 also inhibits the DNA-binding activity of STAT3 [87]. In contrast, PIASx and PIASy inhibit STAT4- and STAT1-mediated gene expression without affecting the DNA-binding activities of STAT4 and STAT1 [88,89]. On the other hand, PIAS proteins can affect the activity of transcriptional regulators through acting as SUMO-E3 ligases. A number of transcription factors such as c-Jun, p53, IRF-1 and Sp3 have been shown to be sumoylated by PIAS proteins [90-94]. Sumoylation of transcription factors can either increase or decrease their activities [95]. Furthermore, several reports indicate that STAT1 can also be sumoylated by PIAS proteins. However, this modification is unlikely to play an important role in regulating STAT1 activity [96-98].

1.5.3 Suppressors of cytokine signaling (SOCS)

The SOCS proteins are transcriptionally regulated by cytokines and function in a classic negative-feedback loop to inhibit Jak/STAT signaling [99]. The SOCS family consists of eight members: cytokine-inducible SH2 containing protein (CIS) and SOCS1 through SOCS7. They contain a variable N-terminal region, a central SH2 domain, and a conserved

C-terminal domain termed the SOCS box (Fig. 6) [100-102]. A small kinase inhibitory region (KIR) is present in the N-terminal domain of SOCS1 and SOCS3 [103,104].

Downregulation of signaling pathways by SOCS proteins can occur by at least three distinct mechanisms. First, through their SH2 domains, SOCS proteins are recruited to specific phosphotyrosine residues in activated cytokine receptors (in the case of SOCS2, SOCS3, and CIS) [105-107] or in Jaks (in the case of SOCS1 and SOCS3) [103,104]. Second, the SOCS box is capable of recruiting the elongin BC complex to target proteins for ubiquitination and subsequent proteasomal degradation [108,109]. Third, SOCS1 and SOCS3 contain an N-terminal kinase inhibitory region (KIR) that inhibits the catalytic activity of Jaks as a pseudosubstrate [103,104].

CIS, which was the first member to be identified, was shown to inhibit IL-3 and EPO signaling [105], whereas SOCS1 is essential for downregulating IFN- γ signaling [110,111]. SOCS2 is implicated in growth hormone responses and SOCS2-deficient mice appeared to develop gigantism [106,112]. The crucial role of SOCS3 for the inhibition of IL-6 signaling was recently confirmed in SOCS3-deficient macrophages [113,114]. SOCS3 recruitment to the tyrosine motif Tyr759 of gp130, which is also the binding site for SHP2, was shown to be required for its inhibitory action [107,115]. SOCS3 has been found to bind to the phosphotyrosine motif 974 of LIFR [116]. Although SOCS3 is structurally similar to SOCS1, the mechanisms by which they act appear to differ. Whereas SOCS1 inhibits signaling by binding directly to Jaks, SOCS3 only inhibits Jaks when bound to receptors, such as gp130 through Y759, the leptin receptor through Y985 and the Epo receptor through Y401. However, a recent study has demonstrated that SOCS3 can inhibit OSMR-mediated signaling by binding directly to Jak1, and the inhibition occurs independent of tyrosine motifs within the OSMR [117]. Disruption of the SOCS3 gene in mice results in embryonic lethality due to placental defects caused by dysregulated LIF signaling [118].

The stability of SOCS3 protein has been shown to be regulated post-translationally by ubiquitination and phosphorylation [119,120]. Phosphorylation of Tyr204 and Tyr221 within the SOCS box upon various stimuli decreases SOCS3 protein half-life by disrupting the interaction between SOCS3 and elongin C [120,121]. Furthermore, a naturally occurring N-terminal truncated isoform of SOCS3, generated by alternative translation initiation at Met12, has been shown to be more stable than the full-length protein as the truncated SOCS3 isoform lacks the first 11 N-terminal amino acids including the potential ubiquitination residue, Lys6 and thus is resistant to proteasomal degradation [119].

1.6 Protein arginine methylation

1.6.1 Protein arginine methyltransferases (PRMTs)

Protein arginine methylation is a common post-translational modification in eukaryotes, and is catalyzed by a family of enzymes called protein arginine methyltransferases (PRMTs) that transfer the methyl group from S-adenosylmethionine (AdoMet) to the guanidino nitrogen atoms of arginine residues (Fig. 7). PRMTs are classified in two major classes; type I enzymes promote the formation of asymmetric dimethylarginine (aDMA), and type II enzymes catalyze the formation of symmetric dimethylarginine (sDMA) [122]. Monomethylarginine (MMA) is thought to be an intermediate formed by both enzyme types (Fig. 7). Currently, nine human protein arginine methyltransferases have been identified: the type I enzymes PRMT1, PRMT2, PRMT3, PRMT4/CARM1, PRMT6, and PRMT8, and the type II enzymes PRMT5, PRMT7, and PRMT9. The most highly conserved domain of the PRMT family is the AdoMet-binding domain. PRMTs are ubiquitously expressed and may serve multiple functions. In contrast to phosphorylation or acetylation, arginine methylation does not alter the charge of the arginine residue, but increases its steric hindrance, its hydrophobicity and blocks hydrogen bonding. Therefore, methylation could serve to modulate intra- or intermolecular interactions of target proteins. Arginine methylation is implicated in many cellular processes including RNA processing, transcriptional regulation, and signal transduction [123,124].

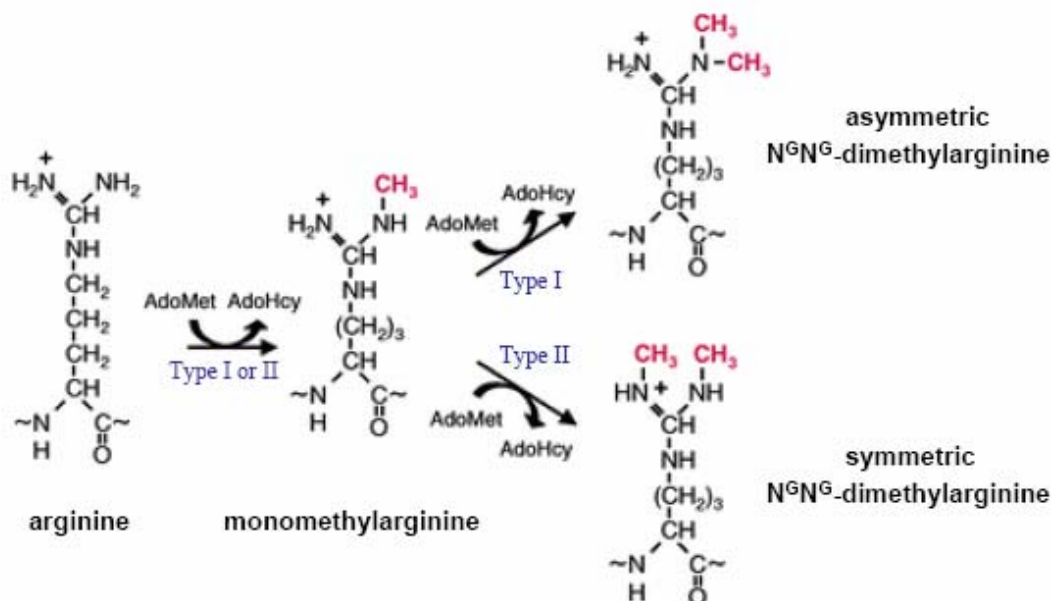


Figure 7. Arginine methylation (adapted from [122]).

Methylation of arginine residues within the context of a protein requires the methyl donor S-adenosylmethionine (AdoMet), which is converted into S-adenosylhomocysteine (AdoHcy).

PRMT1 is the predominant type I enzyme in mammalian cells, accounting for 85% of cellular PRMT activity. It generally recognizes arginines within a glycine-arginine-rich (GAR) region, a motif which is present in many RNA- or DNA-binding proteins [125]. The crystal structure of PRMT1 in complex with the methylation product AdoHcy and a GAR motif has been described [126], and it reveals three different peptide binding channels, possibly reflecting alternate docking orientations for different GAR motif-containing substrates. However, increasing numbers of substrates lacking GAR domain are being discovered [127], including histone H4, which contain no other arginines near the methylation target. PRMT1-deficient mice are embryonic lethal, suggesting that PRMT1 is required for very early stages of mouse development [128].

1.6.2 The role of protein arginine methylation in signal transduction

PRMT1 was the first enzyme in this family to be linked to signal transduction, with the finding that it binds to the cytoplasmic domain of the IFN- α/β receptor, and cells deficient in the methyltransferase are more resistant to growth inhibition by IFN [129]. Consistent with this view, Mowen et al. have reported that a conserved arginine residue (Arg31) in the N-terminal region of STAT1 is subject to methylation by PRMT1 and that STAT1 methylation is important for transcriptional activation induced by IFN- α/β [130]. Methylation of STAT1 reduces the ability of STAT1 to associate with the negative regulatory protein PIAS1, and thus, increases STAT1 DNA binding activity. Later, it was published by the same group that methylation of Arg31 of STAT1 is necessary for its dephosphorylation by the phosphatase TcPTP [131]. Similarly, for STAT6, arginine methylation has been described to be important for STAT6 function [132]. Another PRMT implicated in the same signal transduction pathway is PRMT5, which was first identified as a Jak2-binding protein [133]. PRMT5 has been shown to be involved in regulating the expression of IL-2 in T lymphocytes [134].

1.6.3 Methylation inhibitors

There are two types of small molecules that inhibit the function of AdoMet-dependent methyltransferase (Fig. 8): first, compounds that are structural analogues of AdoMet like 5'-methylthioadenosine (MTA) and thus compete for the cofactor binding site [135,136]. Second, nucleoside inhibitors of S-adenosylhomocysteine (AdoHcy) hydrolase, like *N*-methyl-2-deoxyadenosine (MDA), that cause the intracellular accumulation of AdoHcy, which in turn is a product inhibitor of the methyltransferases [137-139].

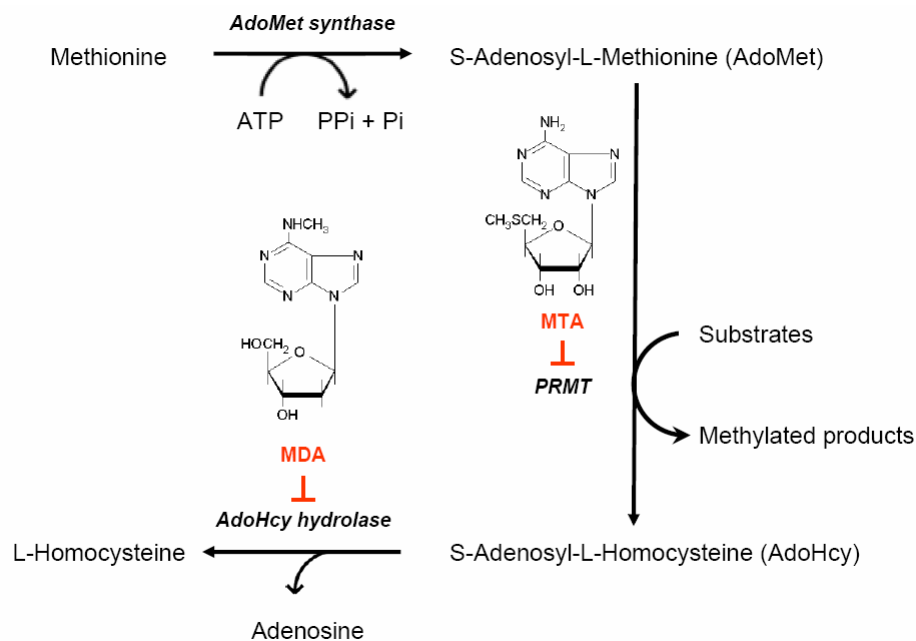


Figure 8. Inhibition of protein methylation.

AdoHcy hydrolase catalyzes the reversible hydrolysis of AdoHcy. Inhibition of AdoHcy hydrolase by MDA leads to an accumulation of AdoHcy and inhibition of methyltransferases.

MTA is produced physiologically as a by-product of the polyamine metabolism. The cellular enzyme metabolizing MTA is methylthioadenosine phosphorylase (MTAP), which is a key enzyme in the methionine salvage pathway. Phosphorylation of MTA by MTAP results in the conversion of MTA into adenine and methylthioribose-1-phosphat. MTAP is expressed abundantly in normal cells and tissues but is frequently homozygously deleted in a wide variety of human tumors and human tumor derived cell lines [140-142]. Reduced activity of MTAP in the cell leads to accumulation of MTA, which acts as an inhibitor of methyltransferases. Based on the finding that arginine methylation of STAT1 is important for STAT1 function and STAT1 is essential for IFN signaling, loss of arginine methylation is proposed to be involved in IFN resistance of cancer cells that have lost expression of MTAP [130].

1.7 Cytokine responses in melanoma

Cytokines play an important role in the growth regulation of melanoma cells. While melanocytes and early stage melanoma cells are growth inhibited by a variety of cytokines including IL-6, OSM, IFN- α , IFN- γ or TNF- α , melanoma cells of advanced tumor stages are often found to be multi-cytokine resistant [24,143,144].

It has previously been shown that STAT3 plays a key role in the IL-6-and OSM-mediated growth inhibition of A375 melanoma cells while STAT1 plays a crucial role in growth inhibition mediated by IFN- γ [57,145]. Cytokine resistance has also been associated with an impaired STAT3 activation [146]. Moreover, lack of receptor expression can contribute to cytokine resistance [147], and it has recently been discovered that melanoma progression coincides with epigenetic silencing of the OSMR β gene [148]. On the other hand, STAT3 has been shown to be constitutively active in melanoma celllines and primary tumors, and inhibition of STAT3 activity induced apoptosis [58]. Thus, STAT3 seems to play a complex role in melanoma.

Interferon resistance of melanoma cells was reported to be associated with defective STAT1 signaling, which correlates either with lack of STAT1 expression or impaired phosphorylation of STAT1 [63,149,150]. Restoration of normal STAT1 levels by IFN- γ induction or by overexpression of STAT1 or IRF-1 is sufficient to augment the responsiveness to IFN- α [149-151]. However, a number of recent studies did not find a correlation between the resistance of melanoma cells to IFN- α and STAT1 expression or activation [152-154]. Thus, the mechanism underlying the cellular IFN- α resistance seems to be complex. Moreover, it has been shown that STAT5 contributes to interferon resistance of melanoma cells by upregulating the cytokine inhibitor CIS and depletion of STAT5 from IFN- α -resistant cells by RNA interference (RNAi) resulted in enhanced sensitivity toward IFN- α -induced growth inhibition [155]. Most recently, STAT5 has been shown to be constitutively active in melanoma, and may function as a survival factor for growth of human melanoma [156].

1.8 Aims of this study

Development of cytokine resistance is an important feature of melanoma cells during tumor progression. Whereas the growth of melanocytes and many early stage melanoma cells can be inhibited by cytokines, late stage melanoma cells have often been reported to be “multi-cytokine resistant”. The aim of this study was to investigate the molecular mechanisms underlying cytokine resistance of melanoma cells.

Mowen et al. have proposed a model suggesting that STAT1 methylation on a conserved arginine residue (Arg31) is functionally necessary since it suppresses the interaction with the negative regulatory protein PIAS1 [130]. Inhibition of STAT1 methylation by the methylation inhibitor MTA increased PIAS1 binding to STAT1, and inhibited STAT1 DNA binding and STAT1-mediated gene transcription. Furthermore, the model proposed by Mowen et al. could provide an explanation for the observed interferon resistance of cancer cells that have lost expression of MTAP, the enzyme that inactivates MTA implicated in inhibition of methylation. It has previously been shown that MTAP expression is reduced in melanoma cells [157]. Since STAT3 is involved in growth inhibition of melanoma cells I wondered whether a potential arginine methylation of STAT3 might be inhibited in an analogous way in MTAP-negative cells which could provide an explanation for cytokine resistance of late stage melanoma cells. Thus, in the first part of this thesis I investigated the potential arginine methylation of STATs.

The role of negative regulators of cytokine signaling, i.e. SOCS proteins, has been investigated in several cancers. Alterations in expression of SOCS proteins were observed in lung cancer, in hepatocellular carcinoma, and in squamous cell carcinoma of the head and neck [158-160]. Those studies revealed that SOCS proteins are implicated in the regulation of cellular proliferation and apoptosis. Interestingly, constitutive expression of SOCS3 has been found in tumor cells from a number of malignancies including acute myeloid leukemia (AML), cutaneous T-cell lymphoma (CTCL), and chronic myelogenous leukemia (CML) [161-163]. Importantly, aberrant SOCS3 expression confers resistance against IFN α treatment [163,164]. Moreover, SOCS1 has also been described to be constitutively expressed in melanoma cells and in primary tissue [165]. In the second part of this thesis I thus investigated SOCS3 expression in melanoma cell lines and evaluated whether SOCS3 could underly cytokine resistance of melanoma cells.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and buffers

All chemicals were of p.a. grade and mainly from AppliChem (Darmstadt), BioRad (München), Carl Roth (Karlsruhe), Merck (Darmstadt), Roche (Mannheim), Serva (Heidelberg) and Sigma (Taufkirchen). The buffers and solutions were prepared with Milli-Q water (Millipore, Schwalbach) and either autoclaved or filter-sterilized prior to use.

2.1.2 Radiochemicals

$[\alpha\text{-}^{32}\text{p}]\text{dATP}$	Hartmann Analytic GmbH (Braunschweig)
<i>S</i> -adenosyl-L- <i>[methyl-¹⁴C]</i> methionine	Amersham Biosciences (Freiburg)

2.1.3 Prokaryotic cells

E. coli JM 83 for general cloning

2.1.4 Eukaryotic cells

2fTGH, U3A	Parental and STAT1-negative human fibrosarcoma cells were kindly provided by Dr. I. M. Kerr (Cancer Research UK, London)
MEF fl/fl, MEF Δ/Δ	Parental and STAT3-negative murine embryonic fibroblasts were kindly provided by Dr. V. Poli (Torino, Italy)
A375	human melanoma cells were purchased from the ATCC (CRL-1619)
WM9, M239	human melanoma cells were received from Dr. R. S. Kerbel (Sunnybrook Health Science Centre, Toronto, Canada)
586, 729, 888, 1102, 1286	human melanoma cells were kindly provided by Dr. Marcin Kortylewski (City of Hope National Medical Center, Duarte, CA)
Mel Im	human melanoma cells were obtained from Dr. Anja Bosserhoff (University of Regensburg, Germany)
NHEM	normal human epidermal melanocytes (NHEM) were purchased from Cambrex Bio Science (Walkersville, USA)

2.1.5 Media and reagents for cell culture

DMEM medium	Gibco (Karlsruhe)
RPMI 1640 medium	Gibco (Karlsruhe)
FCS (fetal calf serum)	Cytogen (Berlin)
Penicillin/Streptomycin	BioWhittaker (Verviers, Belgium)
Trypsin/EDTA (0.05%/0.02%)	BioWhittaker (Verviers, Belgium)
Versene (EDTA) 0.02%	BioWhittaker (Verviers, Belgium)
Trypsin Neutralizing Solution (TNS)	Cambrex (Walkersville, USA)
Melanocyte Medium Bullet Kit	Cambrex (Walkersville, USA)
Geneticin (G418)	Sigma (Taufkirchen)

2.1.6 Cytokines and soluble IL-6R α

Recombinant human IL-6	prepared as described [166]
Recombinant human IFN- α	IntronA (Kenilworth, NJ)
Recombinant human IFN- γ	PeptoTech (London, UK)
Recombinant human OSM	PeptoTech (London, UK)
soluble IL-6R α	prepared as described [167]

2.1.7 Inhibitors

U0126	Calbiochem (Darmstadt)
SB202190	Calbiochem (Darmstadt)
Actinomycin D	Calbiochem (Darmstadt)
Cycloheximide	Sigma (Taufkirchen)
Adenosine	Sigma (Taufkirchen)
D, L-homocysteine	Sigma (Taufkirchen)
N-methyl-2-deoxyadenosine (MDA)	Sigma (Taufkirchen)
5'-methyl-thioadenosine (MTA)	Sigma (Taufkirchen)

2.1.8 Enzymes

T4-DNA-Ligase (Roche, Mannheim)
Taq DNA-Polymerase (Hybaid, Heidelberg)
Restriction endonucleases were purchased from Hybaid (Heidelberg), New England Biolabs (Frankfurt/Main), MBI Fermentas (St. Leon-Rot) and Roche (Mannheim).

2.1.9 Antibodies

Antibody	raised against	Type	Host	Source	Applications
STAT1-pY	phospho-Tyr-701	polyclonal	Rabbit	Cell Signaling Tech. (Frankfurt)	WB
STAT3-pY	phospho-Tyr-705	polyclonal	Rabbit	Cell Signaling Tech. (Frankfurt)	WB
STAT3-pS	phospho-Ser-727	polyclonal	Rabbit	Cell Signaling Tech. (Frankfurt)	WB
STAT1	C-terminus	monoclonal	Mouse	Transduction Lab. (Lexington, USA)	WB
STAT3	N-terminus	monoclonal	Mouse	Transduction Lab. (Lexington, USA)	WB
STAT1 (E-23)	C-terminus	polyclonal	Rabbit	Santa Cruz (Heidelberg)	WB
STAT3 (C-20)	C-terminus	polyclonal	Rabbit	Santa Cruz (Heidelberg)	WB
Phospho-p44/42 MAPK	phospho-Thr-202/Tyr-204	monoclonal	Mouse	Cell Signaling Tech. (Frankfurt)	WB
Erk1 (C-16g)	C-terminus	polyclonal	Goat	Santa Cruz (Heidelberg)	WB
Erk 2 (C-14g)	C-terminus	polyclonal	Goat	Santa Cruz (Heidelberg)	WB
p38 (active) (pTGpY)	dual phosphorylated form of p38 (pTGpY)	polyclonal	Rabbit	Promega (Mannheim)	WB
p38 (C-20)	C-terminus	polyclonal	Rabbit	Santa Cruz (Heidelberg)	WB
Jak1-pY	phospho-Tyr-1022/1023	polyclonal	Rabbit	Cell Signaling Tech. (Frankfurt)	WB
Jak1	residues 551-766 of human Jak1	monoclonal	Mouse	Transduction Lab. (Lexington, USA)	WB
Jak1 (HR-785)	C-terminus	polyclonal	Rabbit	Santa Cruz (Heidelberg)	WB, IP
SOCS3 (M-20)	C-terminus	polyclonal	Goat	Santa Cruz (Heidelberg)	WB
SOCS3 (C204)	C-terminus	polyclonal	Rabbit	IBL (Hamburg)	IHC
SHP-2-pY	phospho-Tyr-542	polyclonal	Rabbit	Cell Signaling Tech. (Frankfurt)	WB
SHP-2 (C-18)	C-terminus	polyclonal	Rabbit	Santa Cruz (Heidelberg)	WB
Phospho-MAPKAPK-2 (Thr222)	phospho-Thr-222	polyclonal	Rabbit	Cell Signaling Tech. (Frankfurt)	WB
anti-Methyl Arginine (ab412)	methyl and dimethyl arginine	monoclonal	Mouse	Abcam (Cambridge, UK)	IP
anti-Methyl Arginine (ab413)	dimethyl arginine	monoclonal	Mouse	Abcam (Cambridge, UK)	IP
GST (B14)	GST	monoclonal	Mouse	Santa Cruz (Heidelberg)	IP
Lamin A/C (346)	Lamin A and Lamin C	monoclonal	Mouse	Santa Cruz (Heidelberg)	IP

Antibody	raised against	Type	Host	Source	Applications
p-Tyr (PY99)	phosphotyrosine	monoclonal	Mouse	Santa Cruz (Heidelberg)	WB
p-Tyr (4G10)	phosphotyrosine	monoclonal	Mouse	Upstate (Hamburg)	WB
PIAS1/3 (N-18)	N-terminus	polyclonal	Goat	Santa Cruz (Heidelberg)	WB
Sam 68 (C-20)	C-terminus	polyclonal	Rabbit	Santa Cruz (Heidelberg)	WB
HA-Tag (262 K)	HA-tagged protein	monoclonal	Mouse	Cell Signaling Tech. (Frankfurt)	WB
HRP-conjugated rabbit anti-goat Ig	goat Ig	polyclonal	Rabbit	DAKO (Hamburg)	WB
HRP-conjugated goat anti-rabbit Ig	rabbit Ig	polyclonal	Goat	DAKO (Hamburg)	WB
HRP-conjugated rabbit anti-mouse Ig	mouse Ig	polyclonal	Rabbit	DAKO (Hamburg)	WB
HRP-conjugated goat anti-mouse Ig	mouse Ig	polyclonal	Goat	DAKO (Hamburg)	WB
HRP-conjugated rabbit anti-chicken IgG	chicken Ig	polyclonal	Rabbit	Rockland, (Gilbertsville, PA)	WB
gp130 (BR3)	extracellular part of gp130 (D2)	monoclonal	Mouse	J. Wijdenes (Besancon, France)	FACS
OSMR (AN-A2)	full length soluble OSMR β chain	monoclonal	Mouse	Santa Cruz (Heidelberg)	FACS
LIFR (C-19)	C-terminus	polyclonal	Rabbit	Santa Cruz (Heidelberg)	FACS
MHC-I (W6/32)	human HLA class I antigen	monoclonal	Mouse	Sigma (Taufkirchen)	FACS
PE-conjugated goat anti-mouse Ig	mouse Ig	F(ab') ₂ Fragment	Goat	Dianova (Hamburg)	FACS
PE-conjugated donkey anti-rabbit Ig	rabbit Ig	F(ab') ₂ Fragment	Donkey	Dianova (Hamburg)	FACS

2.1.10 Plasmids

Plasmids	Description	Source
pCAGGS	Eukaryotic expression vector, Amp ^R , Neo ^R	Pharmacia (Freiburg)
pcDNA3	Eukaryotic expression vector, Amp ^R , Neo ^R	Invitrogen (Karlsruhe)
pGEX-5X-1	glutathione S-transferase fusion vector	Pharmacia (Freiburg)
pGEX-5X-2	glutathione S-transferase fusion vector	Pharmacia (Freiburg)
pCH110- β Gal	Expression vector encoding β -galactosidase	Pharmacia (Freiburg)
pGL3- IRF1-tk-Luc	Luciferase expression vector contains the STAT1-responsive element of the IRF1 promoter upstream of a thymidine kinase minimal promoter	F. Schaper (Aachen)

Plasmids	Description	Source
pXP2-600-c-fos	Luciferase expression vector contains the STAT3-responsive element of the c-fos promoter	F. Schaper (Aachen)
pBS-STAT1	plasmid encoding full-length human STAT1	T. Hirano (Osaka, Japan)
pBS-STAT3	plasmid encoding full-length murine STAT3	T. Hirano (Osaka, Japan)
pCAGGS-STAT1	Expression vector for human STAT1	T. Hirano (Osaka, Japan)
pCAGGS-STAT3	Expression vector for murine STAT3	T. Hirano (Osaka, Japan)
pCAGGS-STAT3F	Expression vector for dominant negative STAT3 (STAT3Y705F)	T. Hirano (Osaka, Japan)
pcDNA3-huSTAT1 wt	Expression vector for human STAT1 wild-type	this thesis
pcDNA3-huSTAT1-R31K	Expression vector for human STAT1-R31K mutant	this thesis
pcDNA3-muSTAT3 wt	Expression vector for murine STAT3 wild-type	this thesis
pcDNA3-muSTAT3-R31K	Expression vector for murine STAT3-R31K mutant	this thesis
pGEX-5X-2 huSTAT1	plasmid encoding a GST fusion protein of full-length STAT1	this thesis
pGEX-5X-1 muSTAT3	plasmid encoding a GST fusion protein of full-length STAT3	this thesis
pcDNA3.1-PRMT1 wt	Expression vector for PRMT1 wild-type	U.M. Bauer (Marburg)
pcDNA3.1-PRMT1 mutant	Expression vector for catalytic inactive PRMT1	U.M. Bauer (Marburg)

2.1.11 Oligonucleotides

All oligonucleotides were synthesized by MWG-Biotech (Ebersberg), except the primers for real-time PCR and siRNA that were purchased from Eurogentec (Seraing, Belgium).

