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The Molecular Cooperation of Stat3 and p14^{ARF} in Hepatocellular Carcinoma Cells

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1. Abstract/Zusammenfassung

1.1. Abstract

Hepatocellular carcinoma (HCC) is amongst the most frequent human cancers and its cure is difficult since resection mostly is not possible. Although there are attempts for systemic therapies, the variety and differences in the underlying genetic alterations and disruptions of signalling pathways makes this a difficult goal to achieve. One well-known oncogenic transcription factor involved in the development of HCC, is Stat3, whose targets drive proliferation and angiogenesis and counteract apoptosis. In addition to these tumor-promoting functions Stat3 was found to act tumor-suppressive under certain circumstances. However, this conversion from pro- to anti-oncogenic Stat3 actions is mechanistically poorly understood. We previously found Stat3 to act tumor suppressive in the absence of p14^{ARF}, a tumor suppressor frequently lost in HCC. Since Janus kinases (Jaks) are the canonical activators of Stat3 we addressed the question whether the absence of p14^{ARF} affects their activation, and which of them induces Stat3 activation. First, we found that *de novo* RNA and protein synthesis is necessary for Stat3 activation irrespective of p14^{ARF} expression. Further, we identified Jak1 as the crucial kinase for IL-6 induced Stat3 phosphorylation. Jak1 was required for Stat3 activation independent of p14^{ARF} expression. Since we found that phosphorylation of Stat3 was circumvented in p14^{ARF}-negative experimental HCC, we assumed that Jak1 activity is blocked by an unknown mechanism. From these data we propose that p14^{ARF} binds to a nuclear protein that can also interact with unphosphorylated-Stat3 (U-Stat3). This interaction is assumed to prevent U-Stat3 from modulating transcription and thus the absence of p14^{ARF} changes the subset of Stat3-modulated genes.

We further observed that Stat3 was required to exert TGF- β 1 induced anti-proliferative effects in hepatoma cells. Thus, the question arose, whether Stat3 is required for the canonical TGF- β /Smad signalling. We observed that TGF- β 1-induced translocation of Smads to the nucleus was not affected by the lack of Stat3, but the overall Smad level was reduced. Thus we propose that Stat3 is crucial for TGF- β 1 functions on a transcriptional level.

1.2. Zusammenfassung

Das Leberzellkarzinom (HCC) gehört zu den am häufigsten vorkommenden Krebserkrankungen. Therapie und Heilung sind schwierig, da eine operative Entfernung meist nicht möglich ist. Es gibt zahlreiche Bemühungen effektive systemische Therapien zu entwickeln, was sich aber aufgrund der Heterogenität der zugrundeliegenden genetischen Veränderungen und Störungen der Signaltransduktion als schwierig erweist.

Stat3 ist ein Transkriptionsfaktor der in den meisten HCC Fällen erhöhte Aktivität aufweist. Seine Zielgene fördern Proliferation und Angiogenese und wirken der Apoptose entgegen. Unter bestimmten Bedingungen kann Stat3 aber auch tumorsuppressiv wirken. Die zugrundeliegenden Mechanismen für diese Wandlung sind jedoch weitgehend unbekannt. Unsere rezenten Untersuchungen haben gezeigt, dass Stat3 in Abwesenheit von p14^{ARF} als Tumorsuppressor fungiert. p14^{ARF} ist ein Tumorsuppressor der in HCC häufig deaktiviert ist. Da Janus Kinasen (Jaks) für die Stat3-Aktivierung verantwortlich sind, wollten wir wissen, ob die Abwesenheit von p14^{ARF} Einfluss auf ihre Aktivität und die Stat3-Phosphorylierung nimmt. Es zeigte sich, dass unabhängig vom p14^{ARF}-Status die *de novo* RNA- und Proteinsynthese für die Stat3-Phosphorylierung notwendig ist. Weiters fanden wir heraus, dass Jak1 für die IL-6-induzierte Stat3-Aktivierung verantwortlich ist, und dass p14^{ARF} auf die Aktivität von Jak1 keinen Einfluss hat. Da sich zuvor gezeigt hatte, dass in p14^{ARF}-negativen experimentellen HCC die Phosphorylierung von Jak1 verhindert wird, schließen wir, dass dies durch einen anderen Modulator geschieht und p14^{ARF} selbst weiter downstream agiert. Unsere These ist, dass p14^{ARF} im Nukleus an einen Faktor bindet und diesen inhibiert, der auch an unphosphoryliertes Stat3 bindet, wodurch ein anderes Set an Genen transkriptionell moduliert wird.

Weiters konnten wir beobachten, dass Stat3 für die TGF- β 1-induzierten anti-proliferativen Effekten nötig ist. Daher stellte sich die Frage, ob Stat3 für die Aktivierung von Smads und deren Translokation in den Nukleus verantwortlich ist. Dies war nicht der Fall. Da wir jedoch reduzierte Mengen von Smads fanden, gehen wir davon aus, dass Stat3 auf transkriptioneller Ebene die Ausführung TGF- β 1-induzierter Effekte moduliert.

2. Introduction

2.1. Malignant transformation

To develop cancer multiple mutations and/or disruptions of signalling pathways and control mechanism are necessary (1). There are a few characteristics typical for cancer cells. The most prominent and crucially important one is the loss of growth and proliferation control resulting in unlimited growth (1). Insensitivity to growth limiting or apoptotic signals and independence of growth promoting signals contribute to this feature. The tumor microenvironment and particular cells of the immune system lead to a tumor-promoting inflammatory environment rather than tumor surveillance (1). Furthermore, influencing its environment in a way to induce neovascularisation is a means of the tumor to ensure its supply and thus, survival and further growth (Fig. 1) (1). A late step in tumorigenesis is metastasis, thus the ability to detach from the original tumor cell assembly and invade other tissues (1).

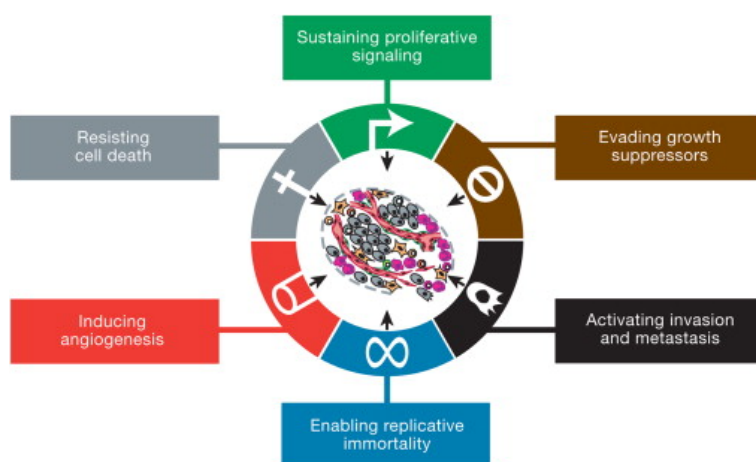


Figure 1: Typical features of cancer cells (1).

2.2 Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the fifth most common cancer among solid tumors worldwide and the third leading cause of cancer-related mortality with 500.000 deaths annually (2). It is a complex disease, which develops over years and is clinically diagnosed often only at late stages (3). The development is a multistep process with accumulation of aberrant genetic and epigenetic changes (Fig. 2) (3, 4). Common risk factors are infection with hepatitis B or C virus, alcohol abuse and fatty liver disease, where progression of disease is associated with fibrosis and cirrhosis in

most cases (3-5). Aflatoxin intoxication and genetic disorders like tyrosinosis contribute to development of HCC without concomitant fibrosis or cirrhosis (3). HCV, HBV, Aflatoxin together are responsible for 80 % of HCC development in humans (5).

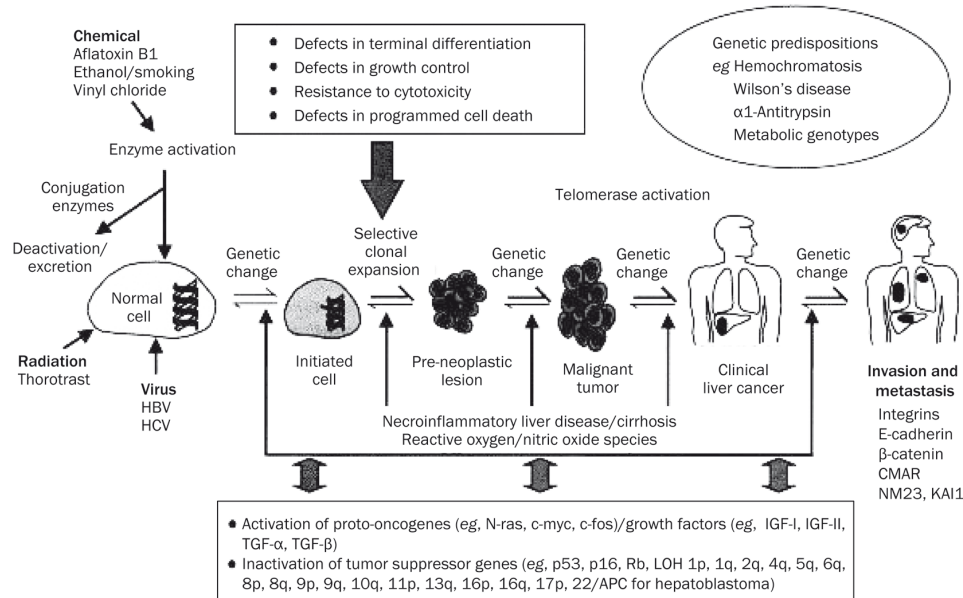


Figure 2: Triggers for HCC and molecular changes during progression (3).

There are some typically affected genes and regulatory circuits, however, the pattern of disruptions and changes is very heterogeneous among the individual HCCs (4, 5). Typical alterations are mutations, allelic deletions and epigenetic changes like abnormal promoter methylation, leading to activation of oncogenes and inactivation of tumor suppressors (4, 5). Besides this, the accelerated proliferation of hepatocytes and selective growth of monoclonal cell populations are typical already in preneoplastic phases, and are even enhanced in HCC (5). The accumulation of genetic changes and the increasing genetic instability is accompanied by characteristic alterations of liver tissue and affected hepatocytes (Fig. 3) (5). Fibrosis and inflammation play key roles in this development, since they trigger changes of the matrix and the microenvironment (5). During these processes many hepatocytes die and thus, inflammatory cells infiltrate the liver and connective tissue is deposited (5). In the course of further progression foci of phenotypically altered hepatocytes develop into dysplastic nodules finally resulting in full blown HCC (5). Inflammation is considered to participate in the development and progression of many tumors, but is especially important in the course of hepatocarcinogenesis, since fibrotic and cirrhotic stages most frequently precede HCC (6). Overexpression of inflammatory cytokines

and aberrant inflammatory responses can enhance the proliferation of malignant cells and contribute to generating a tumor-promoting environment (6-8).