Primers for mutagenesis-PCR

STAT1R31K forward: 5'-CCATGGAAATCAAACAGTACCTGGCACAG-3'

STAT1R31K reverse: 5'-CTGTGCCAGGTACTGTTTGATTTCCATGG-3'

STAT3R31K forward: 5'-CCATGGAGCTGAAGCAGTTCCTGGC-3'

STAT3R31K reverse: 5'-GCCAGGAAGTCTTCAGCTCCATGG-3'

Primers for RT-PCR [168]

SOCS3 forward: 5'-CTCAAGACCTTCAGCTCCAA-3'

SOCS3 reverse: 5'-TTCTCATAGGAGTCCAGGTG-3'

SOCS6 forward: 5'-TCTCACCATTGCTACCTCCA-3'

SOCS6 reverse: 5'-GAGTCCCTGATTGAATGCTC-3'

CIS forward: 5'-GATCTGCTGTGCATAGCCAA-3'

CIS reverse: 5'-ACAAAGGGCTGCACCAGTTT-3'

GAPDH forward: 5'-TGATGACATCAAGAAGGTGG-3'

GAPDH reverse: 5'-TTACTCCTTGGAGGCCATGT-3'
β-actin forward: 5'-GGGAGAGCGGGAAATCGTGCGTGA-3'
β-actin reverse: 5'-GATGGAGTTGAAGGTAGTTTCGTG-3'

Primers for real-time PCR

SOCS3 forward: 5'-CACCTGGACTCCTATGAGAAAGTCA-3'
SOCS3 reverse: 5'-GGGGCATCGTACTGGTCCAGGAA-3'
β-actin forward: 5'-CCCTGAGGCACTCTTCCAG-3'
β-actin reverse: 5'-TGCCACAGGACTCCATGCCC-3'

siRNA against human SOCS3

siRNA1 sense: 5'-AGAGCCUAUUACAUCUACUdTdT-3'
siRNA1 antisense: 5'-AGUAGAUGUAAUAGGCUCUdTdT-3'
siRNA2 sense: 5'-AGACCCAGUCUGGGACCAAdTdT-3'
siRNA2 antisense: 5'-UUGGUCCCAGACUGGGUCUdTdT-3'
siRNA3 sense: 5'-CCAAGAACCUGCGCAUCCAdTdT-3'
siRNA3 antisense: 5'-UGGAUGCGCAGGUUCUUGGdTdT-3'
siRNA negative control: Cat.No.OR-0030-NEG05

2.1.12 Commercial kits

QIAGEN Plasmid Mini Kit (Qiagen, Hilden)
HiSpeed Plasmid Maxi Kit (Qiagen, Hilden)
QIAquick Gel Extraction Kit (Qiagen, Hilden)
QIAquick Nucleotide Removal Kit (Qiagen, Hilden)
RNeasy Mini Kit (Qiagen, Hilden)
QIAGEN OneStep RT-PCR Kit (Qiagen, Hilden)
Luciferase Assay Kit (Promega, Mannheim)
Cell Proliferation Kit II (XTT) (Roche, Mannheim)
QuickChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA)
TOPO TA Cloning® Kits (Invitrogen, Karlsruhe)
1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche, Mannheim)
SYBR® Green PCR Master Mix (Applied Biosystems, Darmstadt)
PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit (Perkin Elmer, Boston, USA)

2.2 Methods

2.2.1 Culture of bacteria

E. coli JM 83 were grown in LB-medium supplemented with the appropriate antibiotic (Ampicillin: 100 µg/ml) and incubated at 37°C with shaking. For long-time storage, *E. coli* strains were stored in LB-medium containing 20% glycerol at -80°C.

LB-medium:

10 g/L	NaCl
5 g/L	Yeast extract (Difco, Detroit, USA)
10 g/L	Bactotryptone (Difco, Detroit, USA)
10 mM	Tris/HCl pH 8.0
1 mM	MgSO ₄

LB-agar plates: 15 g/L Agar (in LB-Medium)

2.2.2 Preparation of transformation-competent *E. coli* cells

50 ml of overnight culture were added to 500 ml pre-warmed LB medium and shaken at 37°C until an OD₆₀₀ of 0.6 was reached. The cultures were placed on ice for 5 min and cells were collected by centrifugation at 8000 rpm for 5 min at 4°C. Cells were resuspended in 125 ml ice-cold 50 mM CaCl₂ and incubated on ice for 20 min. Cells were collected again by centrifugation at 8000 rpm for 5 min at 4°C and then resuspended in 25 ml ice-cold 50 mM CaCl₂/10%glycerol. Aliquots of 400 µl were frozen immediately in liquid nitrogen. The transformation-competent cells were then stored at -80°C.

2.2.3 Transformation of competent *E. coli* cells

Frozen competent *E. coli* cells were thawed on ice and gently mixed by flicking the tube. 100 µl of the cell suspension were transferred into the microcentrifuge tube with the plasmid DNA (1 ng) or 10 µl of a ligation mixture, mixed carefully and incubated on ice for 30 min. Cells were heat-shocked at 42°C for 90 sec and immediately placed on ice for 2 min. Following the addition of 400 µl of LB medium, cells were incubated at 37°C for 30 min. Cells were plated on LB-agar plates containing the relevant antibiotic. Different volumes of cells were spread in order to obtain single colonies on the plate. Plates were incubated overnight at 37°C.

2.2.4 Purification of plasmid DNA

The Qiagen Plasmid Mini Kit and HiSpeed Plasmid Maxi Kit (Hilden, Germany) were used for the small and large scale purification of plasmid DNA, respectively, according to the supplier's instruction.

2.2.5 Quantification of nucleic acids

The concentration of DNA and RNA were determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer using a quartz cuvette. An absorbance of 1 unit at 260 nm corresponds to 50 $\mu\text{g/ml}$ of double-stranded DNA, 40 $\mu\text{g/ml}$ of RNA. The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA and RNA have an A_{260}/A_{280} ratio of 1.7-1.9 and 1.9-2.1, respectively.

2.2.6 Restriction digestion of plasmid DNA

Restriction digestion was carried out according to the standard protocol [169]. Typically 1-5 μg DNA was digested using 1x optimal buffer and 1 μl restriction enzyme (10 U) in a 20 μl volume and incubated at 37°C for 1 h. Restriction digestion was analysed by agarose gel electrophoresis.

2.2.7 Agarose gel electrophoresis

The DNA fragments were separated on 1% agarose gels (dissolved in TAE buffer containing 0.1 $\mu\text{g/ml}$ ethidium bromide) in TAE buffer at 100 V for 45-60 min. The 1 kb DNA Ladder from Gibco-BRL (Karlsruhe) was used as molecular weight marker. DNA bands were visualised by illumination with UV light and documented with the gel print 2000i (MWG-Biotech, Ebersberg).

6x DNA loading buffer:

15 %	Glycerol
0.25 %	Bromophenol blue
0.25 %	Xylene cyanol blue

1x TAE buffer:

40 mM	Tris base
32 mM	Acetic acid
1 mM	EDTA pH 8.0

2.2.8 DNA purification from agarose gels

DNA fragments were extracted from agarose gels using QIAquick Gel Extraction Kit (Qiagen, Hilden) according to the manufacturer's instructions.

2.2.9 DNA ligation

DNA ligations were performed by incubating DNA fragments with appropriately linearized cloning vector in the presence of buffer, ATP, and T4 DNA ligase. The concentration of DNA for DNA ligation was estimated by agarose gel analysis using molecular weight standards of a known concentration. A ligation reaction was prepared using an approximate 3:1 molar ratio of the insert and vector.

<u>The ligation mixture:</u>	x μ l	Vector DNA (20-50 ng)
	x μ l	Insert DNA
	2 μ l	10x ligation buffer
	1 μ l	T4 DNA ligase
	ad 20 μ l	H ₂ O
<u>10x ligation buffer:</u>	660 mM	Tris-HCl, pH 7.5
	50 mM	MgCl ₂
	10 mM	DTT
	10 mM	ATP

The ligation mixture was incubated at RT overnight. Afterwards, the ligation mixture was used directly for bacterial transformation.

2.2.10 Construction of expression vectors

Construction of expression vectors for wild-type STATs and STAT-R31K mutants

The constructs for human STAT1-R31K and murine STAT3-R31K were generated by site-directed mutagenesis using the QuickChange kit (Stratagene, La Jolla, CA). pBS-STAT1 and pBS-STAT3 were used as a template in PCR. cDNA fragments corresponding to human STAT1wt, STAT1-R31K, murine STAT3wt, or STAT3-R31K were then cloned into the pcDNA3 vector (Invitrogen, Karlsruhe) cut with KpnI and NotI.

Construction of pGEX-5X-2huSTAT1

The plasmid encoding a GST fusion protein of full-length STAT1 was generated by excision of an EcoRV/NotI fragment from pBS-STAT1 and subsequent ligation into the SmaI/NotI sites of pGEX-5X-2 (Pharmacia, Freiburg).

Construction of pGEX-5X-1muSTAT3

The plasmid encoding a GST fusion protein of full-length STAT3 was generated by excision of an EcoRI/NotI fragment from pBS-STAT3 and subsequent ligation into the EcoRI/NotI sites of pGEX-5X-1 (Pharmacia, Freiburg).

Cloning of human SOCS3 cDNA for sequencing

The cDNA of human SOCS3 was synthesized from RNA by RT-PCR using specific primers: SOCS3 forward 5'-CTGGCTCCGTGCGCCATG-3' and SOCS3 reverse 5'-GGAAGCTGAG GAATTGAAGGAGAA-3', resulting in a 1013 bp fragment. RT-PCR products were gel purified using a gel extraction kit (Qiagen, Hilden) and cloned into the pCR2.1-TOPO vector (Invitrogen, Karlsruhe).

All constructs were verified by DNA sequencing.

2.2.11 Automated DNA sequencing

The principle of automated DNA sequencing developed by Sanger *et al.* [170] is based on an excitation and detection of fluorescent dye labeled dideoxynucleotides which are incorporated into DNA extension products in PCR reaction and terminate DNA elongation selectively at A, C, G, or T. In this way, a series of DNA fragments is produced with various lengths that can be separated by electrophoresis. The PRISMTM Ready Reaction DyeDeoxyTM Terminator Cycle Sequencing Kit (Perkin Elmer, Boston, USA) was used for the cycle sequencing. The cycle sequencing reactions were set up as following:

4 µl Terminator premix
1 µg DNA Template
1 µl Primer (10 pmol/µl)
ad 20 µl H₂O (Merck, Darmstadt)

The reaction mixtures were transferred into a thermal cycler using a 3-step-cycling for 25 cycles:

PCR program (25 cycles):

Denaturation	96°C	20 sec
Primer-annealing	50°C	20 sec
DNA-extension	60°C	4 min
Last extension	60°C	4 min

The amplified DNA products were purified by ethanol precipitation. To precipitate the DNA, 250 μ l 100% ethanol and 10 μ l 3 M sodium acetate (pH 5.2) were added to the DNA solution and mixed well. After centrifugation at 13000 rpm for 20 min at 4°C, the DNA pellet was washed with 300 μ l 70% ethanol by centrifugation at 13000 rpm for 5 min at 4°C. The supernatant was carefully removed and the DNA pellet was air-dried at RT.

The DNA precipitates were dissolved in TSR-buffer from Applied Biosystems and denatured at 90°C for 2 min. The denatured DNA fragments were subjected to capillary electrophoresis in a 'Performance Optimized Polymer' and the results were analysed on the ABI Prism[®] 310 Genetic Analyzer (Applied Biosystems, Darmstadt).

2.2.12 Cell culture

Normal human epidermal melanocytes (NHEM) were cultured in MBM-4 medium with the recommended supplements and growth factors (CaCl₂, hFGF-B, PMA, rh-Insulin, hydrocortisone, BPE, FBS and gentamicin/amphotericin-B). Human melanoma cells were maintained in RPMI 1640 medium supplemented with 5% FCS. Human fibrosarcoma cells (2fTGH, U3A cells) and murine embryonic fibroblasts (MEF fl/fl and STAT3-negative MEF Δ/Δ cells) were maintained in Dulbecco's modified Eagle's medium containing 10% FCS. All media were supplemented with 100 mg/L streptomycin and 60 mg/L penicillin. Hygromycin (250 μ g/mL) was added to the medium of 2fTGH and U3A. Cells were grown at 37°C in a water-saturated atmosphere at 5% CO₂.

For subculturing of adherent cell lines, cells were washed twice with PBS and treated with Trypsin/EDTA for 1 min. Cells were then diluted appropriately in pre-warmed medium. For the cryopreservation of cell cultures, cells were resuspended in medium containing 10% DMSO, 20% FCS, frozen overnight at -80°C and then stored in liquid nitrogen at -180°C.

PBS:

200 mM	NaCl
2.5 mM	KCl
8.0 mM	Na ₂ HPO ₄
1.5 mM	KH ₂ PO ₄
	pH 7.4

2.2.13 Cell Transfection

Transient transfections of MEF Δ/Δ and U3A cells were performed using FuGENE (Roche Applied Science, Mannheim) and Superfect (Qiagen, Hilden) according to the manufacturers' instructions.

2.2.14 Generation of stable cell lines

The wild-type and dominant negative STAT constructs in the pCAGGS plasmid were gifts from Koichi Nakajima and Toshio Hirano (Osaka, Japan). For preparation of stable transfectants, 2×10^6 1286 cells were transfected with 5 μg of the respective plasmid DNA using FuGENE 6 transfection reagent (Roche Applied Science, Mannheim) according to the manufacturer's instructions. Transfectants were selected in the presence 1 mg/ml of G418 (Sigma, Taufkirchen). Heterologous expression of STATs was assessed with a monoclonal antibody directed against the haemagglutinin (HA)-Tag from Cell Signaling Technology Inc..

2.2.15 Preparation of cell lysates

Cells were washed twice with cold PBS containing 100 μM Na_3VO_4 and then lysed in Hepes-lysis buffer supplemented with freshly added protease inhibitors. Cells were scraped off the plate and transferred to a centrifuge tube and incubated on ice for 30 min. Lysates were centrifuged at 13000 rpm for 10 min at 4°C, and protein concentration in the supernatant was determined by the Bradford method. The cell lysates were stored at -20°C.

Hepes-Lysis buffer:

20 mM	HEPES, pH 7.4
100 mM	NaCl
50 mM	NaF
10 mM	β -glycerophosphate
1 %	Triton X-100

Inhibitors:

1 mM	Sodium vanadate
1 mM	PMSF
5 $\mu\text{g}/\text{ml}$	Aprotinin
5 $\mu\text{g}/\text{ml}$	Leupeptin
3 $\mu\text{g}/\text{ml}$	Pepstatin

2.2.16 Determination of protein concentration according to Bradford

The protein concentration was determined by the Bradford method [171] using the Bio-Rad protein assay (München). The Bio-Rad protein assay is a colorimetric assay, based on the binding of a dye, Coomassie Brilliant blue, to protein and comparing this binding to a standard curve generated by the reaction of known amounts of a standard protein (BSA). The Bio-Rad reagent was diluted 1:5 with distilled water. For each sample, 2 μl lysates were added to 1 ml diluted Bio-Rad reagent, mixed well and incubated for 5 min at RT. The

absorbance was measured at 595 nm. The protein concentration ($\mu\text{g}/\mu\text{l}$) of the samples is the value of OD_{595} multiplied with factor 8.75.

2.2.17 Immunoprecipitations

1 ml of cell lysates containing 1.5 mg of protein were incubated with 5 μl of monoclonal mono-/dimethylarginine antibody (Abcam, Cambridge, UK) or an isotype control antibody overnight at 4°C. The resulting immune complexes were precipitated with 5 mg protein A-Sepharose (CL-4B, Amersham Biosciences, Freiburg) for 1 h, washed three times with lysis buffer, and boiled in Laemmli buffer for 5 min at 95°C. Proteins were separated by SDS-PAGE, followed by Western blot analysis.

2.2.18 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE), first described by Laemmli [172], separates proteins of different molecular weight under denaturing conditions. Interaction of SDS with the poly-peptide chain of protein results in a net negative charge, and therefore proteins are separated according to the length of the polypeptide chain.

Proteins were separated on 7.5% or 12% separating gels, with a 3% stacking gel at 25 mA/gel using the Biometra apparatus. The Precision Plus Protein Standards (Biorad, München) were used as protein molecular weight standard.

<u>Acrylamide stock solution:</u>	29.0 %	Acrylamide
	1.0 %	Bisacrylamide
<u>4x separating gel buffer:</u>	1.5 M	Tris.HCl, pH 8.8
	0.4 %	SDS
<u>4x stacking gel buffer:</u>	0.5 M	Tris.HCl, pH 6.8
	0.4 %	SDS
<u>10x elektrophoresis buffer:</u>	0.25 M	Tris base
	1.92 M	Glycine
	1 %	SDS
<u>2x Laemmli sample buffer:</u>	125 mM	Tris.HCl, pH 6.8
	20 %	Glycerol
	4 %	SDS
	0.02 %	Bromphenol blue
	5 %	2- β -Mercaptoethanol

2.2.19 Western blotting

Following electrophoresis, proteins in a polyacrylamide gel were transferred to PVDF membrane (PALL, Germany) by semi-dry electroblotting as described [173]. An appropriately sized PVDF membrane was pre-soaked in methanol for 30 sec and then incubated in anode buffer II for 15 min. The gel was soaked in cathode buffer for 5 min. On the bottom of the blotting apparatus (cathode) 5 sheets of Whatman paper (soaked in anode buffer I) were placed, followed by 2 sheets of Whatman paper (soaked in anode buffer II), the gel, the PVDF membrane, 5 sheets of Whatman paper (soaked in cathode buffer), and finally the anode. The electroblotting was carried out at a current of 0.8 mA/cm² for 1 h.

<u>Anode buffer I:</u>	0.30 M	Tris base
	20 %	Methanol
<u>Anode buffer II:</u>	0.025 M	Tris base
	20 %	Methanol
<u>Cathode buffer:</u>	0.40 M	6-Aminohexanoic acid
	20 %	Methanol

2.2.20 Immunodetection

After Western blotting, the PVDF membranes were blocked with 10% BSA (w/v) in TBS-N buffer for 30 min. The membranes were washed twice for 5 min each with TBS-N buffer and incubated with the primary antibody (1:1000) for 1 h at RT with gentle agitation. The blots were washed once for 15 min and twice for 5 min with TBS-N buffer and then incubated with HRP-conjugated secondary antibody (1:2000) for 30 min at RT with agitation. The blots were washed once for 15 min and twice for 5 min with TBS-N buffer. Signals were detected using the ECL system (Amersham Biosciences, Freiburg). Before re-probing, blots were stripped in stripping buffer for 20 min at 75°C.

<u>TBS-N buffer:</u>	20 mM	Tris·HCl, pH 7.4
	137 mM	NaCl
	0.1 %	Nonidet P-40
<u>Stripping buffer:</u>	62.5 mM	Tris·HCl, pH 6.7
	100 mM	2-β-Mercaptoethanol
	2 %	SDS

2.2.21 Flow cytometry

Cells were washed twice with cold PBS and detached from the dishes by treatment with PBS containing 10 mM EDTA for 10 min at 37°C. Cells were resuspended in cold PBS supplemented with 5% FCS and 0.1% sodium azide (PBS/azide). 5×10^5 to 1×10^6 cells in 100 μ l of PBS/azide were incubated with 1 μ g/ml of primary antibody for 30 min at 4°C. Cells were then washed with cold PBS/azide and subsequently incubated in darkness with a 1:100 dilution of respective R-phycoerythrin-conjugated secondary antibodies (Dianova, Hamburg) for 30 min at 4°C. Cells were again washed with cold PBS/azide, and then 10^4 cells per sample were analysed by flow cytometry using a FACSCalibur (Becton Dickinson) equipped with a 488 nm argon laser.

2.2.22 Growth inhibition assay

The assay is based on the cleavage of the 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT) to formazan by the mitochondrial enzyme, "succinate-tetrazolium reductase" which is only active in metabolically intact cells. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture.

3×10^3 of viable cells were seeded in triplicates into 96-microwell plates and incubated with various concentrations of cytokines in medium containing 1% FCS. After four days of culture an XTT colorimetric assay (Roche) was performed. Cells were incubated for 3 h at 37°C with a tetrazolium salt XTT reagent in the presence of an electron-coupling reagent. The absorbance of the colored formazane product was measured at 450 nm by an ELISA reader. The percentage of growth inhibition was calculated in relation to the growth of untreated control cells. For the experiments on time-dependence, 1.5×10^3 viable cells/well were seeded with or without cytokines and XTT tests were performed after different periods of incubation.

2.2.23 Labeling of oligonucleotides

A double-stranded mutated SIE-oligonucleotide from the c-fos promoter (m67SIE: 5'-GAT CCG GGA GGG ATT TAC GGG AAA TGC TG-3') was labeled by filling in 5' protruding ends with the Klenow enzyme, using [α - 32 P]dATP (10 mCi/ml, 3000 Ci/mmol). The labeling reaction was incubated for 30 min at 37°C. The labeled oligonucleotides were purified using the QIAquick Nucleotide Removal Kit (Qiagen, Hilden) according to the manufacturers' instructions.

The measurement of the radioactivity was carried out on a PerkinElmer Betascout liquid scintillation counter.

<u>Labeling reaction:</u>	2.5 μ l	DNA (1 pmol/ μ l)
	6 μ l	10x Restriction buffer M (Boehringer Mannheim)
	1 μ l	dCTP (0.5 mM)
	1 μ l	dGTP (0.5 mM)
	1 μ l	dTTP (0.5 mM)
	2 μ l	BSA (1%)
	42 μ l	H ₂ O
	2.5 μ l	[α - ³² P]dATP
	2.0 μ l	Klenow enzyme (1 U/ μ l)

2.2.24 Nuclear extract preparation

Nuclear extracts were prepared as described previously [174]. Cells were washed twice with cold PBS containing 100 μ M Na₃VO₄ and incubated with 400 μ l buffer A for 10 min on ice. After centrifugation at 2000 rpm for 1 min, the pellet was collected and incubated with 40 μ l buffer C for 20 min on ice. After centrifugation at 14000 rpm for 5 min, the supernatant was collected as nuclear extract and stored at -80°C.

<u>Buffer A:</u>	10 mM	Hepes-KOH, pH 7.8
	1.5 mM	MgCl ₂
	10 mM	KCl
	0.5 mM	DTT
	0.2 mM	PMSF
	1 mM	Na ₃ VO ₄

<u>Buffer C:</u>	20 mM	Hepes-KOH, pH 7.8
	1.5 mM	MgCl ₂
	420 mM	NaCl
	0.2 mM	EDTA
	25 %	Glycerol (v/v)
	0.5 mM	DTT
	0.2 mM	PMSF
	1 mM	Na ₃ VO ₄

2.2.25 Electrophoretic mobility shift assay (EMSA)

Nuclear extracts containing 5 µg of protein were incubated with 10 fmol (10.000 cpm) of a α -³²P-labeled double-stranded oligonucleotide in gel shift incubation buffer containing 0.1 mg/ml of poly(dI-dC) and 1 mg/ml BSA for 10 min at room temperature. The protein-DNA complexes were separated on a 4.5% polyacrylamide gel containing 7.5% glycerol in 0.25-fold TBE at 20 V/cm for 4 h. Gels were fixed in a water solution of 10% methanol and 10% acetic acid for 30 min, dried and autoradiographed. Data were further analysed using a Personal FX Phosphorimager with the Quantity One software (Bio-Rad). EMSAs were performed using the m67SIE probe which binds to STAT1 and STAT3.

<u>5x Gel shift buffer:</u>	50 mM	Hepes-KOH, pH 7.8
	5 mM	EDTA
	25 mM	MgCl ₂
	50 %	Glycerol (v/v)
	25 µM	DTT
	3.5 µM	PMSF
<u>Reaction mixture</u>	4 µl	5x gel shift buffer
<u>for one sample:</u>	0.2 µl	0.5 M DTT
	0.2 µl	36 mM PMSF
	1 µl	Poly(dI-dC) (1 mg/ml)
	2 µl	BSA (10 mg/ml)
	ad 9.5 µl	H ₂ O
<u>Gel preparation:</u>	6.75 ml	Acrylamide mix (40%)
	4.5 ml	Glycerol (99%)
	3 ml	5x TBE
	45.75 ml	H ₂ O
	200 µl	APS (10%)
	40 µl	TEMED
<u>5x TBE:</u>	1 M	Tris-aminomethane
	0.83 M	Boric acid
	10 mM	EDTA
	ad 1000 ml	H ₂ O, adjust pH to 8.3 with HCl
<u>Running buffer:</u>	0.25x	TBE

2.2.26 Reporter gene assay

U3A cells were transfected with 3 μ g of IRF-1 luciferase reporter construct, 1 μ g of β -galactosidase control plasmid pCH110, and 100 ng of plasmid encoding STAT1wt or 1 μ g of plasmid encoding STAT1R31K using Superfect (Qiagen). MEF Δ/Δ cells were transfected with 3 μ g of *c-fos* luciferase reporter construct, 1 μ g of β -galactosidase control plasmid pCH110, and 100 ng of plasmid encoding STAT3wt or 1 μ g of plasmid encoding STAT3R31K using FuGENE 6 transfection reagent (Roche Applied Science). Twenty-four hours after transfection, cells were stimulated with 1000 units/ml IFN- α or 20 ng/ml recombinant human IL-6 for 16 h, harvested, and subjected to assays for luciferase and β -galactosidase activity as described below.

Luciferase assay

Cell lysis and luciferase assays were performed using Promega's dual luciferase kit (Promega, Madison, WI). Cells were washed twice with cold PBS and lysed with 200 μ l of reporter gene lysis buffer. Lysates were incubated for 20 min on ice and clarified at 13000 rpm for 10 min at 4°C. 10 μ l of the cell lysate were loaded into each well of a 96-well plate. Then 50 μ l of luciferase assay reagent were added, and luciferase activity was immediately measured in a luminometer (EG&G Berthold, Bad Wildbad), which was programmed to read for 10 sec. Luciferase activity values were normalised to transfection efficiency monitored by the co-transfected β -galactosidase expression vector pCH110 (Amersham Biosciences, Freiburg). Assays were carried out in triplicate and the experiments were repeated at least three times.

reporter gene lysis buffer: 25 mM Tris-phosphate, pH 7.8
 2 mM EDTA
 2 mM DTT
 10 % Glycerol
 1 % Triton X-100

β -Galactosidase assay

To measure the β -galactosidase activity, 100 μ l of cell lysate were incubated with 500 μ l β -galactosidase-buffer and 100 μ l ONPG (1 mg/ml), a substrate of β -galactosidase, at 37°C. When the solution became yellow, the reaction was stopped by adding 250 μ l 1 M Na₂CO₃ and the absorbance of *o*-Nitrophenol as the end product was measured at 420 nm.

β -galactosidase buffer: 60 mM Na_2HPO_4
 40 mM NaH_2PO_4
 1 mM KCl
 1 mM MgCl_2
 386 μl β -Mercaptoethanol per 100 ml solution

ONPG: 1 mg/ml *o*-Nitrophenyl- β -D-galactopyranoside

2.2.27 siRNA transfection

All siRNAs used in this study were synthesized by Eurogentec (Seraing, Belgium) and received as desalted, pre-annealed duplexes in desalted-purified formats. 3 different siRNAs as indicated in 2.1.11 were used for targeting human SOCS3. A nonspecific siRNA, scrambled negative control siRNA (catalog no. OR-0030-neg05) was used as a control. Transient transfection of siRNA oligonucleotides was performed at 50 nM with jetSI-ENDO Transfection Reagent (Eurogentec) according to the manufacturer's protocol.

2.2.28 Preparation of RNA and RT-PCR analysis

Total RNA was isolated from melanoma cell lines using the RNeasy Mini kit from Qiagen (Hilden) as described by the manufacturer. Expression of SOCSs transcripts was determined by RT-PCR analysis using specific primers as indicated in 2.1.11. RT-PCR was performed with 1 μg of total RNA using the OneStep RT-PCR kit from Qiagen (Hilden). The amplification program consisted of one cycle at 50°C for 30 min, 95°C for 15 min, followed by 35 cycles at 94°C for 40 sec, 58°C for 30 sec, 72°C for 30 sec with a final extension at 72°C for 10 min. PCR products were separated electrophoretically on 1.5% TAE-agarose gels and visualised by ethidium bromide staining and documented with the gel print 2000i (MWG-Biotech, Ebersberg).

2.2.29 Quantitative real-time PCR

Real-time PCR was performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Darmstadt). Relative quantification of SOCS3 expression was carried out using SYBR Green PCR reagents from Applied Biosystems (Darmstadt). Briefly, 1 µg of purified RNA was reverse transcribed using the 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche, Mannheim) with random hexamers primers according to the manufacturer's description. The cDNAs for SOCS3 and internal control β-actin were amplified using specific primers for SOCS3 or for β-actin as indicated in 2.1.11. The PCR reaction was performed in a final volume of 25 µl containing 2.5 µl cDNA, 100 nM of each primer, 12.5 µl SYBR Green PCR buffer (Applied Biosystems, Darmstadt). After denaturing for 15 min at 94°C, amplification was performed by 40 cycles of 15 sec at 94°C, and a combined annealing/extension step of 60 sec at 60°C using the ABI prism 7000 (Applied Biosystems, Darmstadt). cDNAs of SOCS3 and β-actin were amplified independently in separate reaction wells in triplicate. The real-time PCR efficiencies were determined for each primer/probe set from standard curves generated from serial dilutions of a cDNA sample of OSM-stimulated human melanoma A375 cells. The quantification of gene expression was calculated using a mathematical model including the PCR efficiencies [175]. The level of SOCS3 gene expression in the test samples was normalised to the corresponding β-actin level and is reported as the -fold difference.

$$ratio = \frac{E_{target}^{\Delta CP_{target}(control-sample)}}{E_{ref}^{\Delta CP_{ref}(control-sample)}}$$

E_{target} is the real-time PCR efficiency of target gene transcript; E_{ref} is the real-time PCR efficiency of a reference gene transcript; ΔCP_{target} is the CP deviation of control – sample of the target gene transcript; ΔCP_{ref} = CP deviation of control – sample of reference gene transcript.

2.2.30 GST fusion protein expression

GST fusion proteins were expressed in *E. coli* strain BL21 using the pGEX vector system (Amersham Biosciences). Purification of GST fusions from crude bacterial lysates was performed by immobilization on glutathione-sepharose (Amersham Biosciences). Subsequently, fusion proteins were eluted from the beads in the presence of 25 mM glutathione/50 mM Tris-HCl (pH 8) solution. Eluates were dialysed against PBS/10% glycerol.

2.2.31 **In vitro methyltransferase assays**

Eluted and dialyzed GST-PRMT1 was incubated with eluted/dialysed GST-GAR and GST-STAT1, respectively, in the presences of 1 μ l of *S*-adenosyl-L-[*methyl*-¹⁴C]methionine (60 mCi/mmol) and PBS at 37°C for 2 h. Reaction products were resolved by SDS-PAGE, blotted onto nitrocellulose, and visualised by autoradiography.

2.2.32 **Molecular modeling of the N-terminal domains of STAT1 and STAT3**

For molecular modeling and graphic representation of the protein structures, the programs WHAT IF [176], Ribbons [177], and Rasmol [178] were used. The structure of the N-terminal domain of STAT4, Brookhaven data bank entry code 1BGF [179], was used as a template for the model structures.

2.2.33 **Immunohistochemistry**

Human melanoma specimens (n=10) derived from patients undergoing routine surgery for therapeutic or diagnostic reasons were examined. Analysis and processing of these samples was previously approved by the ethical committee of the University of Münster, Münster, Germany. The specimens included early primary cutaneous melanomas with Clark level II (n=2), Clark level III (n=3), Clark level IV (n=2), Spitzoid melanoma (n=1, Clark level IV) and melanoma metastases (n=2). Specimens were fixed in 7% buffered paraformaldehyde, dehydrated, embedded in paraffine and mounted on Tissue-Tek (Mikrom, Walldorf, Germany). Paraffineembedded sections were subsequently deparaffinized by routine methods followed by epitope unmasking with proteinase K (Danko, Carpinteria, CA) for 10 min at room temperature in a wet chamber. After washing with PBS sections were stained at 1:100 with a polyclonal antibody against SOCS3 (C204, IBL, Hamburg, Germany) for 45 min. Negative controls consisted of the isotype control IgG incubated at the same protein concentration as the primary antibody. After washing sections were further processed for immunoperoxidase staining using the Polylink system from DCS (Hamburg, Germany) and AEC as substrate. Sections were finally counterstained with Mayer's haematoxylin (Merck, Darmstadt, Germany). Immunostaining steps were semi-automatically processed by the Autostainer 480 (Mikrom, Walldorff, Germany).

3 Results

3.1 Methylation of STATs

3.1.1 No evidence for specific recognition of STAT1 and STAT3 by dimethylarginine antibodies

Mowen et al. have reported that STAT1 is methylated on arginine 31 and that STAT1 methylation is important for STAT1 function [130]. Arg³¹ at the N-terminus of STAT1 is conserved in all STAT family members. Therefore I examined whether STAT3 undergoes methylation by the method described by Mowen et al. [130]. I used the mono/dimethyl-arginine-specific antibody ab412, which has been employed to detect methylation of STATs [130-132,180-183], for immunoprecipitation experiments. I first investigated STAT3 methylation in murine embryonic fibroblasts (MEF fl/fl), and used MEFs (Δ/Δ) lacking STAT3 as a negative control.

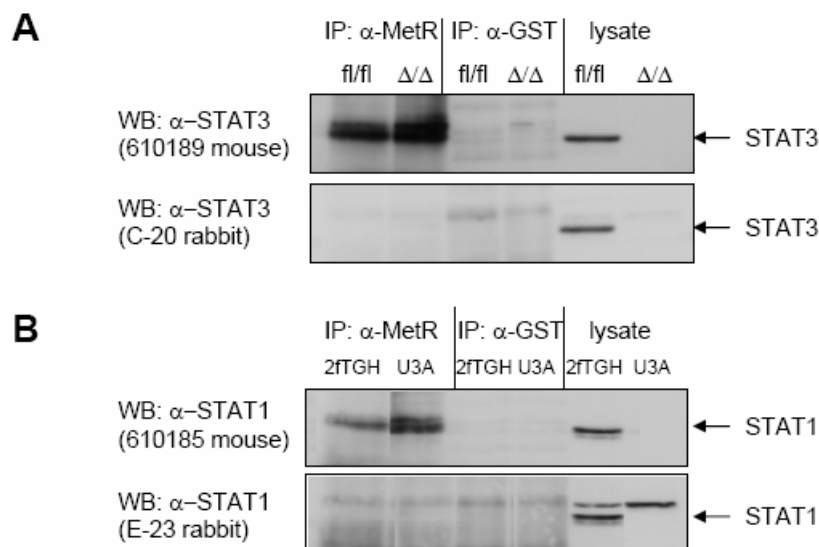


Figure 9. No evidence for specific STAT immunoprecipitation by anti-methylarginine antibodies.

(A) Immunoprecipitations were performed with the mono-/dimethylarginine-specific antibody ab412 or with the isotype-matched anti-GST antibody from lysates of parental STAT3-positive MEFs (fl/fl) or of STAT3-negative MEFs (Δ/Δ). Western blots of the immunoprecipitates and aliquots of the lysates were detected with monoclonal STAT3 antibody (Transduction Laboratories, 610189) or with a polyclonal STAT3 antibody (Santa Cruz Biotechnology, C-20). (B) Immunoprecipitations were performed with the mono-/dimethylarginine-specific antibody ab412 or with the isotype-matched anti-GST antibody from lysates of parental 2fTGH fibrosarcoma cells or STAT1-negative U3A cells. Western blots of the immunoprecipitates and aliquots of the lysates were detected with monoclonal STAT1 antibody (Transduction Laboratories, 610185) or with a polyclonal STAT1 antibody (Santa Cruz Biotechnology, E-23).

Cell lysates were subjected to immunoprecipitations with either anti-mono/dimethylarginine antibody (MetR) or isotype control IgG, and Western blots were probed with antibody against STAT3. As shown in Fig. 9A, a monoclonal STAT3 antibody recognized a band in the immunoprecipitate of wild-type MEFs (fl/fl) cell lysate co-migrating with STAT3, which was not precipitated by an anti-GST antibody, the isotype-matched specificity control (Fig. 9A, *lanes 1 and 3*). However, a band with the same apparent molecular weight could be seen in the lane with immunoprecipitates of MEFs (Δ/Δ) cells lacking STAT3 (*lane 2*), indicating that these bands can not correspond to STAT3. In contrast, a polyclonal anti-STAT3 antibody did not reveal STAT3 precipitated by the anti-methylarginine antibody, whereas in the lysate lanes STAT3 was readily detected by this antibody (Fig. 9A, *lower panel*).

To test the specificity of the mono-/dimethylarginine antibody, I re-examined methylation of STAT1 in fibrosarcoma 2fTGH cells, and used STAT1-deficient 2fTGH-derived U3A cells as a negative control. Similarly, the mono-/dimethylarginine-specific antibody ab412 precipitates a protein, which is recognized by a monoclonal STAT1 antibody in 2fTGH cell lysates (Fig. 9B, *upper panel*). However, a protein co-migrating with STAT1 was also precipitated from lysates of STAT1-deficient 2fTGH-derived U3A cells and recognized in STAT1-specific Western blots, indicating again a specificity problem. Using another STAT1-specific antibody for detection I observed no band corresponding to STAT1 neither in parental 2fTGH cells nor in STAT1-deficient U3A cells (Fig. 9B, *lower panel*). Furthermore, the dimethylarginine-specific antibody ab413 did not lead to a specific precipitation of STAT1 or STAT3 (data not shown).

Taken together, these data do not provide any evidence for arginine methylation of STATs but indicate a specificity problem of the mouse anti-STAT antibodies and underline the importance of negative control cells (i.e. STAT1/3-deficient cells).

3.1.2 Methylation inhibitors affect multiple signaling pathways

Previous studies addressing the potential methylation of STATs largely relied on the use of methylation inhibitors. Whereas in the publication by Mowen et al. (2001), MTA was used to inhibit arginine methylation, a combination of *N*-methyl-2-deoxyadenosine, DL-homocysteine and adenosine (termed MDA treatment) has been employed as an alternative inhibitor of methylation reactions in follow-up papers [131,132,184].

According to Mowen et al., inhibition of STAT1 methylation by MTA increased PIAS1 binding to STAT1 and subsequently reduced STAT1 DNA binding after IFN- α stimulation, resulting in a decrease of STAT1 transcriptional activity without affecting STAT1 tyrosine phosphorylation. I first investigated the effects of MTA on STAT1 activation in 729 melanoma cells. Cells were pretreated with 0.3 mM MTA prior to stimulation with IFN- α . DNA binding activity was analysed by EMSA using the radiolabeled SIE-probe, which binds to activated STAT1 and STAT3. As shown in Fig. 10A, MTA reduced the IFN- α -mediated STAT1 DNA binding activity. In addition, I could confirm that MTA inhibits IFN- α -mediated induction of IRF-1 promoter-driven reporter gene expression (Fig. 10B).

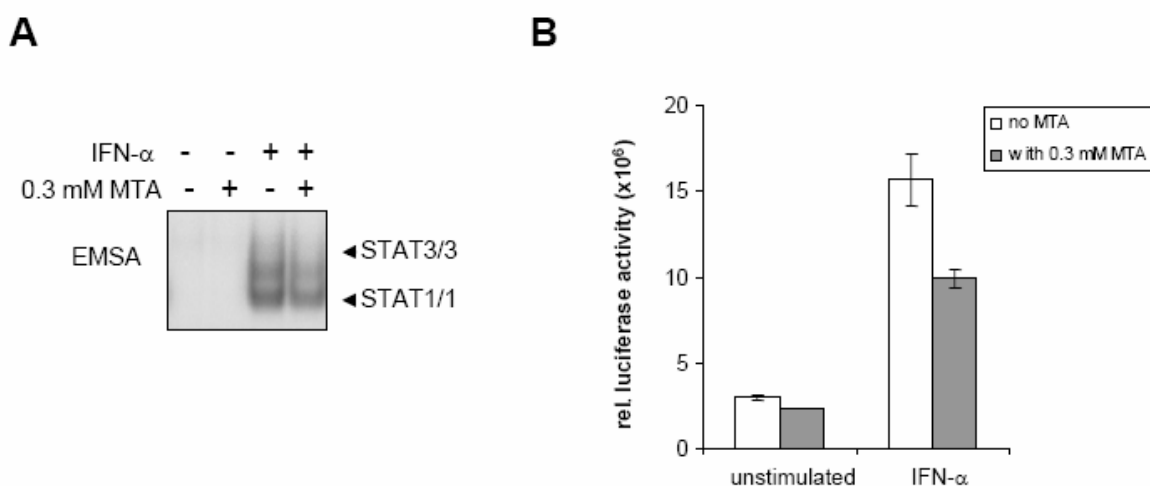


Figure 10. MTA reduces STAT1 DNA binding activity and transcription activity of STAT1.

(A) 729 melanoma cells were pretreated or not for 3 h with 0.3 mM MTA and then stimulated for 30 min with IFN- α (1000 U/ml) or left untreated. Nuclear extracts were analysed by EMSA for binding activities to the SIE probe. (B) 729 melanoma cells were transfected with a luciferase construct under the control of the STAT1-responsive IRF-1 promoter and a β -Gal expression vector to normalize for transfection efficiency. 24 h after transfection, cells were pretreated or not for 3 h with 0.3 mM MTA and then stimulated for 18 h with IFN- α (1000 U/ml) or left untreated. Analysis of luciferase activity was performed as described in *Materials and Methods*.

I then investigated the effects of MTA on tyrosine phosphorylation of STAT1 and STAT3 in 729 melanoma cells. As shown in Fig. 11, MTA inhibited IFN- α - and OSM-inducible phosphorylation of STAT1 and STAT3, which is in striking contrast to the findings by Mowen. The inhibitory effect of MTA on STAT phosphorylation was more evident after long-term incubation (Fig. 11B). However, the phosphorylation of p38 and Erk1/2, members of the MAPK (mitogen-activated protein kinase) family, was not affected by MTA treatment.

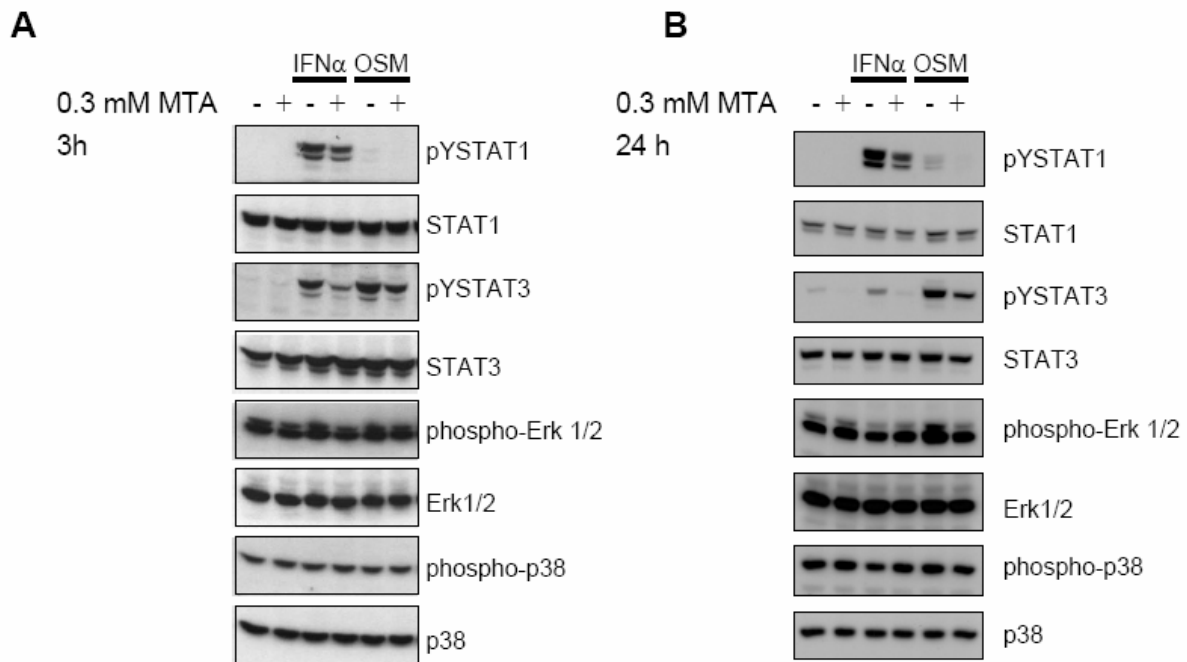


Figure 11. MTA treatment reduces STAT tyrosine phosphorylation.

729 melanoma cells were pretreated or not (**A**) for 3 h or (**B**) for 24 h with 0.3 mM MTA and then stimulated for 30 min with IFN- α (1000 U/ml) or with OSM (10 ng/ml) or left untreated. Total cellular lysates were analysed by Western blotting for the presence of phosphorylated STAT1, STAT3, Erks and p38 before reprobing of the blots with antibodies to STAT1, STAT3, Erk1/2 and p38.

Taken together, the methylation inhibitor MTA inhibits STATs phosphorylation, DNA binding and function.

I next investigated the effects of the MDA methylation inhibitor cocktail on the STAT signaling pathway in WM9 and 729 melanoma cells. Cells were pretreated with 0.3 mM MDA for 3 h prior to stimulation with IFN- α or with OSM. As shown in Fig. 12, MDA reduced the IFN- α - or OSM-mediated DNA binding activity and STAT1 and STAT3 phosphorylation (Fig. 12, *first* and *second* to *fifth* panel, respectively). Moreover, MDA treatment abrogated inducible SOCS3 expression in WM9 cells (Fig. 12, *sixth* panel). Interestingly, when reprobing the blots with antibodies against phosphorylated p38 and Erks, I

found that unlike MTA, MDA strongly affected p38 and Erk1/2 phosphorylation (Fig. 12, *seventh* and *tenth panel*). Moreover, phosphorylation of MAPKAPK2, a downstream target of p38 MAPK, was reduced (Fig. 12, *ninth panel*).

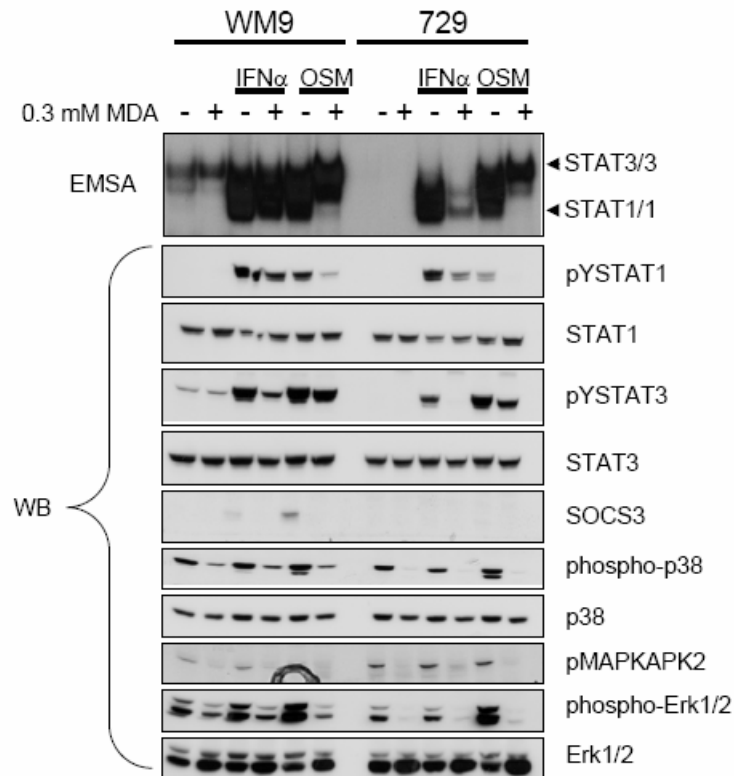


Figure 12. MDA treatment affects multiple signaling pathways.

WM9 or 729 melanoma cells were pretreated or not for 3 h with 0.3 mM MDA and then stimulated for 30 min with IFN- α (1000 U/ml) or with OSM (10 ng/ml) or left untreated. Nuclear extracts were analysed by EMSA for binding activities to the SIE probe. Total cellular lysates were analysed by Western blotting for the presence of phosphorylated STAT1, STAT3, p38, MAPKAPK2 and Erks before re-probing of the blots with antibodies to STAT1, STAT3, SOCS3, p38 and Erk1/2.