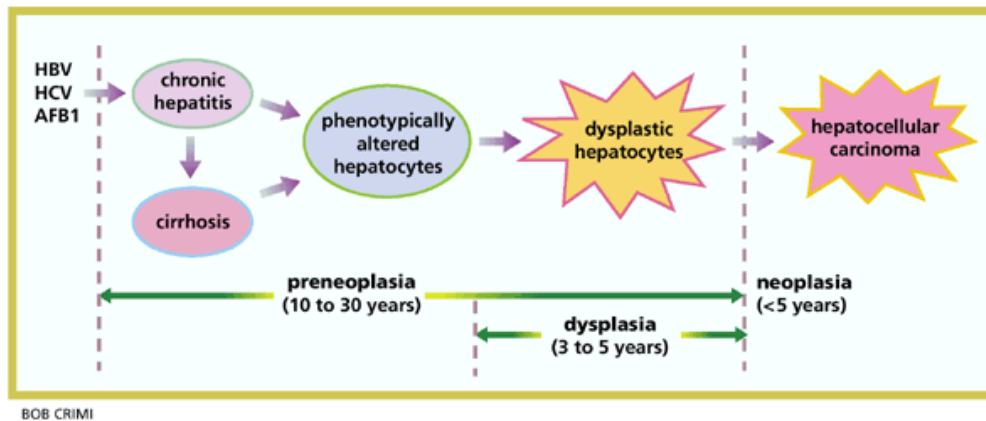


Figure 3: Changes of hepatocytes in the typical time course of HCC (5).

Structural aberrations occur only in a few genes and slowly during early stages, but are increased in dysplastic hepatocytes and HCC (5). Allelic deletions were found in 30-50 % of patients suffering from chronic hepatitis or cirrhosis, while 70–80% of dysplastic nodules and almost all investigated HCC livers showed this alteration (5, 9-11). Some chromosomal regions are especially prone to allelic deletion, loss or gain of chromosomal regions. Within these often affected regions lie the tumor-suppressor genes *p53*, *Rb*, and *CDKN2A* which are often inactivated by deletion or mutation, while the oncogene *c-myc* is frequently overexpressed and was found to be amplified in 30% of HCCs (4, 5, 11). Upon impaired control and repair mechanisms it is likely that structural aberrations accumulate during accelerated proliferation (4, 5). Although aberrant promoter methylation is a frequent and early event in hepatocarcinogenesis, changes in expression levels precede epigenetic alterations (4, 5). Expression of cytokines such as transforming growth factor α (TGF- α) and growth factors like insulin-like growth factor II (IGF-II) is induced by cytokines secreted by inflammatory cells or viruses, or in response to cell death and results in enhanced proliferation of hepatocytes and thus, development of monoclonal cell populations (5). Still, hypermethylation of CpG islands is already found in hepatic cirrhosis (4, 5). Epigenetic alterations increase in the progression from pre-malignancy to HCC, in which hypo- or hypermethylation of promoters is a frequent and common condition (4, 5). Accordingly, the expression of DNA-methyltransferases (DNMT-1, DNMT-3) is enhanced in chronic hepatitis and cirrhosis, and strongly

increased in HCC (5, 12). Interestingly, especially in cirrhotic, HBV- or HCV-associated HCC the methylation frequency was found to be enhanced compared to other HCCs (4). Methylation in HCC affects genes involved in apoptosis, cell adhesion, DNA repair, mismatch repair, inhibition of several proteins like cell cycle regulators such as cyclin-dependent kinases (CDK), cell cycle regulators p16^{Ink4a}, p14^{ARF}, MDM2, metalloproteases (MMP) or cytokines and regulators of signal transduction like SOCS (5). Still, early epigenetic changes and structural aberrations are not sufficient to induce malignant phenotypes in hepatocytes, although some epigenetic changes act indirect and generate conditions for development of cell populations with structurally and functionally aberrant genes (5). Some epigenetic changes also directly precede structural aberrations, like in the cases of c-myc and *CDKN2A* (5). The overexpression of c-myc is associated with promoter hypomethylation in early stages and with following gene amplification later on (5).

2.3. Altered signal transduction in HCC

As every type of cancer, hepatocarcinogenesis shows positive or negative changes in various signalling pathways and regulatory circuits (1, 5, 13). They can get disrupted or hyperactivated by different mechanisms (1, 5, 13). These intracellular signalling networks are complex, often redundant and crucial for many cellular processes like maintenance of cell number homeostasis, growth and proliferation control, the decision between survival or apoptosis, and motility (Fig. 4) (1, 5, 14). Since there are multiple crosstalks and interconnecting nodes between them, the disruption of one gene or the aberrant activity of one protein can affect several events and have severe consequences (5).

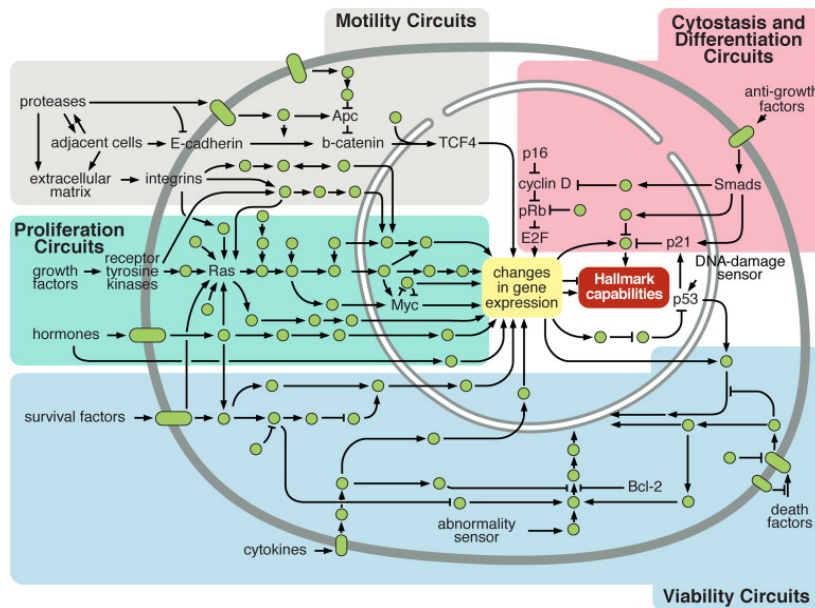


Figure 4: Signalling circuits contributing to tumorigenesis (1).

While strictly controlled in normal cells, tumor cells frequently show aberrations in signalling cascades, either being disrupted or overactivated, but mostly resulting in elevated proliferation (1, 13). Growth factor and cytokine levels can be enhanced due to elevated secretion by the tumor cell itself or by cells of the surrounding microenvironment, which got triggered to do so by the tumor cells (1, 6). Further enhanced expression or mutations in receptors can lead to hyperresponsibility (1). Mutations in receptors which entail ligand-independent signalling or mutations constitutively activating downstream components render the cell independent of growth promoting signals (1). Importantly, also the disruption of negative feed-back loops can be responsible for sustained and thus over-active signalling (1). Also in HCC, mutations, deregulated expression or uncontrolled activity of growth factors and cytokines, their receptor tyrosine kinases or intracellular tyrosine kinases further downstream in the signalling cascade are involved in malignant progression, namely survival, proliferation, angiogenesis, inflammation and invasion (5, 14-16).

Due to underlying liver diseases, resection is suitable only for few HCC patients and was shown to be effective only at early stages (13, 17). Thus, more than 80% of patients suffer from inoperable HCC with very poor prognosis (13). Even if resection is possible, there is a very high recurrence rate of over 60% due to metastasis or new development (13, 17). Furthermore, HCC is highly resistant to available systemic therapies (13, 17). Since there is no effective first-line treatment for advanced HCC, research is focussed on the development of novel targeted therapies (13, 17).

Recent findings proved aberrant proliferative signalling pathways, including Raf/MEK/ERK, PI-3K/Akt/mTOR, and Jak/Stat pathways accountable for tumor initiation and progression, and thus, signal transduction and tyrosine kinases became putative targets for intervention (13, 17).

2.4. Receptor tyrosine kinases (RTK) in HCC

Receptor tyrosine kinases are crucially involved in the control of survival, proliferation, angiogenesis, inflammation and invasion (7, 13). Epidermal growth factor receptor (EGFR) is expressed in a high proportion of HCCs and the proliferation of HCC cells was found to depend on stimulation of EGFR by TGF- α or EGF (13, 18). Hepatocyte growth factor (HGF) and its receptor c-Met have been implicated in HCC playing a role in growth, survival and invasion (13). Point mutations in MET have been detected and elevated HGF levels have been identified as potential predictor of poor prognosis (13). Vascular endothelial growth factor (VEGF), Platelet-derived growth factor (PDGF) and basic FGF (bFGF; FGF-2) are three important pro-angiogenic factors and thus, play important roles in neovascularization, invasiveness, and the metastatic potential of HCC (13). Activation of the VEGF-receptor family, particularly activation of VEGFR-2 by VEGF plays a primary role in tumor angiogenesis and accordingly, elevated levels of VEGF are associated with postoperative recurrence and poor prognosis (13). VEGF expression is detected in dysplastic nodules, and increases during the course of hepatocarcinogenesis (13). PDGF overexpression has been linked to increased metastatic potential of HCC, and also acts angiogenic, while bFGF is a mitogen for HCC cell proliferation via an autocrine mechanism (13). It enhances the development and progression of HCC (13). Upon binding to FGFR-1 it stimulates the release and activity of collagenases, proteases and integrins on the extracellular membrane leading to the development of microvascular networks (13). Furthermore, bFGF has been shown to synergistically augment VEGF-mediated HCC development and angiogenesis (13). High serum levels of preoperative bFGF appeared to be predictive of invasive tumorigenesis and early postoperative recurrence (13).