I next examined the dose- and time-dependency of the MDA effect in 729 melanoma cells. As shown in Fig. 13A, the inhibitory effect of MDA was detectable at a concentration of 0.2 mM, which is below the concentrations that have previously been used to inhibit methylation (0.3 mM up to 2 mM [131,132,184]). The inhibition of signal transduction by preincubation with MDA occurred rapidly, i.e. in less than 1 h (Fig. 13B).

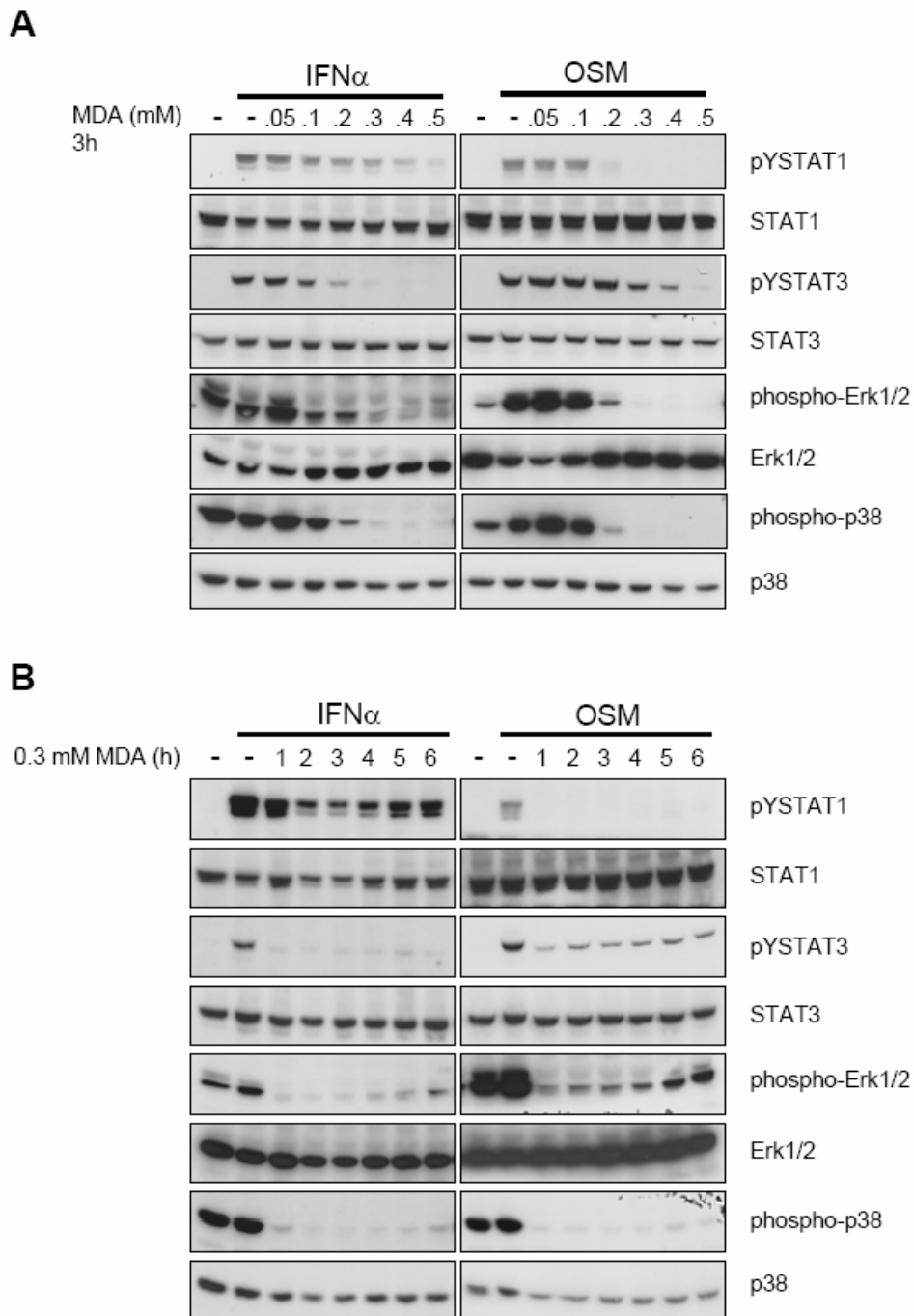


Figure 13. Dose dependence and time course for MDA effects.

(**A**) 729 melanoma cells were pretreated with MDA at different concentrations or (**B**) for different periods of time, as indicated, before they were stimulated for 30 min with IFN- α (1000 U/ml) or with OSM (10 ng/ml) or left untreated. Total cellular lysates were analysed by Western blotting for the presence of phosphorylated STAT1, STAT3, p38, and Erks before re-probing of the blots with antibodies to STAT1, STAT3, p38, and Erk1/2.

The inhibitory effects of MDA were also observed in human fibrosarcoma cells. As shown in Fig. 14, MDA pretreatment similarly attenuated IFN- α - and OSM-mediated STAT phosphorylation as well as p38 and SHP-2 phosphorylation. In this cellular system, however, MDA treatment stimulated Erk1/2 activation. Furthermore, the inhibitory effects of MDA on p38 MAPK and SHP-2 phosphorylation were identical in 2fTGH and STAT1-deficient U3A cells, suggesting that MDA exerts its inhibitory effects independent of STAT1.

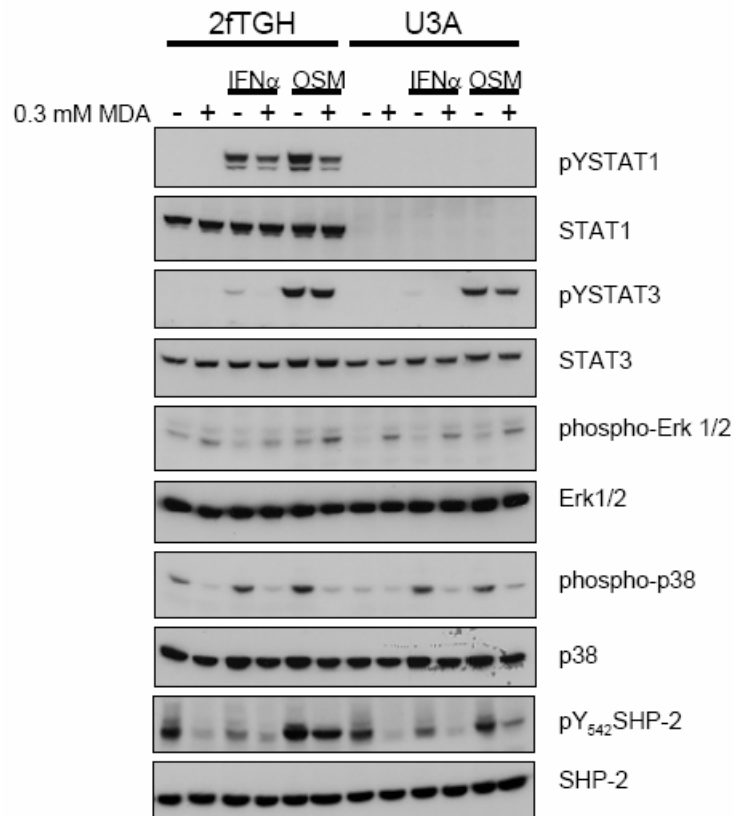


Figure 14. MDA treatment affects multiple signaling pathways.

2fTGH or STAT1-negative U3A fibrosarcoma cells were pretreated or not for 3 h with 0.3 mM MDA and then stimulated for 30 min with IFN- α (1000 U/ml) or with OSM (10 ng/ml) or left untreated. Total cellular lysates were analysed by Western blotting for the presence of phosphorylated STAT1, STAT3, p38, SHP-2, and Erks before re-probing of the blots with antibodies to STAT1, STAT3, SHP-2, p38, and Erk1/2.

I further investigated the effects of the MDA methylation inhibitor cocktail on STAT1-mediated gene transcription. As shown in Fig.15, MDA inhibits IFN- α -inducible reporter gene activity under the control of IRF-1 promoter.

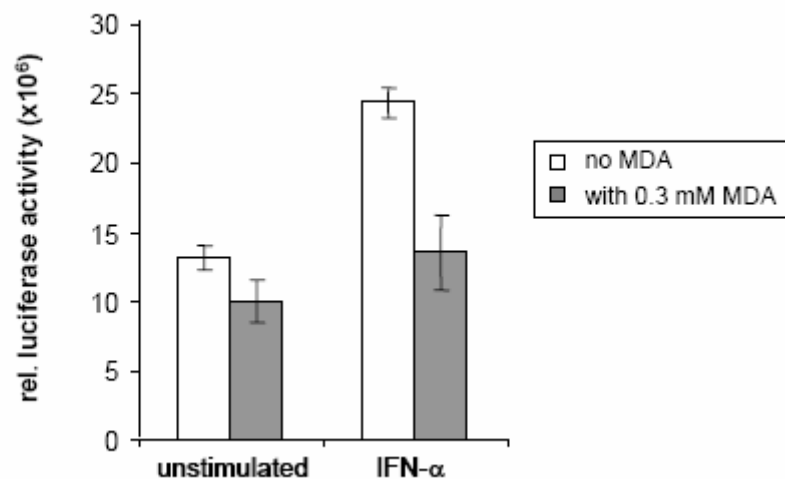


Figure 15. MDA inhibits IFN- α -mediated induction of IRF-1 promoter-driven luciferase expression.

A375 melanoma cells were cotransfected with a luciferase construct under the control of the STAT1-responsive IRF-1 promoter and a β -Gal expression vector to normalize for transfection efficiency. 24 h after transfection, cells were pretreated or not for 3 h with 0.3 mM MDA and then stimulated for 18 h with IFN- α (1000 U/ml) or left untreated. Analysis of luciferase activity was performed as described in *Materials and Methods*.

It can be concluded that the methylation inhibitors, MTA and MDA, clearly affect STAT phosphorylation, DNA binding and function. However, MDA (at the concentrations used) also drastically affects other signaling pathways involving Erks, p38, and SHP-2, which are known to cross-talk with the Jak/STAT signaling cascade [6,185-190]. Therefore, effects of MDA treatment on STAT function do not necessarily mean that STAT arginine methylation is affected.

3.1.3 Mutation of Arg³¹ to Lys led to destabilization of STAT1 and STAT3, implying an important structural role of Arg³¹

Mowen et al. used R31A or R31E mutants to mimic STAT1 methylation [130] whereas Chen et al. used R27A to study STAT6 methylation [132]. However, they reported that replacement of this conserved Arg³¹ by alanine or by glutamic acid rendered the proteins unstable. Moreover, the solved structure of the STAT4 N-terminal region [179] suggests that Arg³¹ plays a structural role as it has multiple contacts with other amino acids (W4, E39, E112) in the N-terminal domain, which are also conserved in STAT1 and STAT3 (Fig. 16, A and B). To generate a mutation that would minimize structural effects we decided to exchange Arg³¹ by Lys. Structural models of the N-terminal regions of STAT1 and STAT3 indicated that this residue could mimic at least one of the two salt bridges built up by Arg³¹ (Fig. 16, B and C).

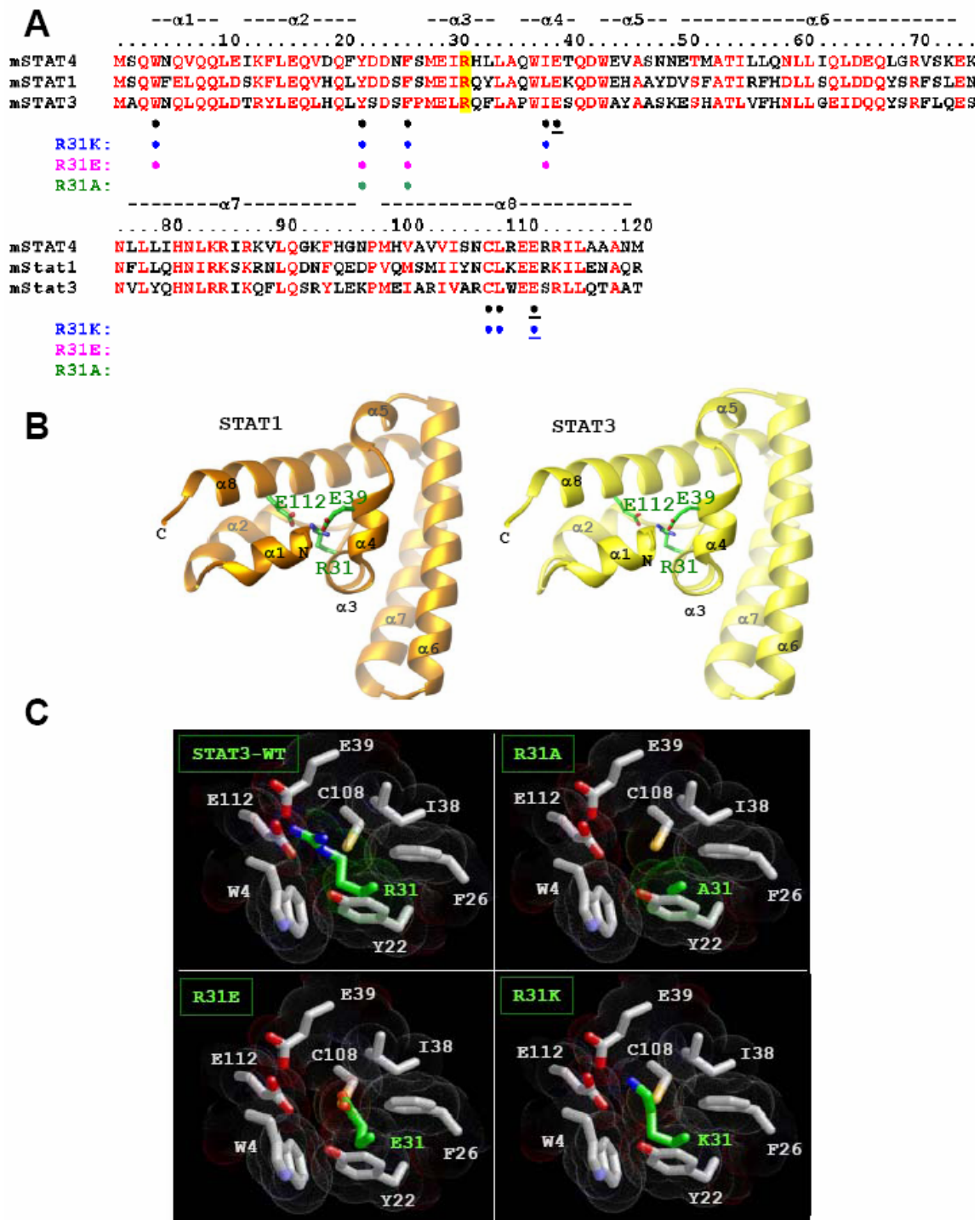


Figure 16. Effects of exchange of STAT3 Arg³¹ on amino acid contacts and STAT3 activity.

(A) Alignment of the N-terminal regions of STAT1, STAT3 and STAT4. Secondary structure characteristics (taken from the solved crystal structure of STAT4 [179]) and amino acid numbers are given on top. Residues that are highly conserved within the sequences are shown in red. R31 is highlighted in yellow. Important residues making side chain contacts with R31 are indicated by closed black circles. Contacts remaining upon mutation of R31 to lysine, glutamate or alanine are highlighted by closed circles (colored in blue, magenta or green, respectively). Contacts involving the formation of a salt bridge (with E39 or E112) are underlined. (B) Ribbon representation of the model structures of the N-terminal domain of STAT1 and STAT3. R31, E39 and E112 which form salt bridge contacts are shown as rod models. (C) Contacts of R31 of STAT3 and its mutants R31A, R31E, and R31K with surrounding amino acid residues. The side chains of the represented amino acids are shown as rod models and Van der Waal's surfaces are represented as dotted surfaces. Protein structure analysis was done by Dr. Serge Haan.

In order to investigate the exogenous expression of STAT1 and STAT3, I again took advantage of human fibrosarcoma U3A cells lacking endogenous STAT1 as well as murine embryonic fibroblasts MEFs (Δ/Δ) lacking STAT3. STAT1 wild-type and the STAT1-R31K mutant were transiently expressed in U3A cells. As shown in Fig. 17A, *upper panel*, the expression of the STAT1-R31K mutant was lower than the one of STAT1 wild-type. It turned out that 10-fold higher DNA amounts of the mutant were needed to achieve the same expression level as observed for STAT1 wild-type. Similar results were obtained for the STAT3-R31K mutant in MEF Δ/Δ cells (Fig. 17B, *upper panel*). Because the expression of R31K mutants was very low compared with wild-type STATs, I determined their stability by cycloheximide treatment. As shown in Fig. 17, *lower panel*, the R31K mutants are extremely unstable contrary to wild-type STATs. These results suggest that the conserved Arg³¹ residue contributes considerably to the protein stability of STATs.

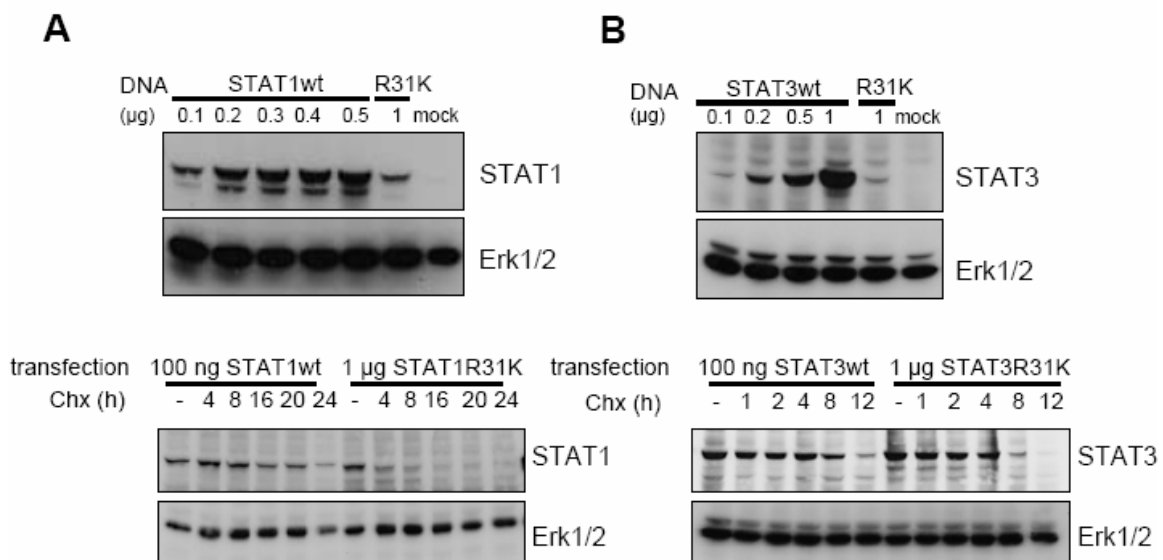


Figure 17. Reduced expression levels of STAT proteins upon exchange of Arg³¹ to Lys.

Upper panels, (A) STAT1-negative U3A cells or (B) STAT3-negative MEF Δ/Δ were transiently transfected with the indicated amounts of expression vectors encoding STAT1 or STAT3 wild-type or the R31K mutants thereof. After 24 h, total cellular lysates were analysed for expression levels of STAT1 or STAT3 and of Erk1/2 as loading control by Western blotting. *Lower panels*, 24 h after transfection with expression vectors encoding wild-type STATs or R31K mutants, U3A cells or MEFs were incubated with cycloheximide (50 $\mu\text{g}/\text{ml}$), and the expression levels of STATs and Erk1/2 were monitored by Western blotting.

I next investigated whether mutation of Arg³¹ affects the STAT function. In the case of STAT1, the R31K mutation slightly reduced STAT1-mediated gene expression but had no effect on IFN- α -mediated STAT1 phosphorylation or induction of STAT1 DNA binding activity (Fig. 18A). The R31K mutation in STAT3 led to a decrease in STAT3-mediated gene

expression and possibly in IL-6/sIL-6R-mediated STAT3 phosphorylation (Fig. 18B). Of note, STAT3-negative MEFs display an enhanced activation of STAT1 in response to IL-6 stimulation as indicated by the strong STAT1 band in the gel shift assay (Fig. 18B, *middle panel*), as described previously [191].

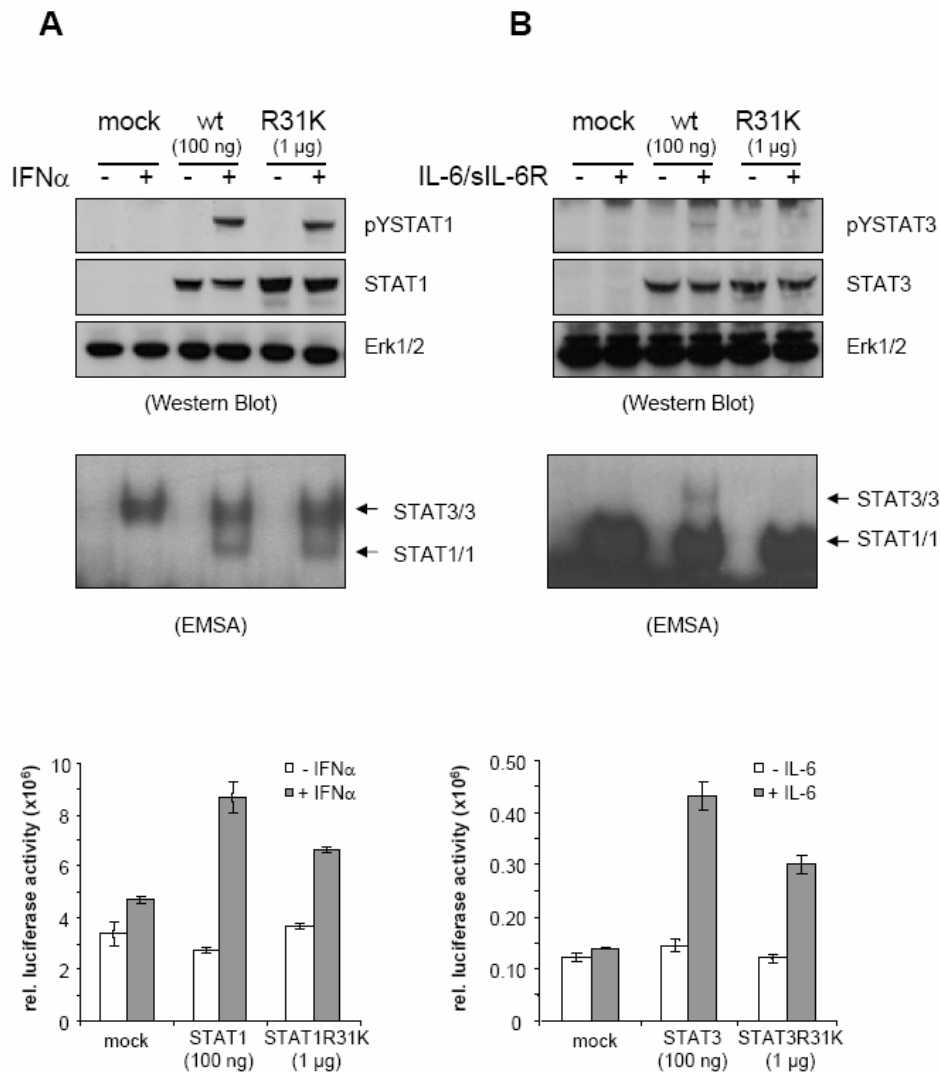


Figure 18. Effects of R31K mutations on STAT phosphorylation and function.

(A) STAT1-negative U3A cells were transiently transfected with expression vectors for STAT1 wild-type (100 ng), for STAT1-R31K (1 μ g) or with the empty vector (1 μ g). After 24 hrs, cells were stimulated with 1000 U/ml IFN- α for 15 min before total cellular lysates and nuclear extracts were prepared. Western blots were detected with antibodies against pY-STAT1, STAT1 and Erk1/2. The nuclear extracts were analysed by EMSA using the SIE probe. *Lower panel:* STAT1-negative U3A cells were additionally cotransfected with a luciferase construct under the control of the STAT1-responsive IRF1-promoter and a β -Gal expression vector to normalize for transfection efficiency. Two days after transfection cells were harvested for reporter gene assay. **(B)** STAT3-negative MEFs were transiently transfected with expression vectors for STAT3 wild-type (100 ng), for STAT3-R31K (1 μ g) or with the empty vector (1 μ g). After 24 hrs, cells were stimulated with 20 ng/ml IL-6 and 1 μ g/ml sIL-6R for 15 min before total cellular lysates and nuclear extracts were prepared. Western blots were detected with antibodies against pY-STAT3, STAT3 and Erk1/2. The nuclear extracts were analysed by EMSA using the SIE probe. *Lower panel:* STAT3-negative MEFs were additionally co-transfected with a luciferase construct under the control of the STAT3-responsive *c-fos*-promoter and a β -Gal expression vector to normalize for transfection efficiency. Two days after transfection cells were harvested for reporter gene assay.

However, the relevance of these effects is questionable considering the 10-fold excess of DNA encoding the R31K mutants of STAT1 or STAT3, which was needed to obtain approximately equivalent expression levels of the wild-type and mutant proteins. Thus, it can not be concluded whether the observed decreased transcriptional activity is due to the loss of a functional activity mediated by arginine 31 or simply to a higher protein turnover.

3.1.4 No evidence for *in vitro* methylation of STAT1 and STAT3 by PRMT1 and for effects of PRMT1 on STAT1-mediated gene expression

To assess arginine methylation directly, *in vitro* methylation assays were performed using purified PRMT1 as well as GST-STAT1 and GST-STAT3 fusion proteins. Although PRMT1-mediated ^{14}C -labeled methyl group incorporation was readily detectable for GST-GAR (glycine-arginine-rich domain of fibrillar) and histone H4, which were used as positive controls, we failed to detect methylation of the STAT fusion proteins (Fig. 19 and Fig. 20).