The glycoprotein130 receptor (gp130) subunit binds to various ligands and is mainly involved in inflammatory responses (6). Its downstream signalling can enhance or suppress inflammation, since it triggers the recruitment of immune cells to sites of inflammation and induces the switch from acute to chronic inflammation by

recruiting different cell types (6). Upon binding with a member of the multifunctional IL-6 family of cytokines it associates with the IL-6 receptor α (IL-6R α) subunit into a heterotrimer and activates the Jak-Stat-pathway (6). Since this receptor subunit has no intrinsic receptor tyrosine kinase activity, constitutively bound cytoplasmatic kinases (Janus kinases, Jaks) get activated upon ligand binding, which in turn phosphorylate signal transducers and activators of transcription (Stats 1,3,5), with Stat3 being the main mediator of IL-6 (8, 19). Also the Ras-Raf-Mek-ERK1/2, MAPK and Ras-PI3K/AKT pathways can be activated by gp130 engagement (8, 20). IL-6 family members are multifunctional cytokines and besides the fact that they are crucial mediators of inflammatory responses, they take part in the regulation of various cellular oncogenic processes, such as proliferation, apoptosis and angiogenesis (7, 21, 22).

Constitutive activation of IL-6/gp130-Jak-Stat was shown to contribute to inflammation-induced tumorigenesis, and since HCC is linked to chronic inflammation it is not surprising, that this signalling pathway is often crucially involved (3, 6). One example is IL-6 induced acute-phase-response via enhanced expression of Stat3 target genes in HCC cells, and without Stat3 the acute-phase response was found impaired. (22). Also mutations of gp130 leading to constant signalling activity of Jak/Stat without cytokine binding were found in inflammation associated HCCs (6, 23). Besides its contribution to inflammation, IL-6-induced Stat3 activation is involved in circumventing apoptosis and growth-stimulation in various cancer cells and was further shown to be involved in src-induced transformation (15, 21).

2.5. Intracellular tyrosine kinases in HCC

Signal transduction from activated cytokine or growth factor receptors is mediated by complex cascades of intracellular signalling pathways and molecules including the Ras/Raf/MEK/ERK, Ras/PI3K/Akt/mTOR and Jak/Stat pathways (1, 13, 16). Some growth factor receptors, such as EGFR, PDGFR and VEGFR transmit their signals via src kinase (24). Ras/Raf/MEK/Erk, Ras/PI3K/Akt/mTOR and Jak/Stat-pathways mostly transmit proliferation promoting signals (16). Upon phosphorylation of various transcription factors gene expression is modulated and posttranslational phosphorylation of signalling molecules affects their activity (16). By modulating the transcription of Bcl-2 family members or phosphorylation of Bcl-2, Mcl-1 or caspase 9 they influence apoptosis regulating pathways (1, 16). Generally, their overactivation

leads to upregulation of molecular players involved in and frequently found in human cancer (1, 16). Notably, both hepatitis B and C viruses are able to induce Ras/MAP kinase and Jak/Stat pathways (13, 25-28).

Ras regulates various signalling pathways involved in survival, growth and proliferation (Fig. 5) (29). While it is tightly controlled normally it is frequently deregulated in cancer (29). Almost all HCCs show hyperactivation of the Ras signalling cascade, either due to enhanced upstream signalling via growth factor receptors, downregulation or promoter hypermethylation of Ras GTPase activating proteins (GAPs) such as Rasal-1, NF1 and DAP2IP, or aberrantly overactive modulators downstream of Ras (30, 31). Oncogenic mutations of Ras, rendering them insensitive to GAPs and thus persistently activated, are rare (31).

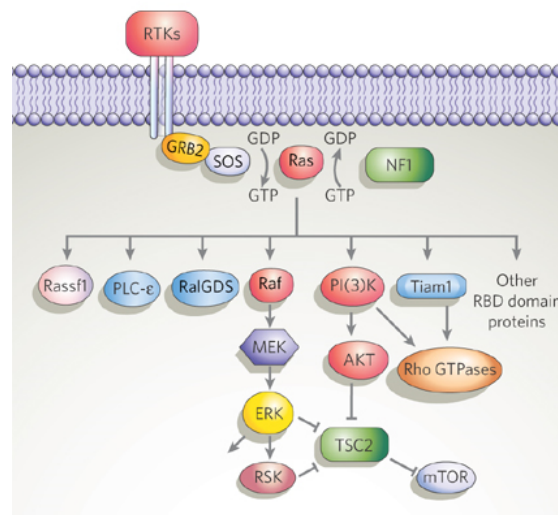


Figure 5: Activation and deactivation of Ras and various mitogenic signalling pathways operating downstream of Ras (29).

The Raf/MEK/ERK signalling cascade is crucially involved in the development of HCC and enhanced activity of this pathway is very frequent (13, 16, 32). Upon ligand binding to RTKs such as VEGFR, PDGFR, EGFR and c-Met it transduces growth-promoting signals to the nucleus (13). Also HCV core protein and HBx can directly activate this signalling cascade (13, 33). Raf-1 is frequently overexpressed in HCC, and the lack of Raf kinase inhibitor protein was associated with enhanced proliferation and migration (13). Since Erk1/2 has hundreds of targets it is not surprising that its over-activation influences a variety of cellular programmes like cell cycle progression, apoptosis resistance, extracellular matrix remodelling, cellular

motility, angiogenesis and resistance to drugs (13, 16). Among transcription factors activated by Erk are some for growth-factors and growth-inducing cytokines as well as for factors impeding apoptosis (16). In general, Erk activation was associated with poor prognosis (16, 28). There exist feed-back loops within the pathway, and deregulation mostly contributes to augmented proliferation (16).

The PI3K/Akt/mTOR protein cascade is another major signalling pathway involved in HCC and forwarding signals from RTKs (13). Its activation was correlated with poor prognosis, enhanced proliferation and angiogenesis (13, 28). Among mammalian target of rapamycin (mTOR) regulated targets is the translational repressor protein PHAS-1/4E-BP, which regulates the expression of VEGF and the translation of proteins involved in proliferation and angiogenesis, such as c-myc, cyclin-D1, and hypoxia-induced factor 1- α (HIF1 α) (13). The enhanced expression of HIF1 α entails the expression of VEGF, PDGF and thus contributes to angiogenesis (34, 35). Akt activates multiple transcription factors and regulators of transcription, like CREB, E2F and forkhead transcription factors (16). It further affects murine double minute 2 (MDM2), which controls the activity of the tumor suppressor p53 (16). Thus, Akt is involved in the regulation of survival, proliferation and EMT (16). Also other proteins involved in survival and apoptosis pathways are influenced, like the proapoptotic Bcl-2 associated death (BAD) promoter (16). Also this regulatory pathway has intrinsic feed-back loops to limit its activity. The most important negative regulator of this pathway is the tumor suppressor phosphatase and tensin homologue deleted on chromosome ten (PTEN) (13, 16). It is lost or inactive frequently in human cancers, which has severe consequences since it restricts growth promoting signalling by degrading phosphatidylinositol-3-kinase (PI3K) products and blocking Akt activation (13, 16). It was found to be affected by deletions, promoter hypermethylations, mutations or phosphorylation (13, 16). Also in HCC, PTEN is frequently lost or at least expressed at diminished levels, and upon PTEN knock-out in hepatocytes, mice developed HCC (36). Further negative regulator of the PI3K/Akt/mTOR signalling pathway are the src homology 2 (SH2) domain-containing inositol 5'phosphatases SHIP-1 and SHIP-2 (16).

2.6. The Jak/Stat-pathway

The family of janus kinases (Jak) consists of the four members Jak1, Jak2, Jak3 and Tyk 2 (14, 22, 37, 38). They are cytoplasmic tyrosine kinases constitutively bound to various receptors via their amino terminal domain (FERM) (14, 22, 37, 39). Among these receptors are interferon (IFN), growth factor, hormone or cytokine receptors, and receptors with gp130- or gamma chain of cytokine receptors (γc) - subunits (14, 22, 37, 39). Jaks forward signalling from receptors that do not have intrinsic tyrosine kinase activity, like the gp130 receptor signalling via Stat (6, 14, 22, 37, 39, 40). The diversity of the FERM domain is thought to provide specificity of receptor-binding, and further has a regulatory catalytic function (22, 37, 39). The regulation of Jaks is not entirely understood, and there are hints that there are still unknown modulators, but it is known that their pseudokinase domain has regulatory functions, and mutations in this domain can entail hyperactivation (22, 37, 39). Probably it is also involved in the recognition of substrates (22, 37, 39). Also the SH2 domain could be involved in Jak regulation (22). The kinase domain possesses catalytic activity for tyrosine phosphorylation, and is further the site for interaction with negatively regulating proteins (Fig. 6) (22, 39, 41).

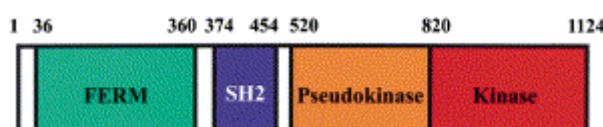
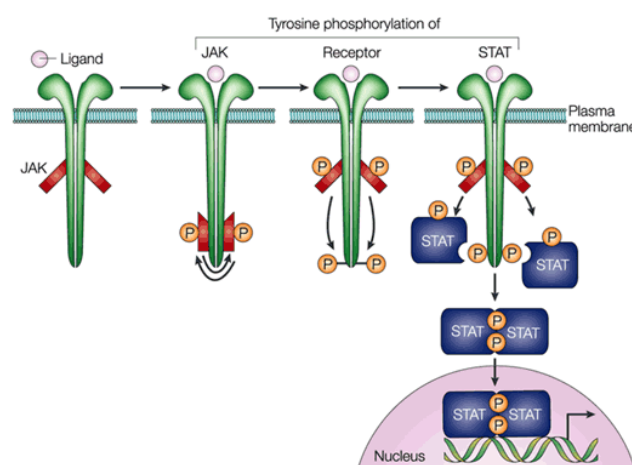


Figure 6: The domains of Janus kinases (39).

However, upon ligand-binding to their cognate receptor and subsequent changes in localization, like receptor subunit dimerization, Jaks can interact with each other and get auto- and transphosphorylated (14, 22, 37, 38). Subsequently, they tyrosine phosphorylate the cytoplasmic tail of the receptor, and thereby create a docking-site for SH2-containing proteins, such as Stats (13-15, 22, 37, 38). Stats then get phosphorylated by Jaks, dimerize and translocate into the nucleus where they modulate transcription of their target genes (Fig. 7) (13-15, 22, 37, 38).



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Figure 7: Activation of the Jak/Stat-pathway (22).

Jaks are crucially important for immunity (14, 39, 42). The lack of Jak1 or Jak3 leads to severe combined immunodeficiency, and mutations that hinder either the binding of Jaks to receptors or inhibit their kinase activity also render the organism with immunodeficiencies (14, 39, 42). Since they forward mainly growth promoting signals, the activation of Jaks is transient and needs to be tightly controlled (14, 15, 22, 37, 39). Aberrations in Jak signalling like activating mutations, permanent ligand binding, enhanced levels of growth promoting signals or disruption of their negative feed-back loops lead to enhanced activity of their substrates and thus changed gene expression, which can contribute to several malignancies, such as cancer (6, 14, 15, 37, 39, 43). Indeed, Jak1, Jak2, mutations were found in several solid cancers and blood malignancies which lead e.g. to autonomous cell-proliferation which is a major characteristics of cancer cells (1, 6, 44, 45). Phosphorylated Jak1, Jak2 and Tyk2 were not detected in healthy liver tissue, but in HCCs (13, 31). Also the Stat3-targets Bcl-xl, Mcl-1, cyclin D1 and c-Myc were found at enhanced levels (13). Interestingly, HBV and HCV can activate Jak/Stat-signalling (13, 26).

The expression of Jak3 was thought to be restricted to the hematopoietic system but more recently was also found in several other tissues, among them liver, and in epithelial cancers (46, 47). Jak3 activating mutations were found in blood malignancies, where they enhanced proliferation, allowed cytokine-independent growth, and triggered Jak3 autophosphorylation and subsequent target gene

activation (46). By modulating the expression of Bax and Bcl-2, and thus contributing to the regulation of apoptosis, Jak3 was found to act anti-apoptotic and induce survival in T-cells (48). But also in breast and gastric carcinomas Jak3 mutations were detected, and both, pharmacological and siRNA-induced Jak3 inhibition could prevent EGF-induced Stat3 and ERK phosphorylation in the SKBR3 breast cancer cell line (45, 49). Furthermore, a Jak3 inhibitor could hinder Stat3 phosphorylation and nuclear translocation upon GH stimulation in C2C12 myoblasts (50). Controversially, Jak3 signaling is thought to be restricted to the γ_c -receptor subunit, which is expressed exclusively in hematopoietic tissues (39, 42). Thus, investigation of Jak3 functions in non-hematopoietic tissues, and to solve the question if Jak3 could bind to receptors beside γ_c , would be highly interesting. Consequently, we did not exclude Jak3 from our experiments.

2.7. Signal transducer and activator of transcription 3 (Stat3)

A prominent oncogene frequently overactivated by aberrant cytokine signalling is the transcription factor Stat3 (14, 15, 39, 51). It is a member of the Stat family of latent, cytoplasmic transcription factors, comprising 7 members (Stat1, 2, 3, 4, 5a, 5b, 6) (14, 15, 39, 51). Stat3 consists of an amino terminal, coiled coil, SH-2 domain, a linker region, DNA binding and the transcriptional activation domain (TAD) (Fig.8) (39, 41, 51).

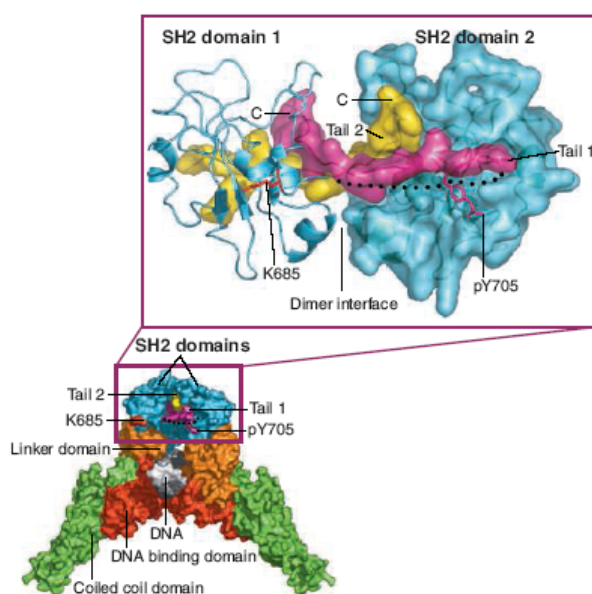


Figure 8: Activated Stat3 dimer bound to DNA. (41)

Stat3 is involved in a variety of cellular functions like differentiation, growth regulation, proliferation, regeneration, inflammation, apoptosis and its functions are tissue-specific. (13-15, 19, 39, 51) Stat3 is essential in early embryonic development, and its inactivation leads to early embryonic lethality (15, 19, 22, 52). Stats are important mediators for forwarding growth factor and cytokine signalling. (15, 51, 53) They forward signals from growth factor and cytokine receptors and their receptor bound Jaks or non-receptor tyrosine kinases (Fig. 9) (15, 51, 53). Stat3 gets quickly activated upon stimulation by a wide variety of cytokines like various interleukins, growth factors such as EGF and HGF, growth hormone, and oncogenes like v-Src which leads to modulation of expression of different gene sets in different tissues (6, 13-15, 19, 39, 51). The IL-6 family members are among the key inducers of Stat3 (6, 14, 54).

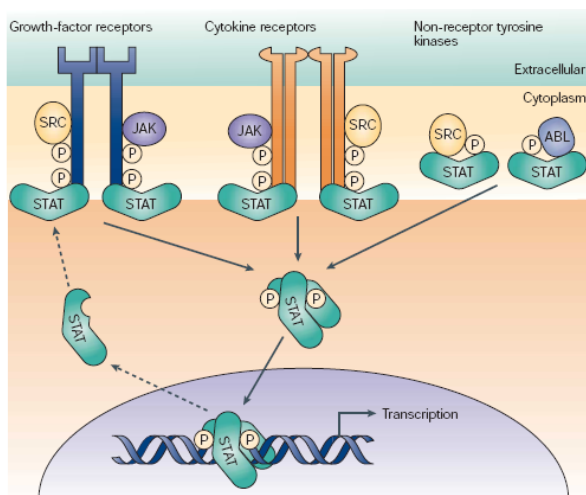


Figure 9: Activation of Stat transcription factors. (53)

Upon binding to a phospho-tyrosine site on the receptor via their SH2-domain, Stat3 gets phosphorylated on its tyrosine 705 (pY-Stat3) by receptor associated Jaks, since most Stat activating receptors, like the gp130 receptor subunit, have no intrinsic tyrosine kinase activity (Fig.9) (6, 14, 15, 39, 53). Still, there are some growth factors signalling via intrinsic receptor tyrosine kinases, such as EGFR and PDGFR (15, 53, 55, 56). Then, Stat3 forms homo- or Stat1/3-heterodimers via reciprocal binding between the phospho-tyrosine and the SH-domains (6, 14, 15, 51, 53). These activated dimers get translocated into the nucleus via importin $\alpha 5$ and Ran, and exported upon dephosphorylation (14, 39, 51, 53). Surprisingly, also

unphosphorylated monomers were found to be translocated into the nucleus, but probably by different mechanisms (39, 57, 58).

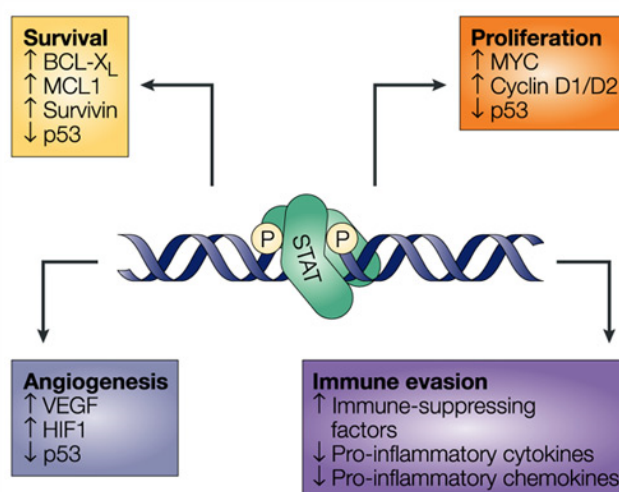
In the nucleus, Stat3 binds to specific DNA sequences and modulates gene expression (14, 15, 39, 51, 53). Mostly it induces transcription, but it can also act repressive (14). Stat3 homodimers and Stat1/3 heterodimers recognize the GAS element, which is an inverted repeat with a 5'-TT(N₄₋₆)AA-3' consensus sequence (14, 39, 54). Stat3 can also recruit co-factors, like co-activators or enhancers, and probably gains its gene specificity via promoter-bound proteins (14, 39, 59). It was shown to interact with Smads, and Stat3 and c-jun interact and cooperate in transcription upon IL-6 stimulation (39, 54, 60). Especially the transcriptional activation domain at the COOH-terminal of Stat3 interacts with transcriptional complexes, and the possibility of Serine 727 phosphorylation in the TAD adds another level of regulation (15, 39, 61). This phosphorylation is performed by MAP kinases and was usually associated with elevated transcriptional activity due to enhanced DNA binding, but it was shown that it can also lead to reduced transcription (39, 61). However, it creates a further possibility for interaction with different transcriptional regulators and the crosstalk with other signalling pathways (39). For example, CBP/p300 and c-jun bind to TAD (54, 59, 60). The coiled-coil domain is another protein-protein interaction site, where Stat3 can interact with transcription factors and regulatory proteins (15, 39, 51, 53, 62).

Stat3 β is a naturally occurring splice variant of Stat3 α that lacks the TAD (19, 63). It was considered as dominant negative factor which it is not actually, since it is able to rescue embryonic lethality of a Stat3 null mutation and to induce expression of Stat3 specific target genes, like acute-phase genes in the liver and cooperate with c-jun to activate transcription (19, 63, 64). Moreover, it can activate and repress genes depending on cellular environment and there is evidence that it counteracts the pro-oncogenic activities of Stat3 α since its overexpression could decrease Bcl_{XL} levels, induce apoptosis and inhibit cell growth in various cancer cells (19). Additionally, it hindered v-src and Stat3-mediated transformation and was able to induce anti-tumorigenic immune responses (19). Still, the distinct functions and mechanisms by which they are executed by Stat3 α and Stat3 β are not entirely clear.