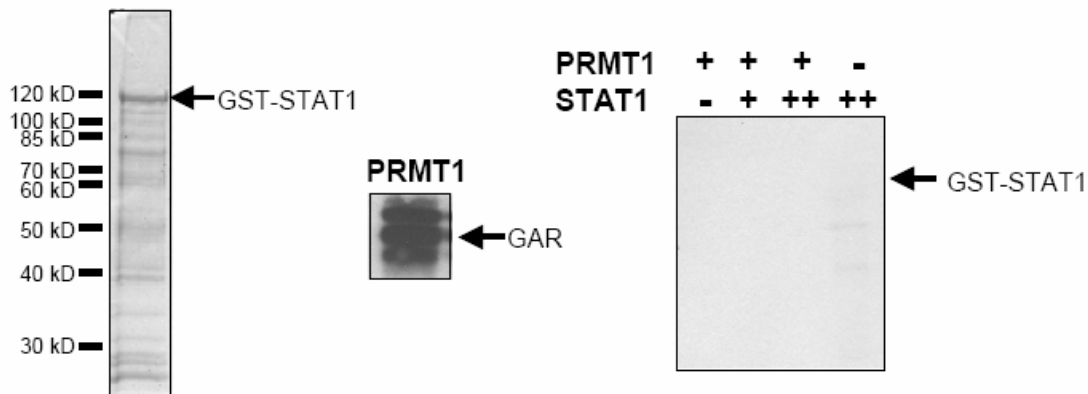


Figure 19. No evidence for *in vitro* methylation of a GST-STAT1 fusion protein by PRMTs.

Left panel, the expression and purification of recombinant GST-STAT1 was analysed by SDS-PAGE and Coomassie-staining. *Middle panel*, purified and eluted recombinant GST-PRMT1 was used in a methylation assay in the presence of the methyl donor [^{14}C]SAM (*S*-adenosylmethionine) and GST-GAR (the glycine-arginine-rich domain of fibrillar) as substrate. Methylated proteins (GST-GAR band occurs as a triplet) were detected by autoradiography (for 16 h) following SDS-PAGE and blotting. *Right panel*, methylation assays were performed using the amount of GST-STAT1 as shown in the *left panel* and the active GST-PRMT1 enzyme preparation as employed for the experiment shown in the *middle panel* in the presence of [^{14}C]SAM. Autoradiography was performed for 2 weeks. The experiments were performed by Dr. Uta-Maria Bauer.

We further tested whether STAT3 could be a substrate for other PRMT family members, like PRMT2, -3, -4, and -6, but could not detect any *in vitro* methylation of STAT3 by these enzymes (Fig. 20C).

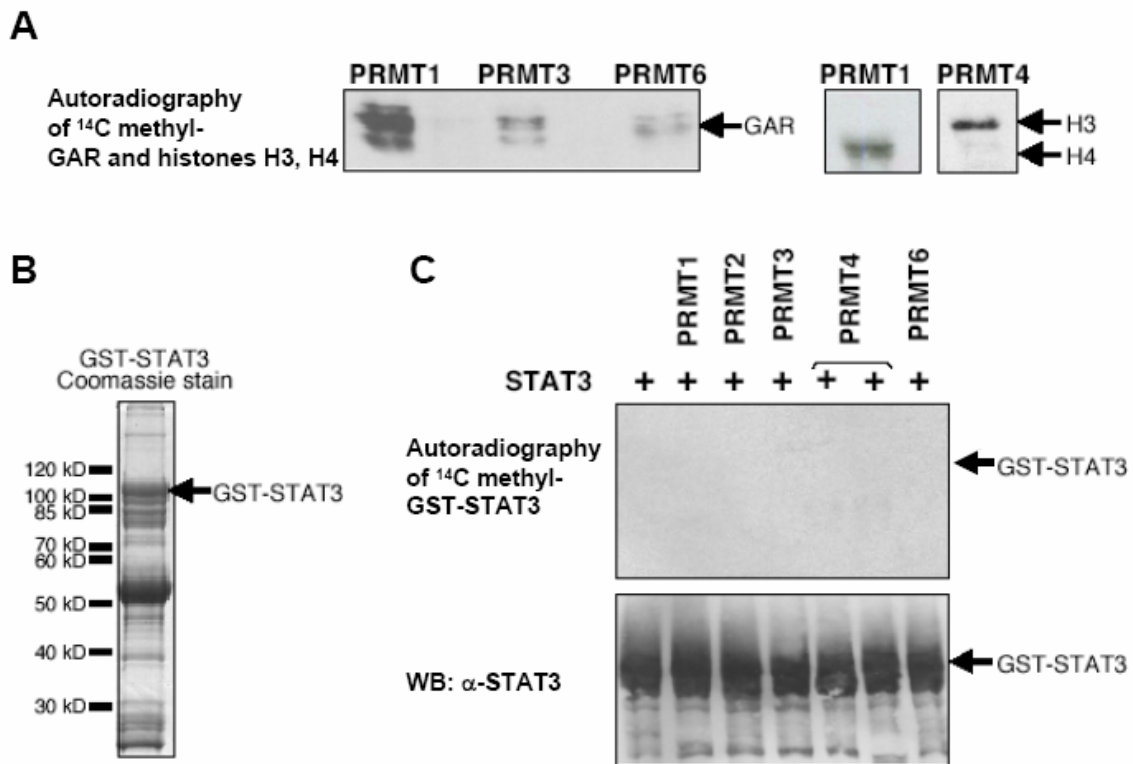


Figure 20. No evidence for *in vitro* methylation of GST-STAT3 by PRMTs.

(A) Recombinant GST-GAR and calf thymus histones, respectively, were *in vitro* methylated by GST-PRMT1, GST-PRMT3, GST-PRMT4 or GST-PRMT6 in the presence of ^{14}C -SAM. Autoradiography was performed for 16 hours. (B) The expression and purification of recombinant GST-STAT3 was analysed by SDS-PAGE and Coomassie-staining. (C) Methylation assays were performed using the amount of GST-STAT3 shown in B. and the active GST-PRMT enzyme preparation as used in A. (except for GST-PRMT2 there is no positive control available, since so far no substrate has been identified) in the presence of ^{14}C -SAM. Subsequently, methylation reactions were subjected to SDS-PAGE, blotting and autoradiography for 2 weeks. As loading control for GST-STAT3, the corresponding blot was stained with anti-STAT3 antibody. The experiments were performed by Dr. Uta-Maria Bauer.

Additionally, we examined whether co-expression of PRMT1 can affect STAT1-mediated gene transcription. For this purpose, we used the catalytic inactive form of PRMT1, which contains point mutations S69A, G70A, and T71A in the catalytic domain [192], as a negative control. We co-expressed PRMT1 or a mutant of PRMT1 lacking catalytic activity together with STAT1 in U3A cells lacking STAT1 along with a STAT1-responsive reporter construct. As shown in Fig. 21, the slight increase in reporter gene activity observed in cells co-expressing PRMT1 was similarly evident in cells expressing catalytically inactive PRMT1. Thus, it is unlikely that the methyl transferase activity of PRMT1 affects STAT function.

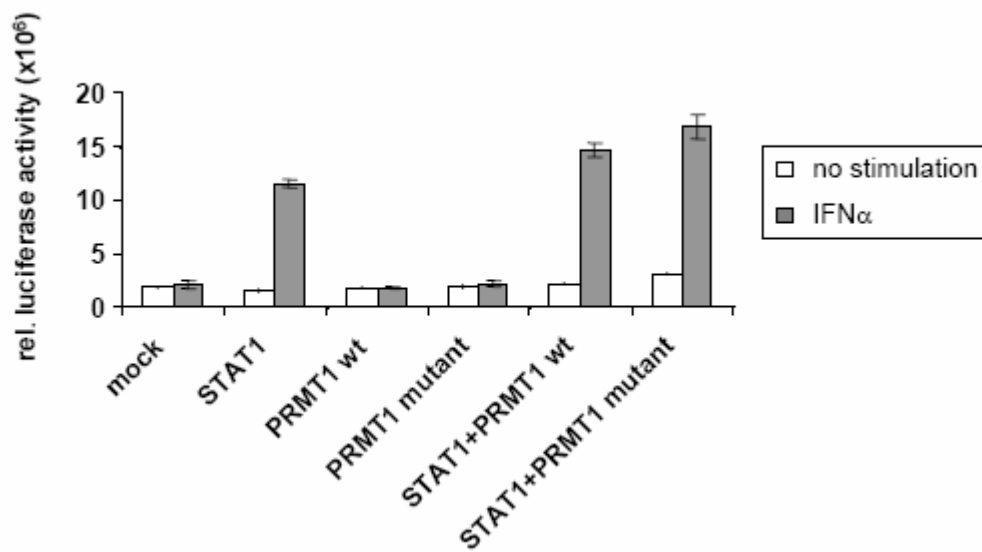


Figure 21. PRMT1 does not affect STAT1-controlled reporter gene activity.

U3A cells were cotransfected with a luciferase reporter gene construct under the control of the STAT1 responsive IRF1-promoter, a β -Gal expression vector to normalize for transfection efficiency, and expression vectors for STAT1, PRMT1 wild-type or a methyl-transferase negative mutant thereof, as indicated. Two days after transfection the cells were harvested for reporter gene assay.

In conclusion, our results do not confirm the occurrence of arginine methylation on STAT1 or STAT3 in cell culture or *in vitro*. Therefore, the data presented here question the validity of the model published by Mowen et al. [130]. In agreement with a report by Meissner et al. [193] we show additional and compelling evidences which suggest the lack of arginine methylation on STATs.

3.2 SOCS3 expression in melanoma cell lines

3.2.1 1286 melanoma cells are resistant to IL-6 and oncostatin M and constitutively express SOCS3

While studying cytokine effects on melanoma cells, I noticed that the growth of 1286 cells was not inhibited by treatment with IL-6/sIL-6R complexes or OSM. However, the same cytokines do dose- and time-dependently inhibit the growth of A375 cells (Fig. 22A and B; see also [27,57]).

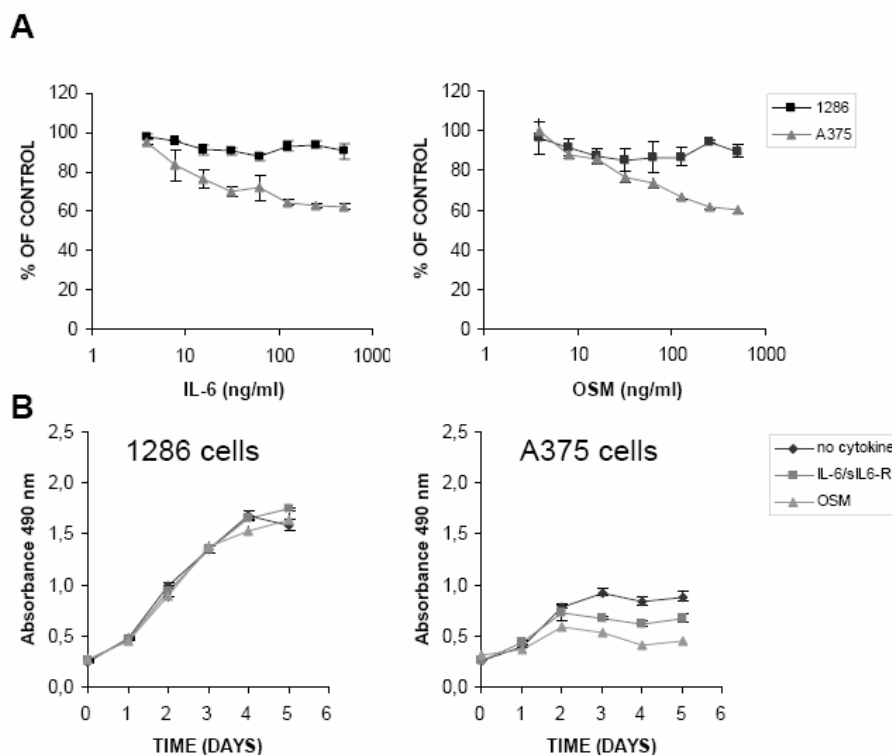


Figure 22. 1286 melanoma cells are resistant to cytokine-induced growth inhibition.

(A) Cells (initial density 3000 cells/well) were cultured for 4 days in the presence of different concentrations of cytokines. Cells were treated with IL-6 in the presence of soluble IL-6R (1 μ g/ml). Growth was assessed by an XTT test, values were re-calculated according to untreated controls. Error bars represent the standard deviation of triplicate samples. (B) Kinetics of growth inhibition. 2000 cells/well were seeded into 96-well plates and cultured for different periods of time in the presence of IL-6/sIL-6R (100 ng/ml and 1 μ g/ml, respectively), OSM (100 ng/ml), or with no cytokine added. Growth was assessed by an XTT test. Error bars represent the standard deviation of triplicate samples.

I next examined whether activation of the Jak/STAT signaling pathway by IL-6-type cytokines is defective in 1286 cells. As shown in Fig. 23A, IL-6 or OSM only induced a marginal STAT3 phosphorylation and no detectable STAT1 phosphorylation in 1286 (and in 888) cells. In contrast, a prominent STAT3 and STAT1 phosphorylation was readily observed

in A375 and WM9 cells. Moreover, 1286 cells demonstrate only a slight Jak1 phosphorylation after stimulation with OSM (Fig. 23B). Most interestingly, when monitoring SOCS3 as a target gene of IL-6-type cytokines, I noticed that 1286 (but not 888) cells constitutively expressed SOCS3 (Fig. 23A and B). Cytokine treatment did not enhance SOCS3 expression in 1286 cells, in contrast to WM9 and 729 cells, which showed a rapid and transient induction with a peak at 60 min (Fig. 23B). Here, the time course of SOCS3 induction correlates with the kinetics of inhibition of Jak1 and STAT3 activation.

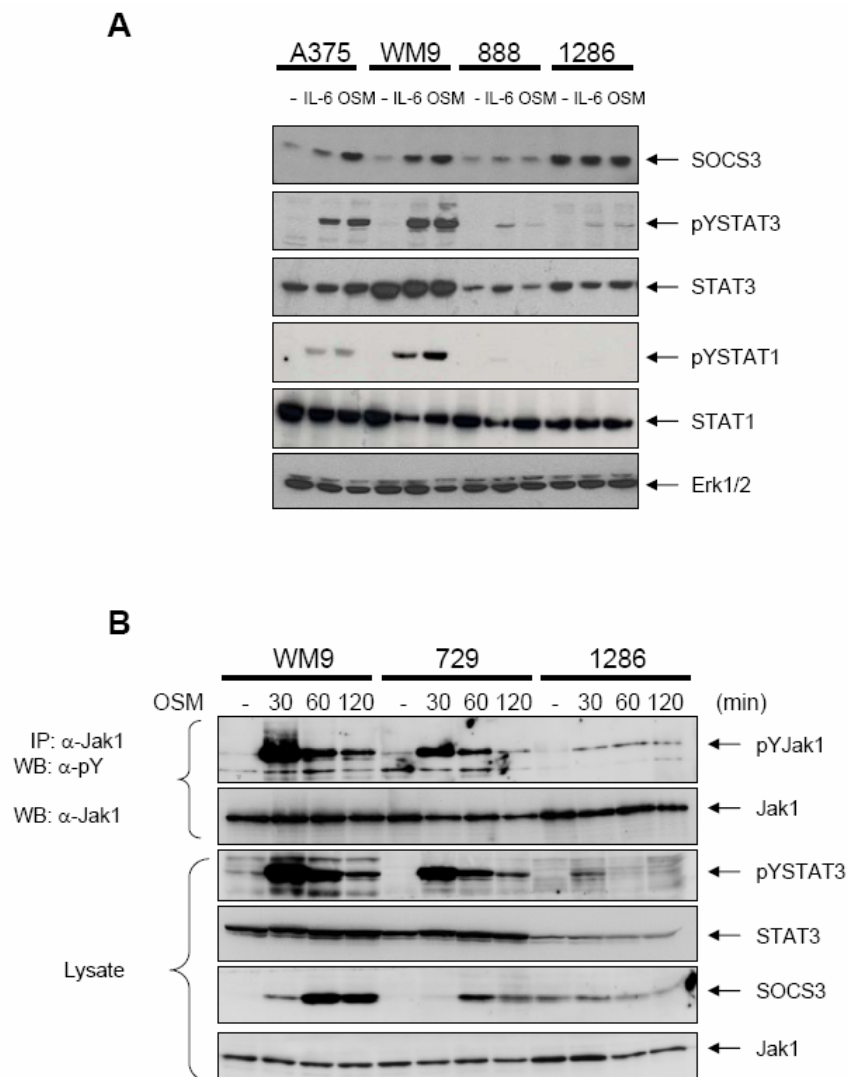


Figure 23. Reduced Jak/STAT signal transduction in 1286 cells.

(A) IL-6 and OSM hardly induce signaling events in 1286 melanoma cells. A375, WM9, 888 and 1286 melanoma cells were stimulated for 30 min with IL-6 (20 ng/ml) and sIL-6R (1 µg/ml) or with OSM (20 ng/ml) or left untreated. Total cellular lysates were analysed by Western blotting for the presence of phosphorylated STAT3, and STAT1, before re-probing of the blots with antibodies to STAT3, STAT1, Erk1/2 and SOCS3. (B) Time-dependence of SOCS3 induction and STAT3 activation in melanoma cells. WM9, 729 and 1286 melanoma cells were stimulated with OSM (10 ng/ml) for different periods of time, as indicated, or left untreated. Jak1 was immunoprecipitated from total cellular lysates, phosphorylation was analysed by staining the Western blot with PY99, before re-probing the blot with Jak1 antibody. Aliquots of the lysates were additionally analysed by Western blotting for the presence of phosphorylated STAT3, before re-probing of the latter blot with antibodies to Jak1, STAT3 and SOCS3.

Lack of receptor expression can contribute to cytokine resistance [147]. I therefore examined the surface expression of IL-6-type cytokine receptors by fluorescence-activated cell sorter (FACS) analysis. As shown in Fig. 24, the relevant receptors gp130 as well as the OSMR β (but not the LIFR, see [194]) were present at the surface of 1286 and A375 cells, although the expression of the OSMR in 1286 cells was somewhat lower than in A375 cells. Thus, impaired Jak/STAT activation in 1286 cells cannot be due to lack of receptor expression.

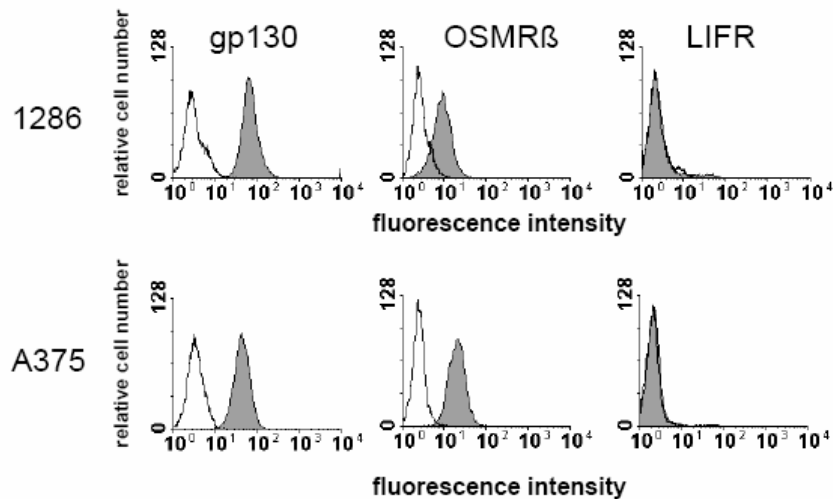


Figure 24. 1286 cells express surface gp130 and OSMR.

Cells were labeled using anti-gp130 monoclonal antibody, anti-OSMR monoclonal antibody, or anti-LIFR polyclonal antibody and the respective R-phycoerythrin-conjugated secondary antibodies (grey histograms). White histograms depict cells treated with secondary antibody alone.

Taken together, 1286 cells have constitutively high levels of SOCS3 and essentially do not respond to IL-6-type cytokines, neither by activation of the Jak/STAT pathway, nor by growth inhibition.

3.2.2 SOCS3 expression in 1286 cells is not due to an altered protein stability and/or a mutation in the coding sequence

To better understand the reason for the constitutive SOCS3 expression, the full-length cDNA of SOCS3 of 1286 cells was cloned and subjected to sequence analysis. No mutations were found (data not shown).

Since a prolonged half-life of SOCS3 could be the reason for the unusual high expression levels, I compared the half-life of SOCS3 protein of 1286 cells with the half-life of SOCS3 inducibly expressed in A375 cells. A375 cells were first stimulated with IL-6/sIL-6R for 60 min to induce the expression of SOCS3 protein. After IL-6 was removed, cells were then

treated with cycloheximide to block *de novo* protein synthesis. As shown in Fig. 25A, the half-life of SOCS3 protein of both cell lines was similar, approx. 2 hours, which is consistent with the reported half-life of this protein [195].

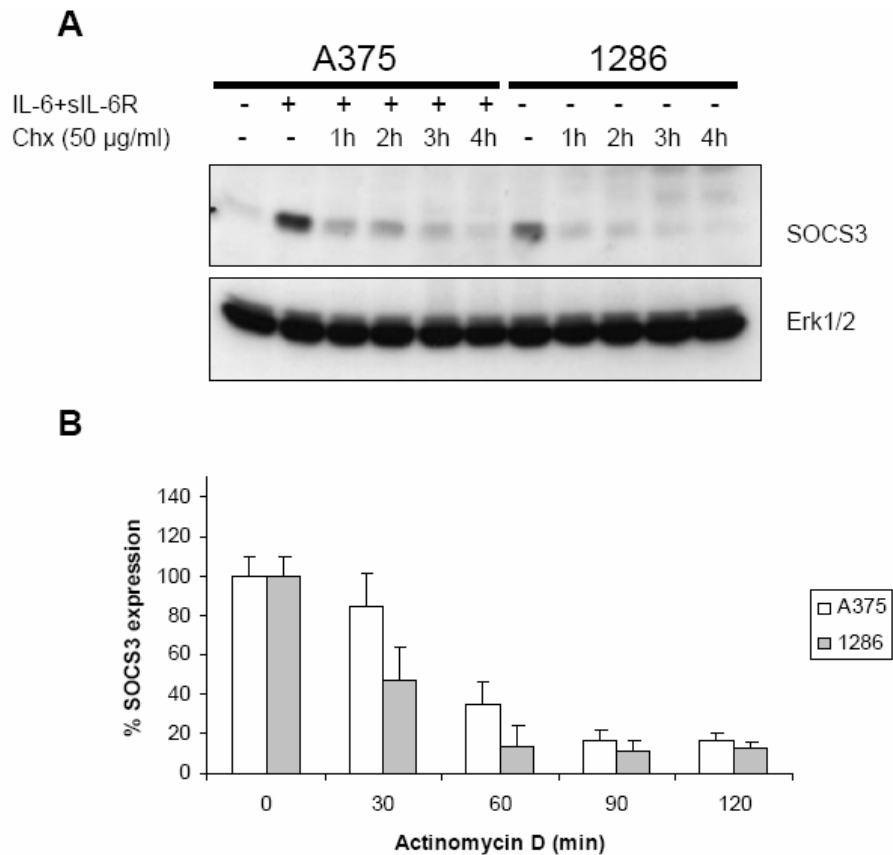


Figure 25. Analysis of SOCS3 protein stability and SOCS3 mRNA stability.

(A) Analysis of SOCS3 protein stability. A375 cells were stimulated for 60 min with IL-6 (20 ng/ml) and sIL-6R (1 µg/ml) to induce SOCS3 expression or left untreated. Thereafter, cells were washed and cultured in fresh medium. A375 and 1286 melanoma cells were then treated with cycloheximide (50 µg/ml) for the indicated periods of time. Total cellular lysates were analysed by Western blotting using α -SOCS3 antibody. The blot was re-probed with α -Erk1/2 antibody to confirm equal protein loading. **(B)** Analysis of SOCS3 mRNA stability. A375 or 1286 melanoma cells were treated with actinomycin D (4 µM) for different periods of time, as indicated, or left untreated. Total RNA was analysed by real-time PCR for expression of SOCS3 mRNA (normalized to mRNA levels of the β -actin housekeeping gene).

To compare the stability of SOCS3 mRNA of 1286 cells with that of A375 cells, cells were treated with actinomycin D to block *de novo* mRNA synthesis, and the decay of SOCS3 mRNA was monitored by real-time PCR. As shown in Fig. 25B, 30 min after treatment with actinomycin D, the level of SOCS3 mRNA of 1286 cells was 50% of the maximal level, indicating that SOCS3 mRNA of 1286 cells has a half-life of 30 min. In contrast, SOCS3 mRNA of A375 cells was significantly more stable than SOCS3 mRNA of 1286 cells (half-life of 50 min). Moreover, the decay of SOCS3 mRNA in A375 cells did not substantially differ from that observed in IL-6-stimulated HepG2 cells (data not shown). These data

implicate that there is a significant cell-specific difference in basal SOCS3 half-life between A375 cells and 1286 cells, albeit the reason for this remains unclear. However, these data indicate that a prolonged mRNA half-life cannot be the reason for the high SOCS3 expression observed in 1286 cells.