Although unphosphorylated Stat3 (U-Stat3) was considered as transcriptionally inactive for a long time, it was found to be translocated into the nucleus even without being phosphorylated and dimerized (39, 58, 65). Surprisingly, it was further found to

modulate transcription in cooperation with different factors such as NFκB but its putative further functions and interaction partners remain to be elucidated (57).

However, Stat3 can activate different sets of target genes in different tissues and cell-types (19, 22). The main targets of Stat3 are proteins involved in cell cycle regulation and proliferation, inhibition of apoptosis, induction of angiogenesis and inflammation, like cyclins D1/D2 and c-myc, Bcl-X_L, Bcl-2 and Mcl-1, MMP-2, MMP-9 and VEGF and interleukins (6, 15, 53, 66). Thus, unrestricted activation contributes to tumorigenesis and Stat activation is transient and must be controlled strictly to prevent unrestricted growth (6, 15, 53).



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Figure 10: The contribution of Stat3-dependent gene expression to tumorigenesis (53).

Normally, the activation of Stat3 is tightly controlled and lasts between some minutes to hours (6, 15). In cancer, Stat3 was frequently found to be upregulated or constitutively active in several types of solid primary tumors, blood malignancies and cells lines, among them HCC (6, 14, 15, 19, 22, 53, 56, 67-71). Mostly, aberrant and persistent activation is due to unrestricted cytokine and growth factor signalling mediated by tyrosine kinases (6, 15, 39, 53). It contributes to development and progression of cancer by inducing enhanced survival (Bcl_{xL}, Bcl-2), growth and proliferation, angiogenesis, tissue invasion and immune evasion and by inhibition of apoptosis (14, 15, 19, 39, 56, 67, 68, 70). Importantly, it is crucially involved in malignant transformation, since constitutively active Stat3 is able to induce

transformation of NIH3T3 fibroblasts (22, 68, 72). Furthermore, Stat3 was found activated in oncogene-transformed cells and even required for src-induced transformation and forwarding signals from activated oncogenic non-receptor tyrosine kinases, such as v-src (7, 14, 15, 39, 54, 56, 73, 74). Stat3 also contributes to cancer-associated inflammation and inflammation-associated tumorigenesis by regulating the expression of interleukins and chemokines important to establish a cancer-promoting inflammatory environment, and further represses genes for anti-oncogenic immune stimulation (6, 55, 69). The whole IL6/gp130-Jak-Stat3 pathway is an important mediator for tumor-promoting inflammation, which was shown e.g. in mice constitutively active in gp130 that developed carcinomas (6). Moreover, in chronic inflammation that contributes to tumorigenesis, changes affecting the Stat3 pathway were found and Stat3-induced expression of IL-6 and growth factors expression generates a feed-forward loop leading to even more Stat3 activation in the microenvironment (6). Also tumor viruses inducing tumor-promoting inflammatory environments, like HBV and HCV activate Stat3 (13, 26).

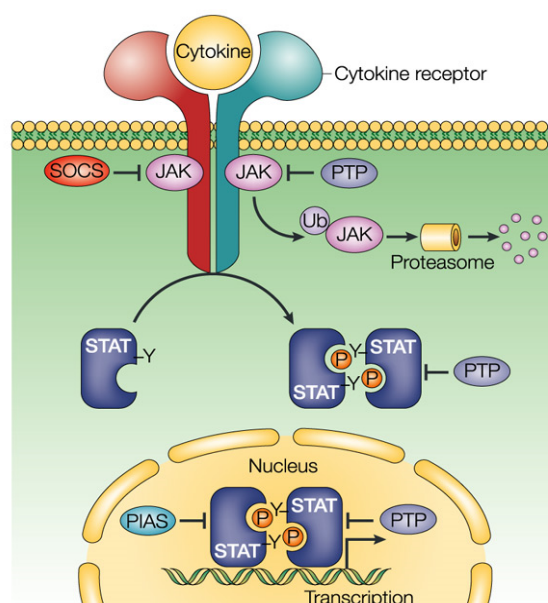
In the liver, Stat3 is important for regeneration by inducing survival and cell-cycle progression and hinder apoptosis (19, 39). Furthermore, it is needed for the induction of inflammatory genes (19). In HCC, Stat3 was found activated in up to 60%, but not in healthy livers, and less in tumor-surrounding liver tissue (13, 31, 69, 71, 75). Stat3-positive tumors were found to be more aggressive and in some studies elevated p-Stat3 levels could be associated with poor prognosis (13, 69, 71).

2.8. Negative regulation of the Jak/Stat-pathway

Negative control of signal transduction, and thus, the limitation of signalling is of highest importance to prevent unrestricted growth-promoting effects (1). The disruption of such control mechanisms required by cancer cells promotes their survival, enhanced proliferation and independence on growth factors and cytokines (1). Also in HCC, disruption of negative signalling pathways contributes to tumorigenesis (5, 14-16).

Concerning gp130-mediated Jak-signalling, this is performed mainly by the family of suppressors of cytokine signalling (SOCS), consisting of the four members SOCS-1, SOCS-2, SOCS-3 and CIS (8, 43, 76). They are cytokine inducible inhibitors of cytokines, containing a SH2 domain and bind to Jaks and/or receptors and thus, inhibit Jak activity or Stat receptor binding (39, 40). Among them, SOCS-1 and

SOCS-3 were found to act specifically on the Jak/Stat-pathway (4, 43, 76). Since their expression is induced by cytokines and Jak/Stat-mediated signalling, they establish a negative feed-back loop (43, 76). By binding to Jaks SOCS inhibit Jak activity and further signalling (43). Since no more Stats get activated, which could in turn induce the expression of SOCS, the negative regulation gets attenuated after a while and the forwarding of cytokine-induced signalling gets possible again (76). CIS and SOCS-3 were found to bind to their receptors and thus, inhibits Stat-binding and activation (8, 77). SOCS family members probably induce ubiquitinylation, followed by degradation of the Jaks or receptors they are bound to (Fig.11) (78, 79).



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Figure 11: Negative regulation of the Jak/Stat-signal transduction pathway (78).

Epigenetic silencing by promoter hypermethylation of SOCS-1 and SOCS-3 was reported in several human cancers, and also in HCC, SOCS-1 was found to be affected in 60% and SOCS-3 in 33% (4, 80, 81). SOCS-1 reintroduction in SOCS-1 silenced cells could suppress Jak-activity and cell growth (43). Interestingly, SOCS-1 hypermethylation is frequent in HCV-induced chronic hepatitis and cirrhosis, with the methylation frequency rising with the stage of fibrosis, the highest found in cirrhosis (4, 82). Upon SOCS-3 reintroduction Stat3 phosphorylation was lost, and proliferation inhibited (43). Also growth and migration were found to be restricted by SOCS-3 signalling via inhibition of Jak/Stat-activity (43). Besides, the knock-out of SOCS-3

under carcinogenic conditions entailed enhanced proliferation, Stat3 activation, inhibition of apoptosis and lead to the development of HCC (43). However, downregulation of SOCS is observed in the majority of HCC (31). Thus, SOCS proteins are considered tumor suppressors involved in HCC (4, 31, 43). Though prolonged pStat3 upon SOCS-3 knock-out and gp130-mediated signalling, the pStat3 signal gets lost, showing that there are still other mechanisms for negative regulation (7). The Src-homology 2 domain-containing phosphatase-1 (SHP-1) also binds to Jaks and inhibits their activity by dephosphorylation, and the protein tyrosine phosphatase (PTP) CD45 was found to dephosphorylate src and Jak family members and thus hinders Stat3 activation, at least in hematopoietic cells (66, 83). Also PTPs acting at receptor level and endocytosis of the receptor contributes to limitation of cytokine-induced signalling as well as E3 ubiquitin ligases inducing proteasome-dependent degradation of kinases (14, 53, 83). In the nucleus, protein inhibitor of activated transcription 3 (PIAS3) recognizes activated Stat3 dimers and hinders them from binding DNA and thus from modulating transcription of their targets (14, 39, 84). PIAS were further found to have E3-ligase activity for sumoylation, so possibly Stat3-sumoylation is another regulatory mechanism (85). Besides, there exist several protein tyrosine phosphatases which limit the activation of Stat3 and thus can induce nuclear export, and also proteolytic degradation of Stat3 is possible (15, 53, 66, 86). PTP1b and PTP receptor –T (PTPRT) are still under investigation (15, 66, 86).

2.9. Targeting Stat3

Like other transcription factors known to contribute to tumorigenesis, Stat3 is a target of inhibition by various means (6). In fact, interruption of the Stat3 signalling cascade by Stat3 knock-down, hindrance of phosphorylation or dimerization entailed diminished or inhibited growth in various diverse *in vitro* and *in vivo* models (6, 15, 70). Also apoptosis was observed as a consequence, in some studies even exclusively in those cells with activated Stat3 (6, 15). Additionally, non-tumorigenic cells or tumor cells without intrinsically activated Stat3 were shown to be less sensitive to the used drugs (15). The multi-kinase inhibitors sorafenib and sunitinib are the only Stat3 inhibitors used in clinics (6, 13). Sorafenib targets VEGFR and thus angiogenesis, PDGFR, FGFR, p38 and Raf kinases and was shown to induce tumor growth suppression, diminished proliferation and apoptosis in preclinical studies (13,

87). They both induce cell cycle arrest and apoptosis, but it is not entirely clear to which extend these effects can be ascribed to the inhibition of Stat3 phosphorylation (6). Moreover, since there are multiple and partly redundant signalling networks involved in tumorigenesis, the potential danger remains that these inhibitions lead to the activation of other oncogenic pathways (1, 6). Stat3 inhibition in tumor cells also affects the tumor microenvironment, since it was observed to change tumor-promoting to tumor-suppressing immune responses (6).

In HCC cells, downregulation of active Stat3 or Stat3 knock-down could enhance chemosensitivity and abolish resistance to ligand-induced apoptosis, the latter effect was achieved by sorafenib (87, 88). Further, the inhibition of dimerization and thus target gene expression entailed diminished growth of various HCC cell lines *in vitro* and in xenografted mice (89).

2.10. Tumor-suppressive effects of Stat3

Though well established oncogenic effects of Stat3, there are hints that it also can act as tumor suppressor in certain genetic backgrounds (90-92). Evidence for this comes from observation in various cancer models. C-myc induced transformation was suppressed by a constitutively activated Stat3 construct in mouse embryonic fibroblasts (MEFs), but had no effect on Ras-induced transformation (91). Moreover, loss of Stat3 in an intestinal cancer mouse model enhanced tumorigenesis but only at later stages of tumor development (92). In glioblastoma, Stat3 can have either pro- or anti-oncogenic effects dependent on the genetic background of the tumor (90). When the tumor suppressor PTEN is missing, knock-out of Stat3 induces transformation of astrocytes, and thus is considered to act as tumor suppressor (90). Furthermore, upon lack of PTEN Stat3 signalling is impaired in murine astrocytes as well as in human glioblastomas (90). On the other hand, Stat3 triggers transformation in PTEN-positive glial cells, by binding to the oncogenic type III variant of EGFR (90). These findings show very clearly how much Stat3 depends on the specific background, and how important the understanding of its putative interactions is to prevent adverse effects in therapy.

2.11. p14^{ARF}

p14^{ARF} and p19^{ARF} (ARF, human and mouse, respectively) are encoded by the CDKN2A locus (human) or INK4a-ARF (mouse) which codes for the two tumor suppressor proteins p14^{ARF} and p16^{Ink4a} both involved in cell cycle regulation (93). They are controlled by distinct promoters but share exon 2 and 3 in alternative reading frames (93, 94). This locus gets activated upon cellular stress, oncogenic and aberrant growth stimulatory signalling and was found to be deleted in various solid and blood cancers (93-96).

p16^{Ink4a} controls the activity of the retinoblastoma protein (Rb) and is involved in inhibition of cell proliferation (93). p14^{ARF} is upregulated upon sustained hyperproliferative signalling, such as oncogenic ras or c-myc and functions as part of check-point response since it restricts proliferation by cell cycle arrest or apoptosis mainly via the murine double minute 2 (MDM2, HDM2 in humans)-p53-pathway, but also by p53-independent pathways (94-97). Besides, the locus is associated with oncogene-induced senescence (94). The best-known function of p14^{ARF} is the stabilization of p53 and thus, induction of cell cycle arrest or apoptosis (93, 96). ARF forms a complex with nucleophosmin (NPM) which enhances its stability and locates in the nucleolus, where ARF cannot interact with MDM2/HDM2 (93, 94, 96). MDM2 is an E3 ubiquitin ligase and negatively regulates the transcription factor p53 by exporting it into the cytoplasm where it undergoes ubiquitin-mediated degradation (93, 94, 96). Upon oncogenic or sustained mitogenic signalling p14^{ARF} binds and translocates MDM2 into the nucleus where it gets sequestered (93, 94, 96, 97). Thus, p53 is released and can initiate cell cycle arrest or apoptosis (Fig. 12) (93, 94, 96, 97).

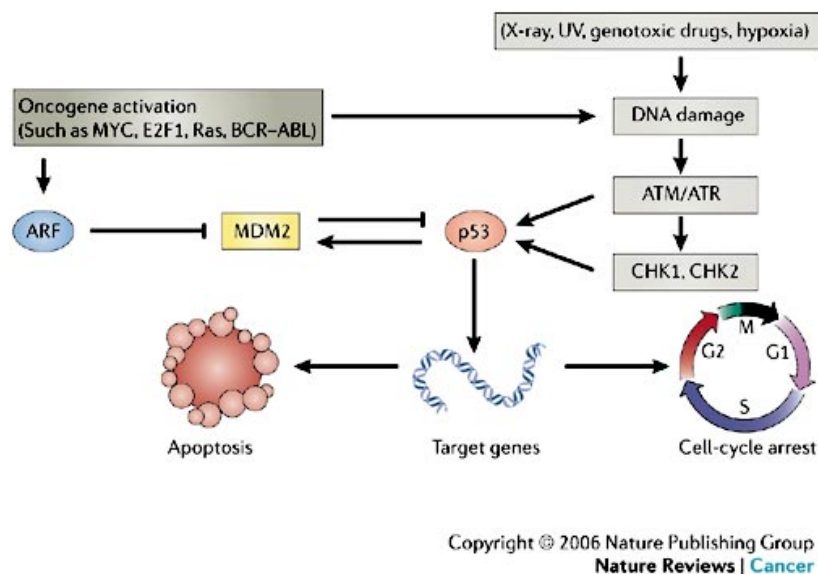


Figure 12: Activation of the tumor suppressive transcription factor p53 to induce cell-cycle arrest or apoptosis (96).

In various cancers the disruption of this ARF-MDM2-p53 axis was found to be impeded frequently (93, 96, 97). Although in HCC the inactivation of p14^{ARF} was only found in cancers with functional p53 and thus loss of ARF and p53 were considered mutually exclusive and functionally equivalent, ARF also has p53-independent tumor suppressor functions (93, 96, 97). It was shown that overexpression of ARF induces cell cycle arrest in p53- and MDM2-negative mouse embryonic fibroblasts and there are speculations that a distinct ARF isoform could trigger autophagy independently of p53, if the p53 pathway is non-functional (96, 98). Moreover, the p53-independent translocation of c-myc and thus the inhibition of its potential to activate transcription was shown in p53-deficient MEFs (99).

Importantly, p14^{ARF} has a variety of interaction partners and by this exerts lots of different functions (93, 95, 96). There are about 30 described binding partners involved in various cellular activities (93, 95, 96). Among them are proteins contributing to transcriptional control, like c-myc, E2Fs and Hif1 α , chromosomal stability and chromatin structure, as well as nucleolar proteins, like NPM and ubiquitin ligases, such as MDM2 and Ubc9, which is required for sumoylation (Figure 13) (95). Among the p14^{ARF}-mediated effects are sumoylation, like MDM2 or NPM, and influencing stability and turn-over of its targets (93, 95, 96). However, binding mostly leads to inactivation of interaction partners (Fig. 13, orange), but also ubiquitin-dependent (Fig. 13, red) and -independent (Fig. 13, pink) degradation, relocalization

and even stabilization and enhanced activation (Fig. 13, green) are possible (95). How $p14^{\text{ARF}}$ influences its targets is not entirely clear, but also its p53-independent anti-oncogenic effects seem to be mainly associated with degradation (95).

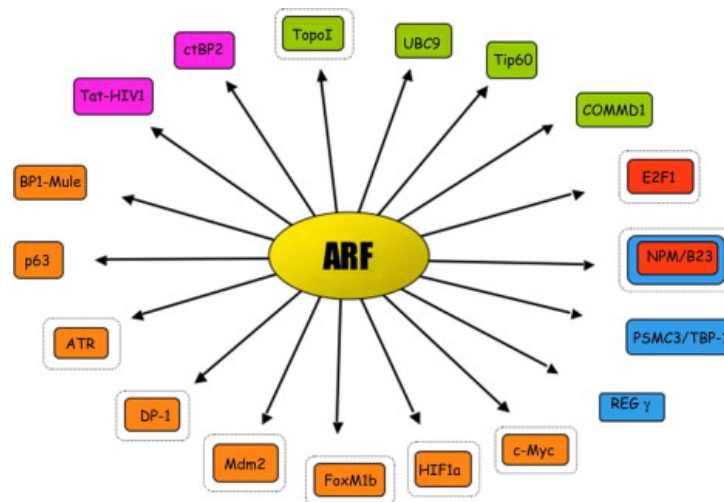


Figure 13: ARF binds to a wide variety of factors and affects them in different ways (95). Orange denotes inactivation, but also ubiquitin-dependent (red) and – independent (pink) degradation, and enhanced activity were observed (green) (95).

Since $p14^{\text{ARF}}$ is a tumor suppressor which is early and frequently altered in HCC, it is considered to be crucially involved in HCC development (100). Biallelic loss, loss of heterozygosity, mutations and promoter hypermethylation were found to be responsible for the lack of functional $p14^{\text{ARF}}$ in HCC (5). Although the CDKN2A locus is located in a chromosomal region frequently deleted in HCC, promoter hypermethylation is the underlying cause rather than loss or mutation (5, 93, 100). Loss occurs seldom in HCC, but hypermethylation was detected in up to 40 % (4, 100). The $p14^{\text{ARF}}$ promoter gets silenced independently of the $p16^{\text{Ink4a}}$ promoter and hypermethylation may directly precede deletion (5, 100). Interestingly, methylation or mutation of $p14^{\text{ARF}}$ are associated with HCV and cirrhosis (43). Promoter hypermethylation of $p14^{\text{ARF}}$ was found in 6% of HCCs with underlying HCV and 27% with underlying HBV infection, and in even 44% cases where alcohol abuse was known (101).

2.12. The TGF- β /Smad-pathway

Transforming growth factor (TGF)- β is a powerful cytokine which is mainly important for tissue homeostasis and tumor suppression at early stages of tumor development (102, 103). TGF- β inhibits proliferation and induces apoptosis (103). Yet, at later stages of tumor progression it can exert opposing effects and promote tumorigenesis (103). TGF- β has the potential to enhance the invasive and metastatic potential of tumor cells (102, 103). Furthermore, TGF- β can influence the tumor microenvironment by generating tumor promoting settings, e.g. by induction of angiogenesis or immune suppression (102-104). Besides, TGF- β represents the most potent fibrogenic factor (105). Since TGF- β plays crucial roles in proliferation and differentiation, it is not surprising that it is required for embryogenesis (102).

The TGF- β family consists of TGF- β and bone morphogenic protein (BMP) branches (106). While the first signal via Smad 2 and Smad3, the latter signal via Smad1, 5 and 8 (106). All of those and the common Smad (Smad4) contain Mad homology regions 1 and 2 (MH1 and MH2), and a linker region between them (106). There exist 3 isoforms of TGF- β , namely TGF- β 1, - β 2 and - β 3, with TGF- β 1 being the one mostly implicated in tumorigenesis (102). TGF- β receptors type I (T β RI) and II (T β RII) possess serine/threonine kinase activity (103). Upon binding of TGF- β to the TGF- β type II receptor, TGF- β type I receptor (also known as ALK5) gets phosphorylated and in turn phosphorylates receptor-associated Smads (R-Smads, Smad2, Smad3) at 2 serine residues in a conserved sequence at the very end of their C-terminal (102, 106-109). They are released, associate via MH2 with Smad4 into heterodi- or heterotrimers and translocate into the nucleus where they modulate transcription of genes with TGF- β responsive promoters, among them the inhibitory Smad7 (Fig. 14) (102, 106-109).