3.2.3 SOCS3 expression in 1286 cells does not seem to be STAT3 dependent

SOCS3 is normally expressed in response to STAT3 signaling. To investigate whether a putative low-level and thus for us undetectable STAT3 activity could be the reason for constitutive SOCS3 expression, we stably overexpressed wild-type STAT3 or a dominant-negative form of STAT3 (STAT3F) in 1286 cells. STAT3F contains a phenylalanine substitution of the tyrosine residue at position 705, which results in a reduction of tyrosine phosphorylation of wild-type STAT3 and inhibition of endogenous STAT3 activation [196,197]. As shown in Fig. 26, overexpression of STAT3F diminished IL-6-inducible STAT3 tyrosine phosphorylation compared to mock transfected cells. However, dominant negative STAT3 (STAT3F) did not affect SOCS3 expression in 1286 cells, suggesting that in these cells constitutive SOCS3 expression is mediated by signaling pathways independent of STAT3.

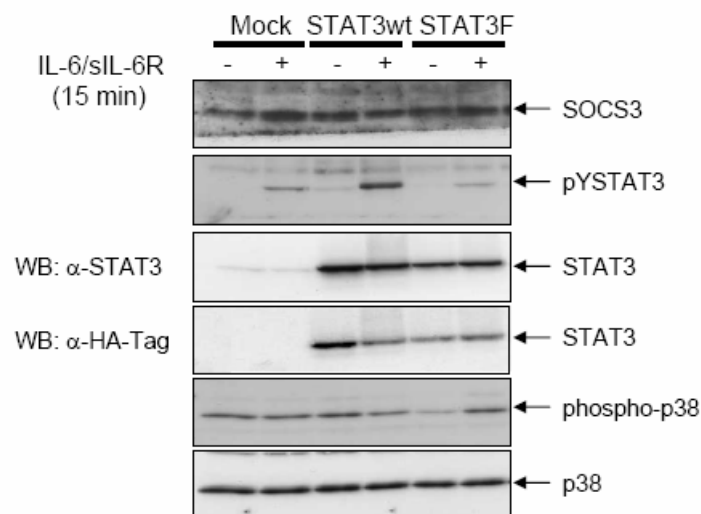


Figure 26. Dominant negative STAT3 does not abrogate the constitutive SOCS3 expression in 1286 melanoma cells.

1286 cells stably expressing STAT3wt, dominant negative STAT3F or mock transfectants were stimulated with IL-6 (20 ng/ml) and sIL-6R (1 μ g/ml) for 15 min or left untreated. Total cellular lysates were analysed by Western blotting for the presence of phosphorylated STAT3 and p38 before re-probing with antibodies against STAT3, p38, HA-tag and SOCS3.

3.2.4 MDA treatment, but not selective inhibition of p38 or Erk abolishes constitutive SOCS3 expression

Several lines of evidence suggest that activation of members of the MAPK family such as Erk1/2 or p38 MAPK cascades are involved in the regulation of SOCS3 expression [198-200]. Therefore, it was investigated whether blocking MAPK signaling pathways by the selective inhibitors was able to block SOCS3 expression in 1286 cells. As shown in Fig. 27, treatment of cells with the MEK inhibitor U0126, which inhibits upstream of Erks, completely inhibited the phosphorylation of Erk1/2 but had no effect on SOCS3 expression. Similarly, treatment with the p38-specific inhibitor SB202190 did not result in down-regulation of SOCS3 expression. It should be noted that the SB202190 inhibitor at 10 μ M also inhibited the phosphorylation of Erk1/2. Taken together, these results suggest that neither Erk activity nor p38 activity are crucial for the constitutive SOCS3 expression in 1286 cells.

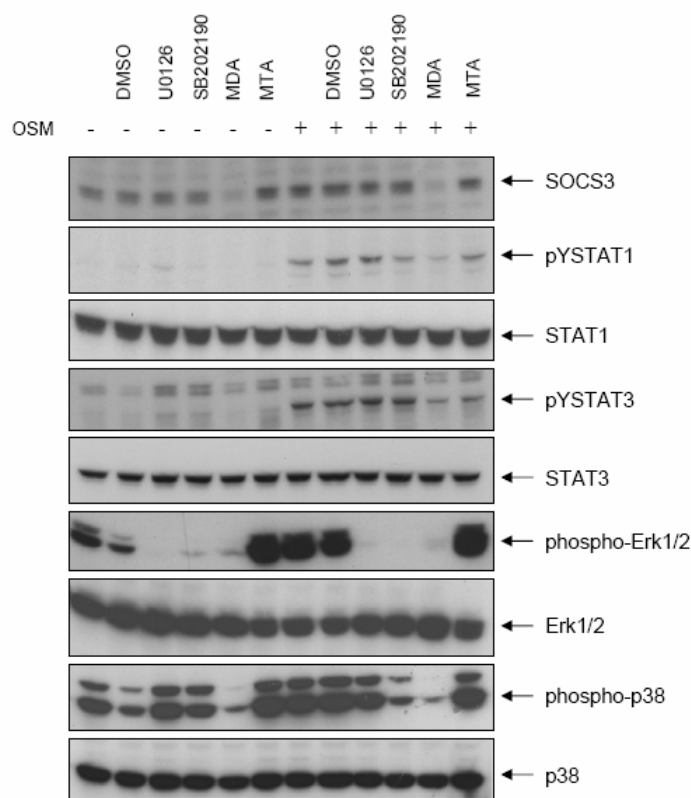


Figure 27. MDA treatment, but not selective inhibition of p38 or Erk abolishes constitutive SOCS3 expression.

1286 melanoma cells were pretreated with DMSO alone or with 10 μ M U0126 or 10 μ M SB202190 for 30 min, with 0.3 mM MDA inhibitor cocktail for 3 h or with 0.3 mM MTA for 18 h prior to stimulation with OSM (20 ng/ml) for 60 min. Total cellular lysates were analysed by Western blotting for the presence of phosphorylated STAT1, STAT3, p38, and Erks before re-probing the blots with antibodies to STAT1, STAT3, p38, Erk1/2 and SOCS3.

Previously, I have shown that treatment with the MDA methylation inhibitor cocktail could abrogate inducible SOCS3 expression in WM9 cells (see Fig. 12). Thus, it was tested whether MDA treatment can also abrogate constitutive SOCS3 expression in 1286 cells. As expected, MDA treatment attenuated the expression of SOCS3 (Fig. 27, *first panel, lanes 5 and 11*). Moreover, real-time PCR analysis indicated that MDA suppressed the expression of SOCS3 mRNA in 1286 cells (data not shown). However, I did not observe a downregulation of SOCS3 expression by treatment with MTA, another methylation inhibitor, suggesting that the observed effects of MDA treatment are unrelated to protein arginine methylation. Thus, the molecular basis for constitutive SOCS3 expression in 1286 cells remains to be elucidated.

3.2.5 Suppression of SOCS3 expression breaks cytokine resistance of 1286 cells

To better understand the correlation between SOCS3 expression and cytokine resistance, SOCS3 protein expression was suppressed by a short interfering RNA (siRNA) approach. As shown in Fig. 28A, transfection of 1286 cells with SOCS3-siRNA almost completely abrogated SOCS3 mRNA expression as measured by RT-PCR. The effect of SOCS3-siRNA is target gene specific, as transfection of SOCS3-siRNA only affected the expression of SOCS3, and not that of SOCS6 and of CIS. Moreover, constitutive SOCS3 protein expression was significantly reduced (Fig. 28B).

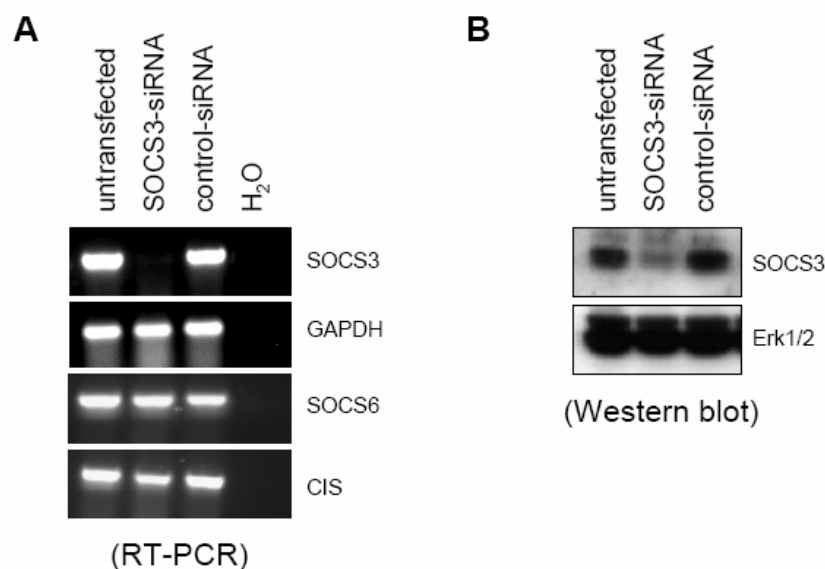


Figure 28. Suppression of SOCS3 expression by siRNA.

SOCS3 expression was suppressed using siRNA transfected with jetSI ENDO transfection reagent. **(A)** Knockdown efficiency at the mRNA level was verified by RT-PCR after 24 h. GAPDH: glyceraldehyde-3-phosphate dehydrogenase, CIS: cytokine-inducible SH2-containing protein. **(B)** Knockdown efficiency at the protein level was verified by Western blotting after 24 h.

I next investigated signaling events in 1286 cells treated with SOCS3-siRNA or with scrambled control-siRNA. As shown in Fig. 29A, a slight increase in IL-6- and OSM-mediated STAT3 phosphorylation was observed in SOCS3-suppressed cells. However, more strikingly, I consistently observed a very strong STAT1 phosphorylation upon treatment with IL-6 and OSM, compared to cells that had been treated with control-siRNA or that remained untransfected. The increased phosphorylation of STAT1 upon SOCS3 suppression correlated with a strong STAT1 DNA binding activity in the gel shift assay (Fig. 29B).

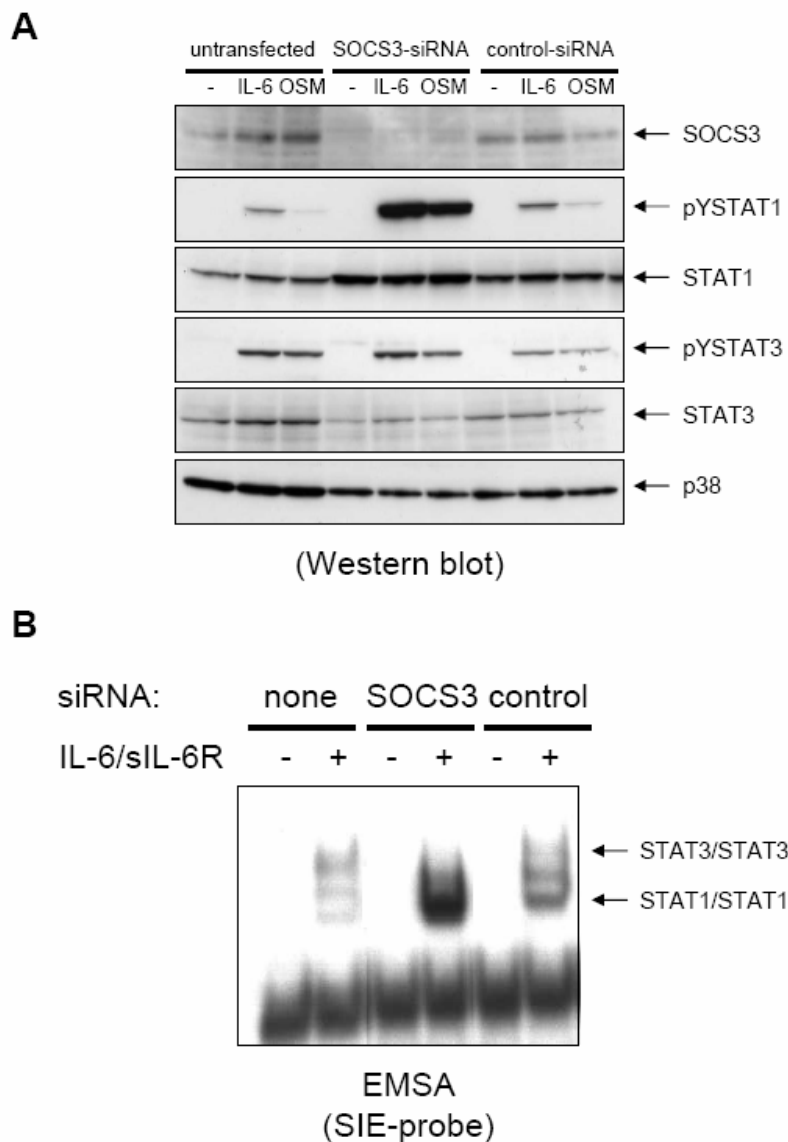


Figure 29. Suppression of SOCS3 expression by SOCS3-siRNA increases cytokine-induced STAT1 and STAT3 activation in 1286 cells.

(A) 1286 melanoma cells were transfected with 50 nM SOCS3-siRNA or control-siRNA for 24 hours and then stimulated for 15 min with IL-6 (20 ng/ml) and sIL-6R (1 μ g/ml) or with OSM (10 ng/ml) or left untreated. Total cellular lysates were analysed by Western blotting for the presence of phosphorylated STAT1 and STAT3 before re-probing of the blots with antibodies to STAT1, STAT3, p38 and SOCS3. (B) 1286 cells were transfected with 50 nM SOCS3-siRNA or control-siRNA for 24 hours and then stimulated for 15 min with IL-6 (20 ng/ml) and sIL-6R (1 μ g/ml) or left untreated. Nuclear extracts were analysed by EMSA for binding activities to the SIE probe.

It was then investigated whether downregulation of SOCS3 expression would break the resistance of these cells towards the growth inhibitory effect of cytokines. 24 hours after siRNA transfection, cells were treated with IL-6 in the presence of soluble IL-6R, and cell growth was assessed 3 days later by an XTT assay (Fig. 30A). Strikingly, SOCS3 suppression increased the growth inhibitory effect significantly: cells treated with control-siRNA were not affected by the cytokine, as shown previously for the untransfected cells (see Fig. 22A). In contrast, cells treated with SOCS3-siRNA were dose-dependently inhibited by IL-6 treatment (Fig. 30A). Similarly, upon SOCS3 suppression, the cytokine sensitivity was also evident in the time-course experiment shown in Fig. 30B and C. I consistently observed values between 20 and 40% of growth inhibition compared with untreated control cells. I often also noted that SOCS3-suppressed cells grew slower than control-siRNA treated cells, even when no cytokine was added (see Fig. 30B).

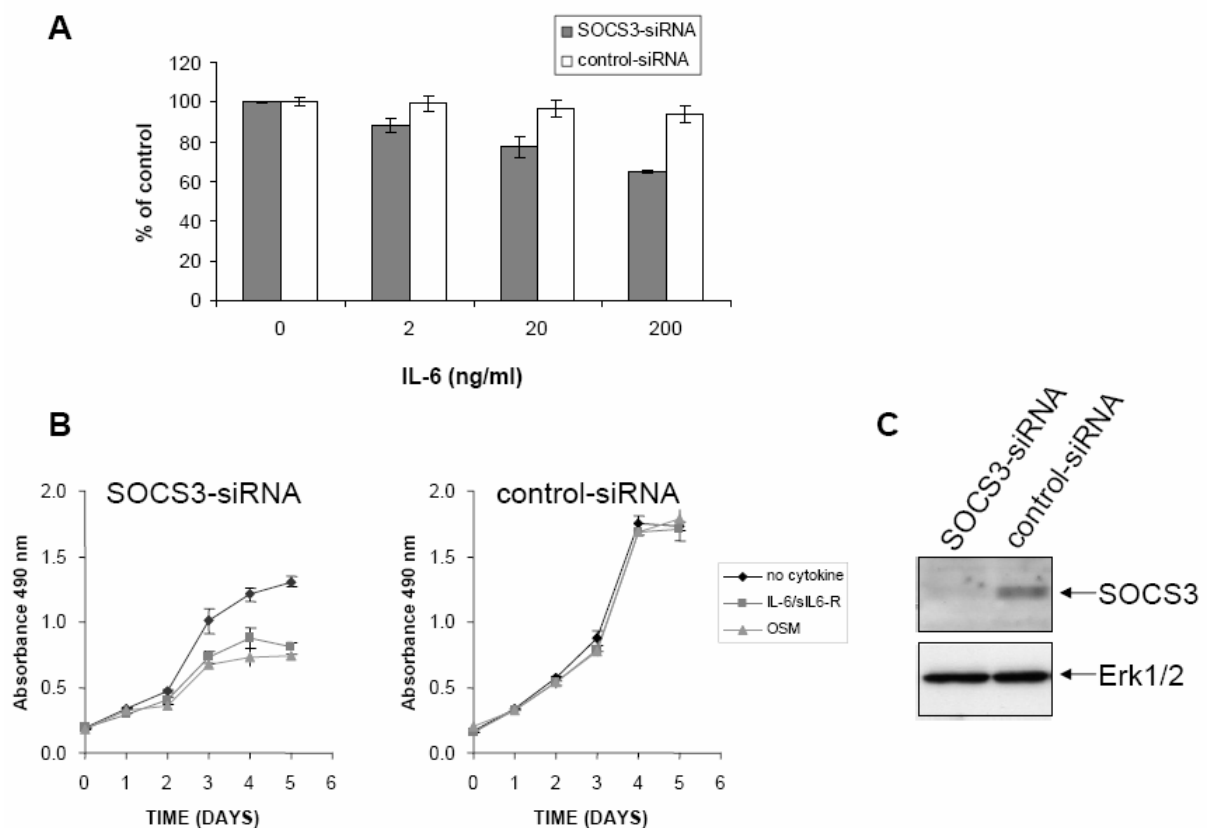


Figure 30. Suppression of SOCS3 in 1286 cells leads to an enhanced sensitivity towards IL-6 and OSM.

(A) 1286 cells were transfected with 50 nM SOCS3-siRNA or control-siRNA. 24 hours after transfection cells were cultured for 3 days in the presence of different concentrations of IL-6 in combination with sIL-6R (1 μ g/ml) or with no cytokine added. Growth was assessed by an XTT test, values were re-calculated according to untreated controls. Error bars represent the standard deviation of triplicate samples. (B) 1286 cells were transfected with 50 nM SOCS3-siRNA or control-siRNA for 24 hours, 2000 cells/well were seeded into 96-well plates and cultured for different periods of time in the presence of IL-6/sIL-6R (100 ng/ml and 1 μ g/ml, respectively), OSM (100 ng/ml), or with no cytokine added. Growth was assessed by an XTT test. Error bars represent the standard deviation of triplicate samples. (C) SOCS3 suppression of the cells used in B. was still evident in Western blot analysis after 4 days of culture.

Two other siRNA oligonucleotides (SOCS3-1 and SOCS3-2) were less effective than SOCS3-3 siRNA, which was used in all experiments shown here. Interestingly, their reduced effect on SOCS3 suppression was paralleled by a weaker “restoration” of STAT phosphorylation (Fig. 31A) and growth inhibition (Fig. 31B), indicating that SOCS3 confers cytokine resistance in a dose-dependent manner.

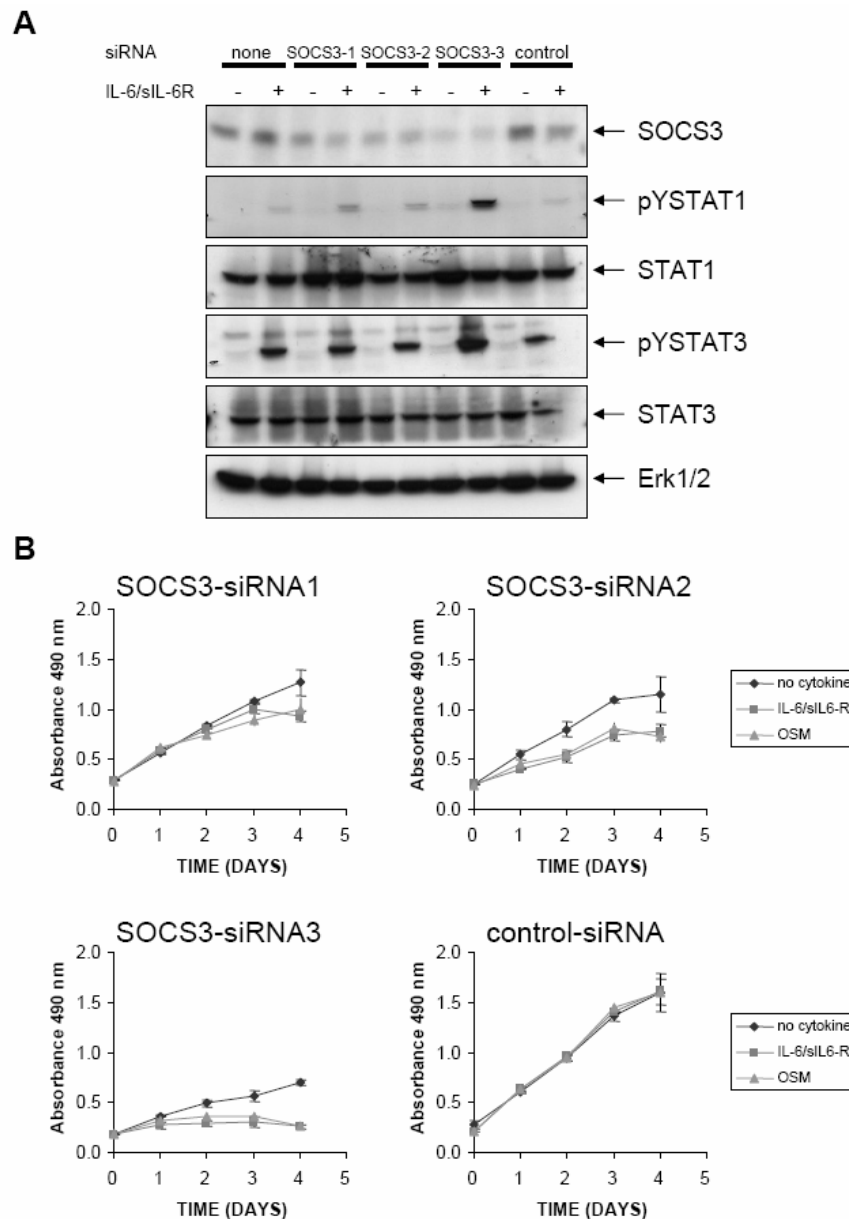


Figure 31. SOCS3 suppression enhances IL-6- and OSM-mediated growth inhibition of 1286 melanoma cells.

(A) 1286 cells were transfected with 50 nM SOCS3-siRNA1, 2 or 3 or control-siRNA for 24 hours and then stimulated for 15 min with IL-6 (20 ng/ml) and sIL-6R (1 μ g/ml) or left untreated. Total cellular lysates were analysed by Western blotting for the presence of phosphorylated STAT3 and STAT1 before re-probing of the blots with antibodies to STAT3, STAT1, Erk1/2 and SOCS3. (B) 1286 cells were transfected with 50 nM SOCS3-siRNA1, 2 or 3 or with control-siRNA for 24 hours and 2000 cells/well were seeded into 96 well plates and cultured for different periods of time in the presence of IL-6/sIL-6R (100 ng/ml and 1 μ g/ml, respectively), OSM (100 ng/ml), or with no cytokine added. Growth was assessed by an XTT test. Error bars represent the standard deviation of triplicate samples.

3.2.6 Suppression of inducible SOCS3 expression increases the growth inhibitory effect of IL-6-type cytokines in sensitive WM239 cells

To address the question whether SOCS3 suppression could also enhance cytokine responsiveness in sensitive melanoma cells, WM239 cells were transfected with SOCS3-siRNA or with control-siRNA. These cells strongly express SOCS3 after cytokine treatment (Fig. 32A). SOCS3-siRNA reduced inducible SOCS3 expression (Fig. 32A). As previously observed in 1286 cells (see Fig. 29A, B), SOCS3 suppression coincided with prominent STAT1 phosphorylation (Fig. 32A). Interestingly, suppression of inducible SOCS3 enhanced the growth inhibitory effect of IL-6/sIL-6R and of OSM (Fig. 32B). Even without cytokine treatment control cells grew better than SOCS3-suppressed cells. Thus, SOCS3 expression also promotes growth in sensitive melanoma cells.

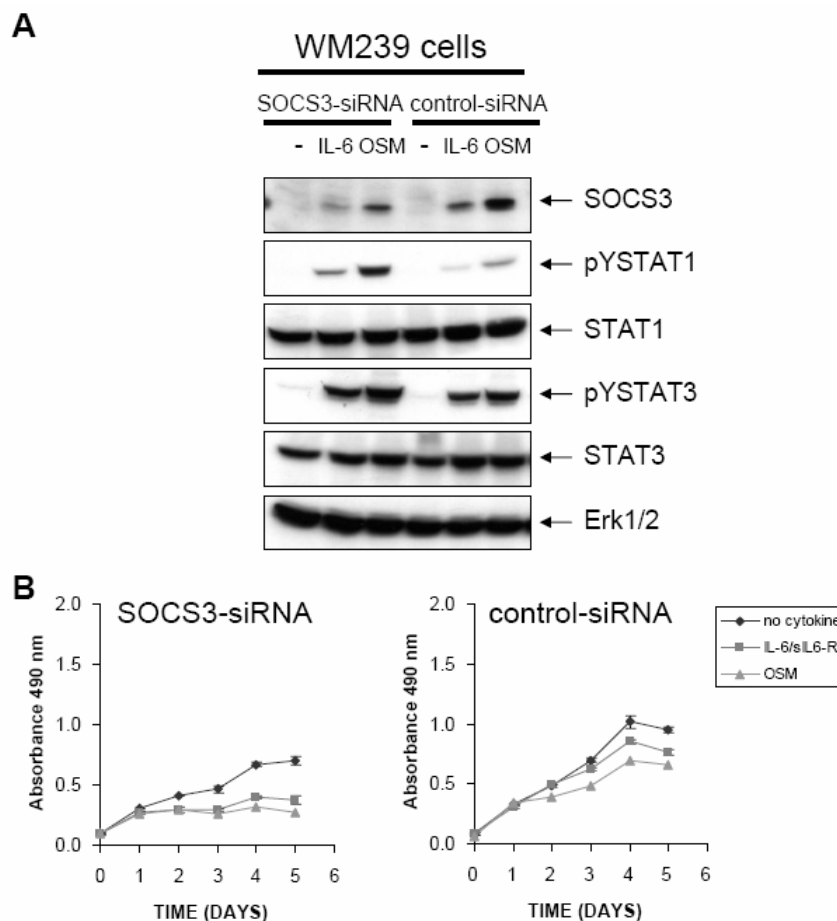


Figure 32. Suppression of inducible SOCS3 expression increases cytokine sensitivity of WM239 cells.

(A) WM239 cells were transfected with 50 nM SOCS3-siRNA or control-siRNA for 24 hours and then stimulated for 60 min with IL-6 (20 ng/ml) and sIL-6R (1 μ g/ml), or with 20 ng/ml OSM or left untreated. Total cellular lysates were analysed by Western blotting for the presence of phosphorylated STAT1 and STAT3 before re-probing of the blots with antibodies to STAT1, STAT3, Erk1/2 and SOCS3. (B) WM239 cells were transfected with 50 nM SOCS3-siRNA or control-siRNA. 24 hours after transfection 2000 cells/well were seeded into 96-well plates and cultured for different periods of time in the presence of IL-6/sIL-6R (100 ng/ml and 1 μ g/ml, respectively), OSM (100 ng/ml), or with no cytokine added. Growth was assessed by an XTT test. Error bars represent the standard deviation of triplicate samples.

3.2.7 SOCS3 suppression does not break resistance of 1286 cells against IFN- α

Type I IFNs play an important role in adjuvant melanoma therapy but only 15% of the patients benefit from a treatment [5]. The reasons for resistance of most patients are not well understood. Therefore, it was investigated whether SOCS3 expression in 1286 cells might also contribute to their resistance against IFN- α . As shown in Fig. 33, suppression of SOCS3 expression enhanced the growth inhibitory effect of IL-6/sIL-6R. However, suppression of SOCS3 expression did not sensitize 1286 cells against the growth inhibitory effect of IFN- α (Fig. 33) and did not enhance IFN- α -mediated STAT1 phosphorylation (Fig. 34). This indicates that constitutive SOCS3 is not the reason for IFN- α resistance of these cells.

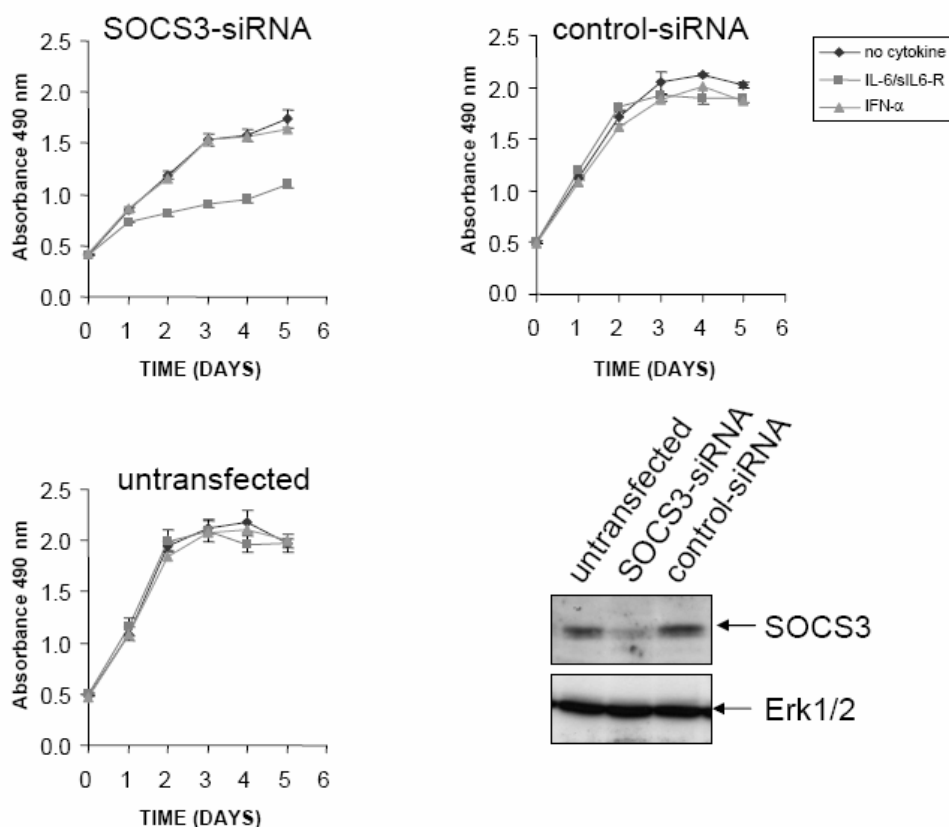


Figure 33. Suppression of SOCS3 does not break resistance of 1286 cells against IFN- α .

1286 cells were transfected with 50 nM SOCS3-siRNA or control-siRNA. 24 hours after transfection 2000 cells/well were seeded into 96-well plates and cultured for different periods of time in the presence of IL-6/sIL-6R (100 ng/ml and 1 μ g/ml, respectively), IFN- α (5000 U/ml) or with no cytokine added. Growth was assessed by an XTT test. Error bars represent the standard deviation of triplicate samples. SOCS3 suppression of the cells used for proliferation assay was still evident in Western blot analysis after 4 days of culture.

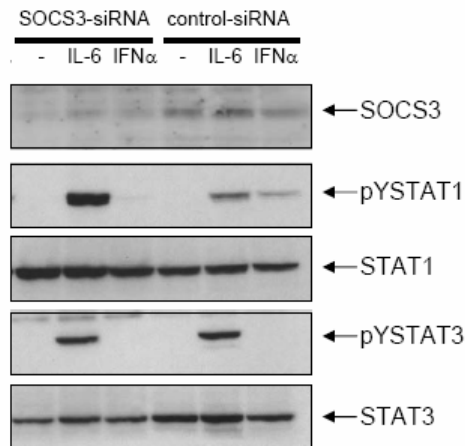


Figure 34. Suppression of SOCS3 does not break resistance of 1286 cells against IFN- α .

1286 cells were transfected with 50 nM SOCS3-siRNA or control-siRNA for 24 hours and then stimulated for 15 min with IL-6 (20 ng/ml) and sIL-6R (1 μ g/ml) or with IFN- α (1000 U/ml) or left untreated. Total cellular lysates were analysed by Western blotting for the presence of phosphorylated STAT1 and STAT3 before re-probing of the blots with antibodies to STAT1, STAT3 and SOCS3.

3.2.8 SOCS3 suppression slightly increased the surface expression of both gp130 and OSMR

To investigate possible explanations for the increased STAT signaling upon IL-6 and OSM stimulation in SOCS3-suppressed cells, I examined the surface expression of IL-6-type cytokine receptors in cells transfected with SOCS3-siRNA or with control-siRNA by FACS analysis. Interestingly, SOCS3 suppression slightly increased the surface expression of both gp130 and OSMR (10-20%, median fluorescence channel), but not of LIFR (Fig. 35).

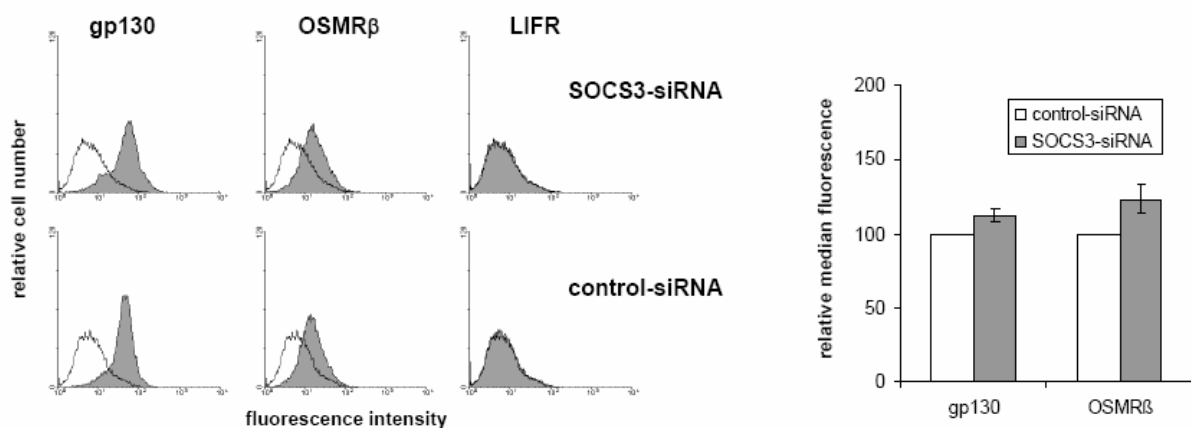


Figure 35. SOCS3 suppression slightly increased the surface expression of gp130 and OSMR.

1286 cells were transfected with 50 nM SOCS3-siRNA or control-siRNA. 24 hours after transfection cells were labeled using anti-gp130 monoclonal antibody, anti-OSMR monoclonal antibody, or anti-LIFR polyclonal antibody and the respective R-phycoerythrin-conjugated secondary antibodies (grey histograms). White histograms depict cells treated with secondary antibody alone. In the diagram (right panel), the gray bar represents the median fluorescence of cells transfected with SOCS3-siRNA relative to the median fluorescence of cells transfected with control-siRNA (white bar), which was set to 100%. The mean values and S.D. of at least three different experiments are depicted.

3.2.9 IL-6 does not elicit a full-blown IFN- γ -like response in SOCS3-suppressed 1286 cells

It has previously been described that SOCS3 can influence the pattern of STAT activation: in the absence of SOCS3, it was observed that macrophages, hepatocytes and murine embryonic fibroblasts respond to IL-6 with a prolonged STAT1 activation (in addition to a prolonged STAT3 activation) [114,201,202], which led to the expression of IFN- γ -responsive genes [114,201]. I next examined whether the excessive phosphorylation of STAT1 induced by IL-6 could also induce an IFN- γ -like response in SOCS3-suppressed 1286 cells. 24 hours after siRNA transfection, cells were treated with IL-6/sIL-6R or IFN- γ for 3 days, and MHC class I surface expression, a hallmark of an IFN- γ response, was assayed by FACS analysis. As shown in Fig. 36, IL-6 stimulation did not lead to an upregulation of MHC class I expression, even in cells treated with SOCS3-siRNA, suggesting that IL-6 does not elicit a full-blown IFN- γ -like response in SOCS3-suppressed 1286 cells.

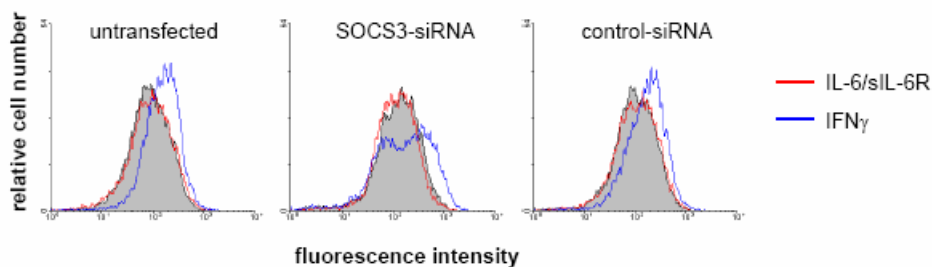


Figure 36. IL-6 does not elicit a full-blown IFN- γ -like response in SOCS3-suppressed 1286 cells.

1286 cells were transfected with 50 nM SOCS3-siRNA or control-siRNA. 24 hours after transfection cells were stimulated with IL-6 (20 ng/ml) and sIL-6R (1 μ g/ml) or with IFN- γ (1000 U/ml) for 3 days, and MHC-I expression was assayed by FACS analysis. Histograms resulting from unstimulated cells stained with the MHC-I-specific antibody are shown in *gray*; those from stimulated cells are shown as a *red line* (IL-6/sIL-6R) or a *blue line* (IFN- γ).

3.2.10 Enhanced SOCS3 expression is not a general phenomenon of melanoma cells *in vitro*

To further assess whether a constitutive SOCS3 expression is frequently observed in melanoma, I analysed nine additional melanoma cell lines and normal human epidermal melanocytes (NHEM). As shown in Fig. 37A, 1286 cells clearly expressed the highest levels of SOCS3 protein. For A375, WM9, and 888 cells, I sometimes, but not consistently, observed a faint signal in the Western blot (see also Fig. 23).

I next examined the expression of SOCS3 mRNA by RT-PCR. Interestingly, most of the cell lines expressed SOCS3 mRNA in a constitutive manner, although SOCS3 mRNA expression was barely detectable in Mel Im cells (Fig. 37B). These data indicate that the SOCS3 gene is not generally silenced in melanoma cells. Quantification of SOCS3 expression by real-time PCR confirmed highest mRNA expression in 1286 cells (Fig. 37C). Compared with melanocytes, 1286 cells express nine times more SOCS3 mRNA. Other cell lines, including A375 and WM9, showed a 2- to 3-fold increase in SOCS3 expression. Three cell lines (WM239, 1287 and Mel Im) expressed lower SOCS3 mRNA levels than melanocytes. Taken together, 1286 cells show highest expression levels of SOCS3, both on protein and on mRNA levels. However, such a high expression does not seem to be a general phenomenon of melanoma cells.

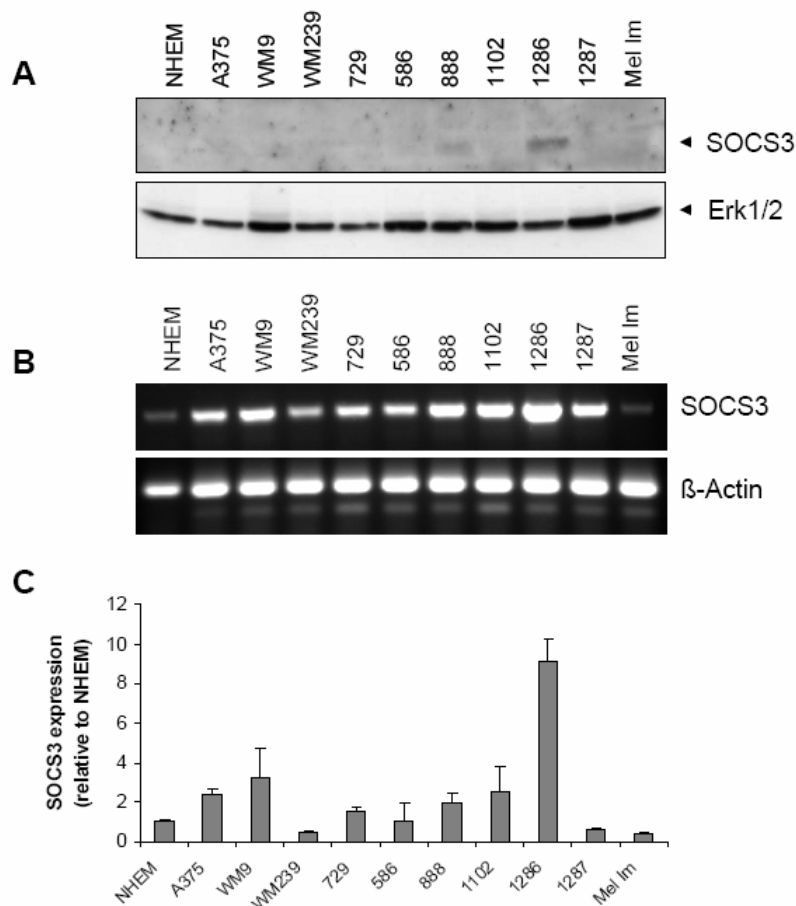


Figure 37. SOCS3 expression in melanoma cell lines.

(A) Western blot analysis of SOCS3 protein expression in melanoma cell lines and NHEM. Whole-cell lysates were subjected to Western blotting using α -SOCS3 antibody. The blot was re-probed with α -Erk1/2 antibody to confirm equal protein loading in each sample. (B) Analysis of constitutive expression of SOCS3 by RT-PCR. Total RNA from NHEM and ten human melanoma cell lines was reversely transcribed and amplified with SOCS3 primers. (C) The relative SOCS3 mRNA levels were determined in cDNA samples by quantitative real-time PCR as described in *Materials and Methods*. The indicated relative gene expression shows expression levels that were normalised to β -actin expression as a standard. Expression levels were determined in three independent experiments. Bars represent the mean \pm SD.

3.2.11 SOCS3 mRNA expression does not correlate with cytokine sensitivity

To address the question whether the level of SOCS3 expression correlates with cytokine sensitivity, the growth inhibitory effect of IL-6, OSM and IFN- α was assessed. As shown in Fig. 38, two cell lines (WM239 and A375) were sensitive to OSM treatment, whereas 586 cells were sensitive to IFN- α treatment. However, there is no clear-cut correlation between SOCS3 mRNA levels and sensitivity to the various cytokines. This indicates that sensitivity of cells to cytokines can be determined by various other factors, including receptor expression.

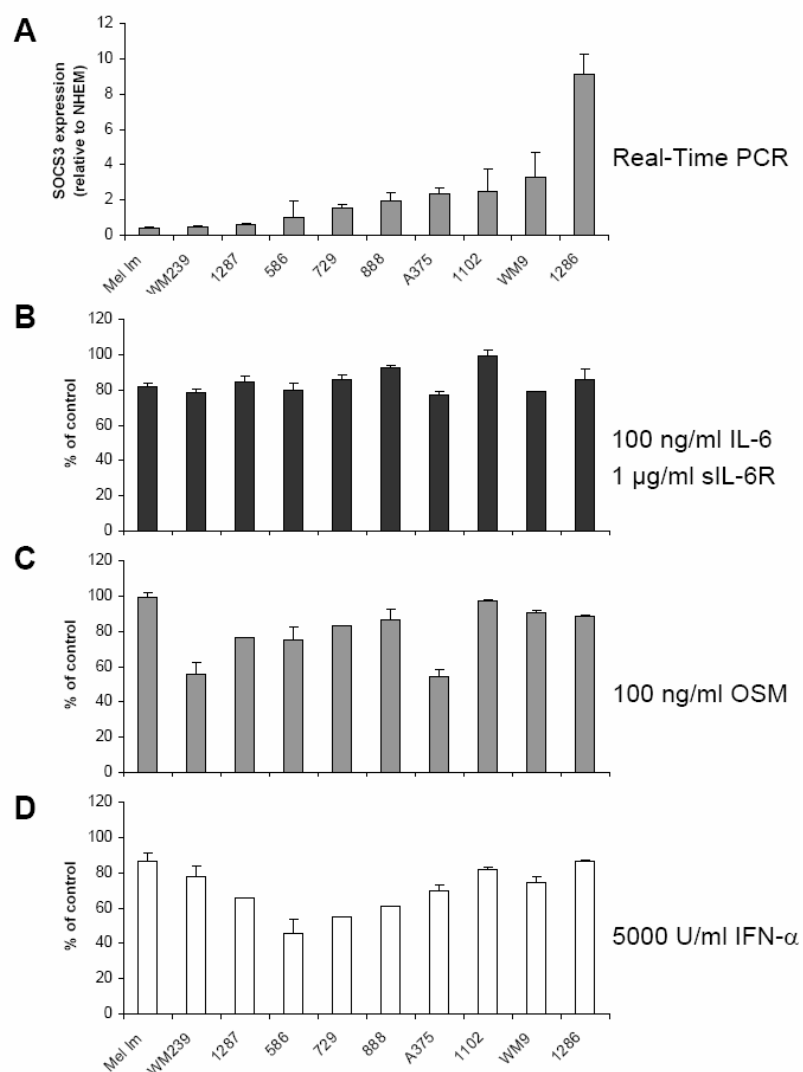


Figure 38. SOCS3 mRNA expression in melanoma cells does not correlate with cytokine sensitivity.

(A) Real-time PCR data shown in (Fig. 37C) are now shown according to SOCS3 mRNA levels. (B-D) Melanoma cells (initial density 3000 cells/well) were cultured for 4 days in the presence of (B) IL-6/sIL-6R (100 ng/ml and 1 μ g/ml, respectively), (C) OSM (100 ng/ml), (D) IFN- α (5000 U/ml) or with no cytokine added. Growth was assessed by an XTT test, values were re-calculated according to untreated controls. Error bars represent the standard deviation from the mean of at least three independent experiments.

3.2.12 SOCS3 immunoreactivity in human melanoma *in situ*

To further investigate whether SOCS3 is expressed in melanoma cells *in situ*, and thus, could mediate IL-6 resistance *in vivo*, we performed immunohistochemical studies on a limited number of human melanoma specimens (n=10) derived from early and advanced primary cutaneous melanomas (n=8) as well as melanoma metastases (n=2). SOCS3 immunoreactivity was detectable in three primary cutaneous melanomas (Clark levels II, III, and IV) as a fine granular cytoplasmic staining of the tumor cells (Fig. 39A and C). The detected SOCS3 immunostaining was not uniformly present in all tumor cells but was confined to cell clones, sometimes located at the periphery of the tumor. In contrast, sections incubated with isotype control IgG did not reveal any immunostaining (Fig. 39B and D). In 4 out of 10 samples, some SOCS3 immunoreactivity was also detectable in basal epidermal keratinocytes, and in 8 out of 10 samples, it was also in selected skin appendages, i.e. in secretory epithelia of eccrine glands and in hair follicle epithelia (data not shown), suggesting that SOCS3 expression is not a melanoma-specific marker.

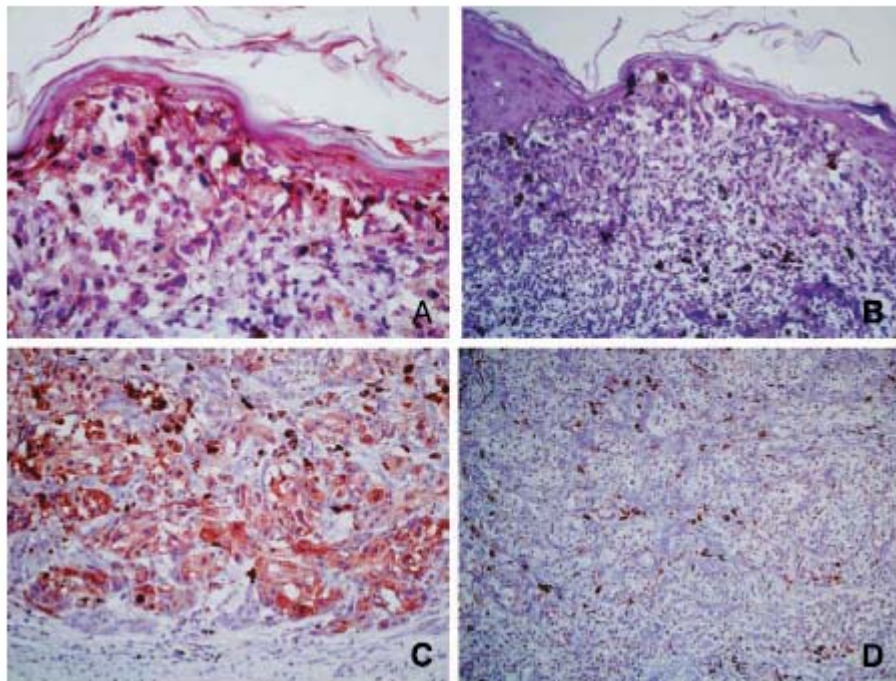


Figure 39. Detection of SOCS3 immunoreactivity in human melanoma *in situ*.

Deparaffinized tissue sections were stained with a polyclonal anti-SOCS3 antibody and bound antibodies were visualized by the immunoperoxidase technique. (A) SOCS3 immunostaining of a primary cutaneous melanoma (Clark level III). Note intense staining of some tumor cells but absence of staining of others (original magnification, x 200). (B) Negative control of (A; original magnification, x 100). (C) Primary cutaneous melanoma (Clark level IV). Note the SOCS3 immunoreactive tumor cell nest, whereas other tumor cells remain negative. The brown staining in contrast is due to melanin (original magnification, x 200). (D) Negative control of (C; original magnification, x 100). The experiments were performed by Dr. Markus Böhm and Dr. Dieter Metzger.

4 Discussion

4.1 Methylation of STATs

Transcription factors of the STAT family are important in signal transduction of cytokines. They are subject to post-translational modification by phosphorylation on tyrosine and serine residues upon activation. Although phosphorylation is a key regulatory modification of STAT factors, other post-translational modifications including acetylation, sumoylation, isgylation, ubiquitination, and methylation have been reported [203]. Lysine acetylation of STAT1 at residues 410 and 413 has recently been demonstrated and is believed to regulate the activity of NF- κ B [204]. Lysine acetylation of STAT3 at residue 685 has been reported to play an important role for STAT3 to form stable dimers and to activate transcription [205,206].

Previously, Mowen et al. have reported that STAT1 is methylated on a conserved arginine residue (Arg31) within the N-terminal region [130]. STAT1 arginine methylation has been described to be important for its function since it suppresses the interaction with the negative regulatory protein PIAS1, and loss of arginine methylation was discussed to be involved in IFN resistance of cancer cells. Similarly, arginine methylation of STAT6 has been reported to be important for STAT6 activity [132]. Mostly, studies describing methylation of STATs have made use of antibodies directed against methylarginine, STAT mutants, and inhibitors of methylation: methylthioadenosine (MTA) [130], or *N*-methyl-2-deoxyadenosine (MDA) in the presence of adenosine and DL-homo-cysteine [131,132]. In this thesis, I provide several lines of evidence questioning the previously proposed arginine methylation of STATs.