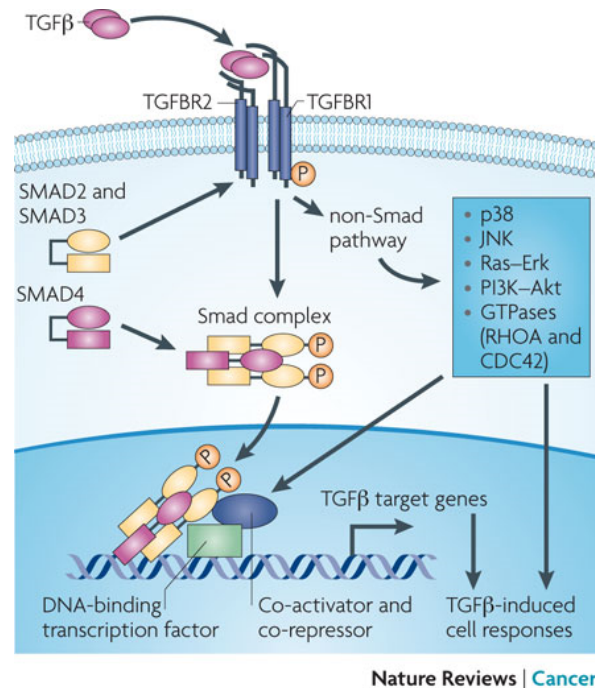


Figure 14: Canonical and non-canonical transduction of TGF- β -induced signalling (110).

Smad3 and Smad4 contain a β -hairpin structure in their MH1 domain which binds the Smad binding element (SBE) 5'-GTCT-3' or 5'-CAGA-3' (108, 111). One or more of these SBEs can be found in Smad-responsive promoters (108, 111). The affinity and specificity of this binding is weak, so the Smads need to assemble into a transcriptional complex to drive gene expression (103, 108, 111). These complexes contain general and specific transcription factors, co-activators or co-repressors (103, 108, 111). The SBEs are located close to the binding sites of these other transcription factors or cofactors, and besides this, Smad-complexes can also bind to some GC-rich promoter sequences (108). Interaction with other transcription factors is mediated via the MH1 domain while Smad4, transcriptional co-activators or co-repressors bind to R-Smads via the MH2 domain (105, 106). Among interacting transcription factors are members of forkhead, zinc-finger, homeobox and bHLH families, stabilizing transcription factors, such as activating transcription factor2 (ATF2), Jun and Sp1, the general co-activator complex CREB-binding protein (CBP)/p300 mediating the interaction with general transcription factors, and more smad-specific co-activators, like SMIF or Swift (106, 108, 112, 113). The binding to various transcription factors with sequence-specific DNA binding and co-factors allows the Smads to form a huge amount of different target-gene activating or repressing transcriptional complexes

(106, 108, 113). Thus, forwarding of a wide variety of TGF- β 1-induced signals leads to different context- and cell-type-dependent cellular responses (106, 108, 113). Since the Smad interaction partners themselves underlie specific regulations, the formation of a functional transcriptional complex is a point of converging with other signalling pathways (113). The transcription factor AP1 forms complexes with Smad that regulate c-jun, collagenase I and MMP-1 (114, 115). An initial event in TGF- β -induced growth arrest is the Smad-mediated expression of the cyclin dependent kinase inhibitors p15^{Ink4B} or p21^{Cip1}, which are normally repressed by the oncogene c-myc (113). The transcription factor c-myc, which acts activating or repressing dependent on its target, is an important repressed target in anti-proliferative TGF- β -signalling, but also cooperates with Smads to induce oncogenic gene expression (103, 106, 116). Also other proteins that are involved in forwarding cell cycle progression like Cdc25A phosphatase or the inhibitors of differentiation 1-3 (ID1, ID2, ID3) are repressed by TGF- β -Smad and thus exert TGF- β anti-proliferative properties (108, 117). On the other hand, the translation inhibitor 4E-BP1 gets expressed upon TGF- β signalling and thus, a halt of proliferation is achieved (118). The action of TGF- β is highly context dependent, even the transcription of the same target can be either activated or repressed by TGF- β dependent on the composition of the Smad transcriptional complex (108, 117). An example is the regulation of ID, where first, activated Smad3/Smad4 induces the expression of ID and ATF3, and upon sustained TGF- β -signalling, the newly expressed ATF3 complexes with Smad3/Smad4, and together they repress ID (108, 117). p38 and JNK Map kinases induce the expression of ATF3 and thus, interfere with this signalling circuit (108, 117). Besides, the PI3/AKT pathway can interfere with the anti-mitogenic activity of the TGF- β pathway (119). Phosphorylation of the transcription factor FoxO inhibits the association into a complex with Smad3/Smad4 to drive the expression of p21 (119). These examples illustrate the lots of opportunities for a crosstalk of TGF β -Smad-signalling with other signalling pathways (119). By binding to certain transcription and co-factors Smad3 can hinder them to perform their function and thus, negatively influence the expression of their target genes (108). Furthermore, Smad3 is capable of recruiting histone deacetylase 4 and by this induce epigenetic repression of its targets (120). TGF- β activates also Smad-independent signalling cascades (Fig.14) (106). It can activate the extracellular-signal-regulated (Erk) kinase, Jun N-terminal kinase (JNK), p38 mitogen-activated protein (MAP) kinase, Src kinase and the

phosphatidylinositol 3'-kinase (PI3K) pathways. Furthermore, TGF- β induces the activation of Ras via Erk/MAPK signalling and via TGF- β associated kinase 1 (TAK1)/p38/MAPK signalling (113). Ras and TGF- β can be rivals or colleagues, since on the one hand, the activation of Erk/MAP kinase pathway by Ras can trigger the inhibition of TGF- β 1-induced growth arrest by negatively affecting Smad3 (106, 121). On the other hand, Ras and TGF- β 1 can act synergistically, since MAP kinase signalling is a non-canonical TGF- β signaling pathway (106, 121). In addition, Smad2 and Smad3 can be activated independent of TGF- β by stress signals, JNK/c-Jun- and p38/ATF2-signalling (113). Since pro-inflammatory cytokines activate JNK, they can induce Smad2/3-mediated signalling (122, 123).

Negative regulation of Smad-signalling adds another level of complexity to the TGF- β pathways (106, 108). Inhibitory Smads 6 and 7 (I-Smads) compete for receptor binding and Smad7 induces receptor I degradation via a smurf-Smad7 complex and ubiquitinylation (124, 125). Both I-Smads are induced by the TGF- β family to establish a negative feed-back loop (124). While Smad 6 mainly targets the BMP-pathways, Smad 7 affects members of both family parts (124). Furthermore, Smad7 is activated by EGF/MAPK- and INF- γ /Jak1/Stat1-pathways and hinders Smad2/3 to form complexes with Smad4 (102, 113). Downstream in the signalling cascade, the oncogenic protein Ski and SnoN can bind to Smad2, Smad3 and Smad4 and by this compete with CBP/p300, disrupt Smad complexes, recruit other co-repressor or hinder the binding of co-activators (106, 112). The expression of the Smad co-repressors Ski and SnoN is induced by TGF- β , and thus causes limitation of TGF- β -signalling (112). Another protein, SNIP1, hinders the formation of functional transcriptional complexes by binding to CBP/p300 and Smad4 (126). In the nucleus, R-Smads get dephosphorylated, which causes the dissociation of the transcriptional complex and export of its components (108). Degradation is mainly mediated by ubiquitinylation (106, 108).

Besides at their conserved C-terminal serine residues, Smads can also be phosphorylated at serine and threonine sites in their more divergent linker region (106, 108). This allows a more selective crosstalk with their upstream signals and other signalling pathways (108). Among the kinases capable of phosphorylating the linker region are the ERK MAP kinases Erk1 and 2, the JNK MAP kinase, CDKs, TAK-1 kinase, calcium-calmodulin-dependent protein kinase II and G-protein-coupled receptor kinase-2 (122, 127-131). The consequences of Smad-linker phosphorylation

are not entirely clear (132). Mostly, diminished translocation and activity were reported, like Erk MAP kinase-mediated linker phosphorylation of Smad2 and Smad3 interfering with nuclear translocation and thus, inhibiting transcription (122, 129, 133). On the other hand, Erk-mediated Smad4 linker phosphorylation is followed by improved p300/CBP binding and thus, enhances Smad transcriptional activity (134). Moreover, linker phosphorylation is associated with oncogenic signalling and cancer (108). Besides this, the linker region is the point of interaction with ubiquitin ligases (106).

The TGF- β -Smad pathway and thus its tumor-suppressive function are frequently in human cancers (103, 132). The deregulation can affect different parts of the pathway. Mutations or loss of T β RI or T β RII, Smad4 or Smad2 were found in pancreatic, colon, breast and ovarian carcinomas, gliomas and T-cell lymphomas (102, 103). Also epigenetic changes of regulatory components, amplification or overexpression of inhibitors or hyperactivation of signalling pathways converging in Smad-signalling contribute to tumorigenesis (135). Once, TGF- β switched to oncogenic activity, its own overexpression, the expression of MMP-2 and MMP-9 and the expression of VEGF induce fibrosis, immune evasion, cell invasion and angiogenesis which mainly contribute to tumor promotion (103, 110, 136, 137). TGF- β is considered to induce epithelial-to-mesenchymal transition (EMT) since characteristic morphological changes typical for this process could be observed first in rat kidney fibroblasts and later in various carcinoma cell lines exhibited to TGF- β (102, 138). TGF- β -induced EMT is accompanied by TGF-mediated expression of Snail and Slug (110, 139). TGF- β is also involved in the regulation of cell adhesion. (103) First, c-myc and Smad complexes cooperate to enhance the expression of snail, and then Smad complexes together with Snail repress epithelial genes, such as E-cadherin (140, 141). The evasion from immune surveillance strongly supports tumorigenesis, and TGF- β acts as immune suppressor by affecting most cells of the innate and the adaptive immune system in their development and function (1, 104, 112). Among them are macrophages, natural killer cells, and regulatory as well as effective T-cells (112). Moreover, TGF- β can induce immune tolerance at high levels (112). Since tumors secrete TGF- β and high TGF- β plasma levels are often found in patients, this seems to be a common mechanism of tumors to escape from destruction by the immune system (102, 112).