Mowen et al. used the dimethylarginine-specific antibody for immunoprecipitations to demonstrate methylation of STAT1 *in vivo*. In addition, STAT3 has been shown to be recognized by an anti-dimethylarginine-specific antibody [180]. Here it is demonstrated that certain STAT antibodies could apparently recognize proteins that were precipitated by anti-dimethylarginine-specific antibodies but that were different from STAT1 or STAT3, since they were also observed in cells lacking STAT1 and STAT3, respectively (Fig. 9). The reason for this unexpected cross-reactivity of potentially arginine-methylated proteins with monoclonal antibodies directed against STAT1 or STAT3 is currently unknown. A potential

unspecific detection of proteins, which are co-migrating with STAT1 and STAT3, makes it difficult to interpret Western blot results [130,131,181,182,207].

It was clearly shown that the methylation inhibitors, MTA and MDA, inhibited tyrosine phosphorylation of STAT1 and STAT3, DNA binding activity, and STAT1-mediated gene transcription. The decrease in DNA binding activity provides a simple explanation for the reduced transcriptional activity of STAT1 upon treatment with MTA or MDA. Importantly, our findings that both methylation inhibitors suppressed tyrosine phosphorylation of STATs are in contrast to the findings by Mowen and David [130,131]. In addition, I found that MDA treatment also affected phosphorylation of p38 and Erk MAP kinases. The inhibitory effect of MDA could be observed at concentrations below those usually used for inhibition of arginine methylation [131,132,184]. The Jak/STAT signal transduction cascade is known to be influenced by other pathways involving p38 and Erk MAP kinases [6,185-188]. Therefore, effects of MDA treatment on STAT function could well be explained by the influence on these and possibly other signaling molecules and do not necessarily imply an involvement of STAT arginine methylation.

N-Methyl-2-deoxyadenine (MDA) is a nucleoside that naturally only occurs in DNA from prokaryotes. Long term incubation with this compound has previously been shown to affect various cell types: it induced differentiation of P19 teratocarcinoma cells and of C6.9 glioma cells, neurite outgrowth of PC12 cells, and also stimulated C2C12 cells to undergo myogenic differentiation. Based on inhibitor studies, a possible involvement of adenosine A2a receptors and cAMP-, MAPK-, and rapamycinsensitive pathways has been discussed [208-210].

Methylthioadenosine (MTA) has also been shown to adversely affect a variety of biological processes: it inhibited FGF-stimulated protein tyrosine phosphorylation in fibroblasts and neurite outgrowth of PC12 cells [211]. Moreover, MTA has been shown to induce apoptosis of HepG2 cells by upregulating Bcl-x_s [212]. Furthermore, MTA has been described to inhibit LPS-induced TNF- α production and inducible NO synthase (iNOS) gene expression by stimulating the production of the anti-inflammatory cytokine IL-10 [213]. Since methylation is involved not only in signal transduction but also in protein synthesis, it could not be excluded whether the general inhibitors of methyltransferases, MTA and MDA under the conditions used would produce a major change of the proteins in the studied cells.

Collectively, the data obtained in this thesis together with the data reported in the literature strongly suggest that the current methylation inhibitors, MTA and MDA, display limited specificity and therefore are not very suitable for this kind of signaling studies.

The data presented in this thesis certainly underline the importance of Arg³¹ for the structural integrity and stability of the STAT protein, a fact already implied by the solved crystal structure of the N-terminal domain of STAT4 [179]. Analysing the three-dimensional structure it is difficult to envisage how this conserved arginine residue can post-translationally be modified. When the DNA amounts of the STAT wild-type and R31K mutant expression vectors were adapted to obtain equal overall expression levels, we observed a suppressive effect of the mutation on STAT-mediated gene expression. Our findings are contrasting those describing a gain-of-function upon mutation of Arg³¹ in GST-STAT1 [130] but would match data describing loss-of-function of STAT1-Arg³¹ and STAT6-Arg²⁷ mutants, which both showed defective nuclear translocation [132,193].

We did not observe an *in vitro* methylation of a full-length GST-STAT1 fusion protein by PRMT1, although the catalytic activity of our enzyme preparation was readily demonstrated for the glycine-arginine rich protein. It should be noted that Mowen et al. [130] used a GST fusion protein of STAT1 truncated after the N-terminal 129 amino acids to demonstrate *in vitro* methylation, which might explain the divergent results. Moreover, we did not observe *in vitro* methylation of a GST-STAT3 fusion protein by active enzyme preparations of PRMT1, -2, -3, -4, or -6, although FLAG-tagged STAT3 had been used as a positive control in a study on *in vivo* arginine methylation of the hepatitis C virus NS3 protein [180]. Furthermore, we did not observe a difference in STAT1-mediated reporter gene activity upon co-expression of PRMT1 or a catalytically inactive form of PRMT1, although in previous reports a similar PRMT1 co-expression substantially enhanced reporter gene activity controlled by NIP45 co-activator and nuclear hormone receptors, respectively [207,214]. Thus, our data indicate that PRMT1 does not affect STAT1 function with respect to transcriptional regulation.

In conclusion, and together with the recently published data [193], our results do not support the proposed model of STAT arginine 31 methylation. Thus, alternative explanations need to be explored in order to understand the molecular mechanisms that underlie cytokine resistance of melanoma cells.

4.2 SOCS3 expression in melanoma cell lines

The major findings of the second part of this thesis are (a) cytokine-resistant 1286 melanoma cells show constitutive expression of SOCS3 and (b) SOCS3 expression confers a growth advantage to the cells as suppression of SOCS3 restores or increases sensitivity against IL-6-type cytokines. IL-6-type cytokines lead to STAT3 and STAT1 tyrosine phosphorylation, although the latter is generally only observed on short-term treatment with a high dose of IL-6 [57, 215]. For A375 cells, it has previously been shown that STAT3 is crucial for IL-6- and OSM-mediated growth inhibition [57]. The signal transduction of IL-6-type cytokines is subject to feedback inhibition by SOCS proteins, with SOCS3 playing the most important role [216]. Overexpression of SOCS3 shuts off cytokine signaling: A375 cells heterologously expressing SOCS3 are resistant to the growth-inhibitory action of OSM [217]. These findings are in full accordance with the results of this thesis.

The “quality” of a STAT response can be modulated by the presence or absence of other proteins, as first described for cells lacking STAT3: in the absence of STAT3, IL-6 mediates a STAT1-dominated IFN- γ -like response [191]. Interestingly, the results of this thesis show that suppression of SOCS3 in 1286 cells modulated the STAT response not only quantitatively but also in a qualitative manner by drastically increasing the STAT1 response to IL-6 (Fig. 29). It has previously been described that SOCS3 can influence the pattern of STAT activation: in the absence of SOCS3, it was observed that macrophages, hepatocytes and murine embryonic fibroblasts respond to IL-6-type cytokines with a prolonged STAT1 activation (in addition to a prolonged STAT3 activation) [114,201,202], which could lead to the expression of IFN- γ -responsive genes [114,201]. An IFN- γ -like response in the absence of SOCS3 was also discussed to be the cause for changing the phenotype of B7-stimulated dendritic cells from immunostimulatory to immunosuppressive [218]. For A375 melanoma cells, it has previously been shown that STAT1 activation can mediate growth inhibition [145]. It is therefore likely that STAT1 contributes to IL-6-mediated growth inhibition in SOCS3-suppressed 1286 cells. However, IL-6 does not elicit a full-blown IFN- γ -like response in SOCS3-suppressed 1286 cells, e.g. I did not observe an upregulation of MHC class I expression, a hallmark of an IFN- γ response (Fig. 36). Taken together, our data underline the role of SOCS3 not only in limiting the extent of a STAT response quantitatively but also in the “sculpting” of the STAT response in a qualitative way.

The expression of SOCS3 was previously described to be altered in cancer cells and either tumor suppressing or tumor protecting functions have been discussed: The SOCS3 gene was found to be silenced by promoter methylation in lung cancer as well as in primary lung cancer tissue samples [158], in hepatocellular carcinoma [159], in squamous cell carcinoma of the head and neck [160], and in cholangiocarcinoma [219]. Restoration of SOCS3 suppressed growth and resulted in apoptosis [158-160,219]. Although SOCS3 protein was virtually undetectable in most melanoma cells we tested prior to cytokine treatment, the SOCS3 gene is probably not silenced in melanoma cells, as SOCS3 mRNA could readily be detected, albeit at varying levels, in all melanoma cells as in melanocytes (Fig. 37). Our data are consistent with the findings of Li et al. [165], who observed that SOCS3 expression is elevated in transformed melanocytes as well (unpublished data). However, methylation-associated silencing of the SOCS3 gene was recently reported in human malignant melanomas [220].

In other cancers such as anaplastic large cell lymphoma [221], breast cancer [222-224] and prostate cancer [225], SOCS3 is overexpressed. Constitutive SOCS3 expression in T-cell lymphoma depended on STAT3 signaling because expression of a dominant negative version of STAT3 inhibited SOCS3 expression [161,164]. Constitutively expressed SOCS3 was shown to protect chronic myeloid leukemia cells against IFN- α treatment [163,226]. Thus, for SOCS3 expressing tumors, SOCS3 may not be a tumor suppressor but rather a protector, which may be of special relevance in tumors that are treated with type I interferons. However, the results of this thesis show that SOCS3 expression selectively mediates resistance of 1286 melanoma cells against IL-6-type cytokines but does not underly their resistance to IFN- α (Fig. 33). There may be several explanations. Firstly, I found that 1286 cells exhibited a diminished STAT1 phosphorylation in response to IFN- α , which is consistent with previous reports [63,149,150]. However, this was not due to reduced STAT1 protein expression. On the other hand, an inhibitory role of other SOCS proteins, particularly SOCS1 and CIS, in IFN- α signaling has been suggested. It has been shown that STAT5 contributes to interferon resistance of melanoma cells by upregulating the cytokine inhibitor CIS and overexpression of CIS in IFN- α -sensitive cells reduced the interferon response of the cells [155]. Interestingly, I noticed that CIS mRNA is constitutively expressed in 1286 cells (Fig. 28) and in the majority of melanoma cell lines examined in this study (data not shown). Thus, it will be of interest to investigate whether constitutive expression of CIS might play a role in the insensitivity to IFN- α of melanoma cells.

Moreover, SOCS1, another member of the SOCS family implicated mainly in IFN signaling, was found to be constitutively expressed in melanoma cells [165]. In this report, SOCS1 expression did not correlate with IL-6-sensitivity, but a potential involvement in growth promotion and/or IFN resistance of melanoma cells was discussed based on the detected SOCS1 immunoreactivity *in situ* which increased as a function of the stage of disease [165]. With regard to SOCS1 expression, melanoma cells are thus different from other cancer cells in which the SOCS1 gene is silenced by methylation, such as in pancreatic cancer [227,228], in gastric cancer [229], in human hepatoblastomas [230,231], and in hematopoietic malignancies [232-235]. Our present analysis of 10 human melanomas indicates that SOCS3 immunoreactivity is likewise present in a portion of tumors. Further studies on a higher number of melanoma specimens are currently underway to precisely clarify whether SOCS3 behaves as a progression marker of melanoma and/or whether its *in situ* expression (alone or in combination with SOCS1) might correlate with IFN- α responsiveness *in vivo*.

In this thesis, I further show that suppression of constitutive SOCS3 expression slightly increased the surface expression of both gp130 and OSMR (Fig. 35). SOCS proteins as ubiquitin ligase components are known to affect the half-life of their interacting proteins. It has previously been shown that SOCS1 and SOCS3 bind to the epidermal growth factor receptor (EGFR) and facilitate its proteasomal degradation [236]. Moreover, SOCS3 has recently been shown to target CD33 and Siglec7 (sialic acid-binding immunoglobulin-like lectin 7) for proteasomal degradation [237,238]. Since SOCS3 binds to gp130, further studies will show whether SOCS3 can target gp130 for degradation. In contrast to gp130, the OSMR does not contain a binding site for SOCS3 [117]. However, the upregulation of OSMR surface expression in SOCS3-suppressed cells could be explained by the fact that OSMR is a STAT3 target gene [239]. Short interfering RNA-mediated inhibition of SOCS3 expression resulted in increased STAT3-dependent transcriptional activity and subsequently leading to enhanced expression of OSMR. Moreover, the upregulation of OSMR was also observed in SOCS3-deficient mouse embryonic fibroblasts (data from our group). It will be important to examine the expression of OSMR at the mRNA level by real-time PCR analysis to confirm that the increased OSMR in SOCS3-suppressed cells results from the transcriptional activation of the OSMR gene by STAT3. Our results further indicate that SOCS3 did not seem to be involved in regulating Jak1 because the steady-state amounts of Jak1 were similar in SOCS3-suppressed cells and in control-siRNA treated cells (data not shown), thereby excluding the possibility of the upregulation of OSMR surface expression being caused by Jak1.

Interestingly, in a recent study loss of OSMR expression correlated with melanoma progression [148]. In keeping with this, I observed a lack of OSMR expression in 888 melanoma cells (data not shown), which showed impaired Jak/STAT activation (Fig. 23A) and were resistant to OSM-induced growth inhibition (Fig. 38C).

The molecular mechanism responsible for elevated SOCS3 gene and protein expression in 1286 cells is unknown. Although constitutive SOCS3 expression in many tumor cells has been shown to be mediated by STAT3 [161,162], constitutive SOCS3 expression in 1286 cells seems to be STAT3 independent since a dominant negative STAT3 (STAT3F) did not affect SOCS3 expression (Fig. 26). However, there is evidence suggesting that STAT5b also regulates SOCS3 expression. It has been shown that growth hormone (GH)-induced expression of SOCS3 mRNA in liver is inhibited in mice lacking STAT5b, and STAT5b can also bind to the STAT1/STAT3 binding elements in the mouse SOCS3 promoter [240,241]. Interestingly, STAT5 has recently been shown to be constitutively activated in melanoma cells [156]. Further studies are necessary to clarify whether STAT5 is involved in mediating constitutive SOCS3 expression in 1286 cells. Besides STAT factors, specificity protein 3 (Sp3) has been shown to be involved in the regulation of SOCS3 expression [242]. In addition, cyclic AMP (cAMP)-induced SOCS3 expression was shown to be mediated by Exchange Protein Activated by cAMP (Epac) [243]. It will be of interest to investigate whether Sp3 and Epac are involved in the regulation of SOCS3 expression in 1286 cells.

The cDNA sequence of SOCS3 of 1286 cells shows no mutations (data not shown). Moreover, enhanced SOCS3 expression in 1286 cells was not due to a prolonged half-life of the protein or mRNA (Fig. 25). By contrast, I found that SOCS3 mRNA of 1286 cells was less stable than that of A375 cells (half life of 30 min in 1286 cells vs. 50 min in A375 cells). This finding further suggests a complex regulation of SOCS3 expression in 1286 cells. Because of the rapid turnover of the SOCS3 mRNA, I speculate that a high rate of transcription must be required to sustain the high levels of SOCS3 mRNA and protein in 1286 cells. Recently, it has been shown that the p38 MAPK pathway is involved in the regulation of the mRNA stability of SOCS3 by antagonizing the action of tristetraprolin (TTP) on the decay of SOCS3 mRNA [244]. As a target for SOCS3 mRNA stability-regulating signals, a region containing three copies of a pentameric AUUUA motif in close proximity of the 3' untranslated region of SOCS3 was identified. The authors have shown that TTP, a *trans*-acting RNA-binding protein, was capable of destabilizing SOCS3 mRNA via this region: TTP

expression enhances SOCS3 mRNA degradation, whereas siRNA-mediated knockdown of TTP strongly enhances the amount of SOCS3 mRNA. Furthermore, deletion of the region, which contains three copies of the pentameric AUUUA motif, rendered SOCS3 mRNA insensitive toward TTP expression. Thus, it will be of interest to determine whether TTP has an influence on mRNA stability of SOCS3 of 1286 melanoma cells.

The regulation of SOCS3 expression is known to occur on the level of protein degradation that is thought to be accelerated by tyrosine phosphorylation of SOCS3 [120]. In fact, I had no evidence for tyrosine phosphorylation of the SOCS3 protein in 1286 cells (data not shown) indicating that the protein stability of SOCS3 was not affected, which is consistent with the results on the half-life of SOCS3 protein of 1286 cells (Fig. 25). I observed a downregulation of SOCS3 expression when treating cells with the MDA methylation inhibitor cocktail (*N*-methyl-2-deoxyadenosine/adenosine/DL-homocysteine) which has been shown to affect multiple signaling pathways (Fig. 12). However, more specific inhibitors of MEK and p38 did not influence SOCS3 expression (Fig. 27), although in other situations these signaling pathways have been implicated in the regulation of SOCS3 expression [198-200]. Thus, it clearly warrants further investigations to identify the molecular basis responsible for constitutive SOCS3 expression in 1286 cells.

5 Outlook

5.1 How does SOCS3 regulate the transcriptional response to IL-6?

The data presented in this thesis underline the important role of SOCS3 in the negative regulation of IL-6-type cytokine signaling. Further investigations are needed to address the following questions.

- How does SOCS3 regulate the response to IL-6 at a genome-wide level, and in particular, whether distinct sets of genes respond differentially to the loss of SOCS3 following stimulation with IL-6?
- How does suppression of SOCS3 qualitatively affect IL-6-type cytokine signaling from a STAT3-dominated response towards a STAT1-dominated (IFN- γ -like) response?

For this study, microarray analysis can be used to profile gene expression.

5.2 Is SOCS3 implicated in the regulation of the expression of IL-6-type cytokine receptors?

The data presented in this thesis show that suppression of constitutive SOCS3 expression slightly increased the surface expression of gp130 and OSMR. It will be of interest to examine whether SOCS3 is responsible for regulating the expression of gp130 and OSMR.

To investigate whether the regulation of gp130 expression by SOCS3 occurs at the level of protein degradation, the following experiments should be performed.

- Comparison of the half-life of gp130 protein in SOCS3-suppressed cells with the half-life of gp130 protein in control-siRNA treated cells upon incubation with the protein synthesis inhibitor cycloheximide.
- Treatment with the proteasome inhibitor MG132 to determine whether the degradation of gp130 is mediated by the ubiquitin-proteasomal pathway.

In addition, it should be investigated whether overexpression of SOCS3 reduces the half-life of gp130. For this purpose, wild-type MEF cells and MEF cells lacking SOCS3 can be used.

To investigate whether the increased OSMR surface expression in SOCS3-suppressed cells resulted from the transcriptional activation of OSMR gene by STAT3, the expression of OSMR at the mRNA level in SOCS3-suppressed cells should be analysed by real-time PCR.

5.3 Does constitutive CIS expression contribute to IFN- α resistance of melanoma cells?

Previously, it has been shown that STAT5 contributes to interferon resistance of melanoma cells by upregulating the cytokine inhibitor CIS [155]. Interestingly, our preliminary data revealed that CIS mRNA is constitutively expressed in most melanoma cell lines investigated in this study. Moreover, CIS expression is elevated in breast carcinoma [222]. It will be interesting to evaluate the effect of suppressing CIS in resistant cells, e.g. 1286 cells and evaluate the changes in cytokine sensitivity. Because CIS is a STAT5 target gene [245], it will be important to determine whether constitutive CIS expression in melanoma cell lines is mediated by STAT5.

5.4 Could SOCS3 expression in 1286 cells be downregulated by SOCS2?

Recently, SOCS2 has been shown to play an important role in regulating the expression of other SOCS proteins, e.g. SOCS1 and SOCS3 [246,247]. Expression of SOCS2 resulted in a marked proteasome-dependent reduction of SOCS3 protein expression. Furthermore, the degradation of SOCS3 by SOCS2 is enhanced by coexpression with Elongin BC and requires an intact SOCS box, suggesting that SOCS2 acts as an ubiquitin ligase towards SOCS3. It is important to note that SOCS2 has not been shown to inhibit the activation of STAT3 in response to IL-6 or OSM. Therefore, it will be interesting to investigate whether overexpression of SOCS2 in 1286 melanoma cells could restore or increase sensitivity against IL-6-type cytokines by accelerating the degradation of SOCS3. To address this issue, stable 1286 cells that inducibly express SOCS2 protein need to be generated. This study is currently under investigation.

5.5 Analysis of SOCS3 promoter of 1286 cells

The molecular mechanism responsible for the constitutive SOCS3 expression in 1286 cells is currently unknown. To better understand the regulation of SOCS3 expression in these cells, the promoter region of the human SOCS3 gene of 1286 cells needs to be analysed. Cloning and characterization of a functional promoter of the human SOCS3 gene can be performed as described in previous reports [248,249].

6 Summary

Cytokines play an important role in the growth regulation of melanoma cells. Whereas the growth of melanocytes and many early stage melanoma cells can be inhibited by cytokines, melanoma cells of advanced tumor stages have often been reported to be “multi-cytokine resistant”. Thus, understanding the molecular mechanisms underlying cytokine resistance is of cardinal importance.

Transcription factors of the STAT family are crucial in signal transduction of cytokines. Previously, it has been reported that methylation of STAT1 on a conserved arginine residue (Arg31) is important for STAT1 function and loss of this modification was proposed to be involved in interferon resistance of cancer cells. In the first part of this thesis I thus investigated the arginine methylation of STAT1 and STAT3. Here I provide several independent lines of evidence that did not support the occurrence of arginine methylation of STAT1 or STAT3. First, it was shown that the anti-methylarginine antibodies did not precipitate specifically STAT1 or STAT3. Second, it was shown that the methylation inhibitors, MTA and MDA, had profound and rapid effects on phosphorylation of STAT1 and STAT3, but MDA additionally also affected the p38 and Erk signaling cascades which are known to cross-talk with the Jak/STAT pathway. Third, it was shown that mutation of Arg31 to Lys led to destabilization of STAT1 and STAT3, implicating an important structural role of Arg31. Finally, the *in vitro* methylation assay using purified catalytically active protein arginine methyltransferases (PRMT1, -2, -3, -4, and -6) demonstrated that STAT proteins are not methylated, and cotransfection of PRMT1 did not affect STAT1-controlled reporter gene activity. Taken together, the data presented in this thesis suggest the absence of arginine methylation of STAT1 and STAT3.

The Jak/STAT signaling pathway is subject to feedback inhibition by members of the suppressors of cytokine signaling (SOCS) family. In the second part of this thesis, I analysed the melanoma cell line 1286, resistant towards the growth-inhibitory effects of IL-6 and OSM, to better understand the mechanisms underlying cytokine resistance. Although the relevant receptors gp130 and OSMR are expressed at the cell surface of these cells, cytokine stimulation hardly led to activation of Jak1, STAT3 and STAT1. I found a high level constitutive expression of SOCS3 that did not further increase after cytokine treatment. Importantly, upon suppression of SOCS3 by short interfering RNA, cells became susceptible

towards OSM and IL-6: they showed an enhanced STAT3 phosphorylation and a dramatically increased STAT1 phosphorylation. Moreover, suppression of SOCS3 rendered 1286 cells sensitive to the anti-proliferative action of IL-6 and OSM, but not of IFN- α . Interestingly, SOCS3-siRNA treatment also increased the growth-inhibitory effect in cytokine-sensitive WM239 cells expressing SOCS3 in an inducible way. Thus, SOCS3 expression confers a growth advantage to these cell lines. Constitutive SOCS3 mRNA expression, although at lower levels than in 1286 cells, was found in nine additional human melanoma cell lines and in normal human melanocytes while at protein level SOCS3 expression was marginal at best. However, *in situ* analysis of human melanoma specimens revealed SOCS3 immunoreactivity in 3 out of 10 samples suggesting that *in vivo* SOCS3 may possibly play a role in IL-6 resistance in at least a fraction of tumors.

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Danksagung

An erster Stelle möchte ich mich ganz herzlich bei Herrn Prof. Dr. Peter C. Heinrich für die Aufnahme in seine Arbeitsgruppe, seine Unterstützung und sein Interesse am Fortgang dieser Arbeit bedanken.

Herrn Prof. Dr. Fritz Kreuzaler danke ich ganz herzlich für die Übernahme des Koreferats.

Mein besonderer Dank gilt Frau Prof. Dr. Iris Behrmann für die ausgezeichnete Betreuung dieser Arbeit. Ihre stete Diskussionsbereitschaft, Anregungen und Hilfestellungen haben wesentlich zum Erfolg dieser Arbeit beigetragen.

Ebenfalls möchte ich unseren Sekretärinnen Elke Broekmeulen und Elke Meyer für ihre sehr freundliche Hilfsbereitschaft und viele nette Gespräche danken.

Dr. Serge Haan danke ich für die Analyse der Proteinstrukturen von STAT1 und STAT3 und seine Hilfsbereitschaft.

Bei Hildegard Schmitz-Van de Leur bedanke ich mich für ihre Unterstützung bei der Klonierung wichtiger Konstrukte und ihre grosse Hilfe bei allen möglichen Dingen.

Allen Mitarbeitern des Instituts für Biochemie und vor allem meinen Kollegen aus Labor 13, Marlies Kaufmann, Monique Wiesinger, Kathrin Borggrebe und Saskia Holdschuh danke ich für die große Hilfsbereitschaft und die nette Arbeitsatmosphäre. Hier gilt mein Dank auch den vielen ehemaligen Mitarbeitern, insbesondere Simone Radtke, Claude Haan, Ulrike Sommer, Xiangping Yang, Angela Jörissen, Sandra Diefenbach, Marcin Kortylewski und Georg Munz.

Außerdem bedanke ich mich bei den Technischen Assistenten für den reibungslosen Ablauf des Laboralltags und die zuverlässige Bereitstellung von Materialien.

Mein herzlicher Dank gilt meinem Freund Ple und meinen Eltern für die liebevolle Unterstützung.

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