In the liver mainly hepatic stellate cells produce and secrete TGF- β 1, which is crucially important in liver regeneration since its anti-mitotic effects terminates unlimited hepatocyte proliferation by inhibition of hepatocyte growth factor (HGF) (142). In the course of HCC development the TGF- β 1-Smad pathway is involved in the molecular mechanisms of chronic liver diseases like chronic hepatitis B infection and fibrosis, the setup of a tumor-promoting environment, angiogenesis, EMT, immune suppression and tumor recurrence (123, 142-144). For example, TGF- β 1 overexpression was observed in fibrosis. (110) The hepatitis B virus protein X was shown to shift the pSmad3C isoform towards a JNK-mediated pSmad3L isoform resulting in enhanced cell growth and activation of the c-myc pathway (144). Additionally to its oncogenic properties, the pSmad3L-pathway acts fibrogenic, since it induces extracellular matrix deposition and thus the development of liver fibrosis (123). The Smad-interacting protein 1, a transcription factor involved in E-cadherin expression control, was found to be silenced in HCC by promoter hypermethylation (143).

2.13. Aim of the study

Stat3 is a transcription factor known to be activated in malignant hepatocytes that contributes to enhanced proliferation and tumorigenesis (31, 51, 69, 71). Since tumor-suppressive properties were observed under certain circumstances, e.g. in the lack of the tumor suppressor PTEN in glioblastomas, it is an open issue if Stat3 can act tumor-suppressive in HCC (90, 91). Because there are clinical efforts to inhibit Stat3, it is of highest importance to understand the mechanisms converting Stat3 to a tumor suppressor.

Unrestricted proliferation is a major hallmark capability of cancer and the suppressor of hyperproliferative signalling termed p14^{ARF} is lost early and frequently during hepatocarcinogenesis (1, 100). Most notably, recent studies revealed that Stat3 exhibits tumor suppressive effects in HCC cells (145). To clarify the underlying molecular changes in Stat3 signalling and characteristics we focused on Stat3 activation by Jak-mediated tyrosine 705 phosphorylation dependent on p14^{ARF}. First, we wanted to know if *de novo* synthesis is required for IL-6-induced Stat3 phosphorylation in both p14^{ARF}-positive and p14^{ARF}-negative HCC cells. Next, we addressed the question whether Jaks are able to phosphorylate Stat3 in the presence or absence of p14^{ARF}. All experiments were performed with two human HCC cell lines, both lacking or expressing p14^{ARF}.

TGF- β and Stat3 are both involved in processes promoting tumorigenesis by regulating apoptosis and EMT (1, 6, 15, 112). While TGF- β induces apoptosis at early stages of tumor development, Stat3 mediates transcription of anti-apoptotic factors of the Bcl-2 family (1, 6, 112). Since the ability to resist apoptotic triggers is an important characteristic of cancer cells, we focused on a putative interaction of these two players (1). Since we previously found that Stat3 is required to exert TGF- β -induced apoptosis, we addressed the question whether Stat3 has an impact in the canonical TGF- β /Smad signalling by analyzing the nuclear translocation of Smads. The cells used for this approach were murine hepatocytes, hepatocytes with deleted Stat3 and those cells with reintroduced wt-Stat3.

3. Materials & Methods

3.1. Cell lines & Cell culture

The human hepatocellular carcinoma (HCC) cells Plc/prf/5 (Plc) and Plc/prf/5-p14^{ARF} (Plc-p14) cells were used as model system. (145) Plc cells lack p14^{ARF} and express mutated p53. Exogenous p14^{ARF} has been reintroduced into these cells by retroviral transmission, generating Plc/prf/5-p14^{ARF} cells. In order to accomplish retroviral transmission, human Plc cells transiently expressing the wzl-receptor were overlaid with a supernatant containing p14^{ARF}-viral particles. (146) Retroviral particles were produced from phoenix cells after transfection of the pMSCV plasmid harbouring wild type (wt) p14^{ARF}. Transient transfection was performed with SatisFfection according to the guidelines of the manufacturer (Stratagene, Santa Clara, USA). Ectopic expression of p14^{ARF} was detected by Western blot analysis as outlined below. Immortalized Plc and Plc-p14 cells were cultivated in DMEM plus 10% fetal calf serum (FCS) and 1% antibiotics (penicillin and streptomycin) at 37° C and 5% CO₂.

Human Hep3B and Hep3B-sh p14^{ARF} hepatoma cells were employed as a further cellular liver cancer model. Parental Hep3B cells express wild type p14^{ARF} while Hep3B-sh p14^{ARF}_2 and Hep3B-sh p14^{ARF}_3 cells show each a stable knock-down of p14^{ARF} after lentiviral transmission of distinct small hairpin (sh) RNA directed against p14^{ARF}. shRNA sequences targeting p14^{ARF} as well as a scrambled control were cloned into the pLKO.1 lentiviral vector and Hep3B cells were infected with a VSV-G pseudotyped virus by spin infection and subsequently selected with 2 µg/ml puromycin. (145) Hep3B cells and their derivatives were cultivated in RPMI 1640 plus 10% FCS and 1% antibiotics (penicillin and streptomycin) at 37°C and 5% CO₂.

Mouse hepatocyte cell lines were generated by isolation of p19^{ARF}^{-/-} hepatocytes from p19^{ARF}^{-/-} mice, which lack the 1β exon of the locus and thus express p16^{INK4a} but not p19^{ARF} (MIM-1-4, designated as MIM in the following). (147, 148) Stable retroviral transmission of these cells with a construct expressing oncogenic Ras yielded MIM-Ras cells (MIM-R). (149)

To obtain cell lines lacking p19^{ARF} and Stat3, p19^{ARF}^{-/-} mice were crossbred with Stat3^{Δhc} mice. (150) Thus, Stat3^{Δhc}/p19^{ARF}^{-/-} double-null mice were generated and hepatocytes were isolated and subjected to single cell cloning, resulting in the cell

lines MIM-Stat3^{Δhc}-1 and MIM-Stat3^{Δhc}-2. (145) By stable retroviral transmission of a construct expressing oncogenic v-Ha-Ras, Ras-transformed MIM-R-Stat3^{Δhc}-2 cells were generated. For Stat3 reintroduction, MIM-Stat3^{Δhc}-2 and MIM-R-Stat3^{Δhc}-2 cells were subjected to stable retroviral transmission of a construct expressing wtStat3 (MIM-Stat3^{Δhc}-2-wtStat3, MIM-R-Stat3^{Δhc}-wtStat3. Retroviral transmissions were performed as described. (91) Cells were cultured on collagen-coated dishes in RPMI plus 10% FCS and 1% antibiotics (penicillin and streptomycin). Additionally, MIM-derived cells not transformed with oncogenic Ras required the supply with 40 ng/ml transforming-growth factor-α (TGF-α) (Sigma, St. Louis, USA), 30 ng/ml insulin-like growth factor (IGF-II) (Sigma), and 1.4 nM insulin (Sigma). (151, 152) All cells were propagated by splitting 1:4 twice a week and were routinely checked for the absence of mycoplasma.

Inhibitor treatment

3 x 10⁵ (Plc, Plc-p14) or 2,5 x 10⁵ (Hep3B, Hep3B-shp14) cells were seeded per 6-well to obtain 60-70% confluency overnight. Cell culture medium was changed and inhibitors and/or growth factors were added at the indicated concentrations and for the indicated time (Table 1). Inhibitors were tested in pilot studies for dose- and time-dependent efficacy. Inhibitors were used twice as high as the inhibitory concentration (IC)₅₀ –values as provided by the suppliers.

Table 1: Overview of inhibitors used in the study

Name	Inhibition	Concentration	Time of treatment	Company
actinomycin D	transcription	0.1 / 1 µg/ml	24 h	Sigma (St.Louis, USA)
cycloheximide	translation	1 / 10 µg/ml	24 h	Sigma
Jak-Inhibitor I	Jak1-3, Tyk2 (pan-Jak)	10 ng/ml	1 h	Calbiochem Merck, (Darmstadt, Germany)
AG490	Jak2	20 µM	24 h	Calbiochem Merck
Jak3 inhibitor VI	Jak3	60 nM	24 h	Calbiochem Merck
AG9	Tyk2	50 µM	24 h	Calbiochem Merck

Table 2: Growth factors used in the study

Growth factor	Name	Concentration	Time of treatment	Company
IL-6	rh-IL-6	20 ng/ml	20 min	R&D Systems, (Minneapolis, USA)

TGF- β 1 treatment and proliferation kinetics

1 x 10⁵ MIM, MIM-Stat3 ^{Δ hc}-1, MIM-Stat3 ^{Δ hc}-2, MIM-Stat3 ^{Δ hc}-2-wtStat3, MIM-R and MIM-R-Stat3 ^{Δ hc}-2 cells were each seeded in triplicate. Cell culture medium was changed and cell number determined every second day in a cell analyser (CASY; Schärfe Systems, Reutlingen, Germany). Cumulative cell numbers were calculated from all counts and dilution factors. (153) To analyze the influence of TGF- β 1, 1ng/ml TGF- β 1 (R&D Systems, Minneapolis, USA) was added to the cell culture medium.

TGF- β 1 treatment for immunofluorescence

For immunofluorescence analysis, 5 x 10⁴ cells (MIM, MIM-Stat3 ^{Δ hc}-2, MIM-Stat3 ^{Δ hc}-2-wtStat3, MIM-R, MIM-R-Stat3 ^{Δ hc}-2, MIM-R-Stat3 ^{Δ hc}-2-wtStat3) were each seeded per flexiperm-slot on a collagen-coated glass slide to obtain 70-80 % confluency overnight. Cell culture medium was changed and 2,5 ng/ml TGF- β 1 were added for 1 hour. Cells were fixed as described below.

3.2. Immunoblotting

Preparation of protein extracts

All steps were performed on ice. After discarding cell culture medium and washing the cells twice with ice-cold phosphate buffered saline (PBS), protein extracts were prepared using 50 μ l RIPA (Radioimmunoprecipitation assay) buffer plus freshly added protease- and phosphatase-inhibitors (composition see below) per 6-well. Cells were scraped into microcentrifuge tubes, snap-frozen in liquid nitrogen, thawed on ice and incubated for 10 minutes prior to centrifugation for 15 minutes at 16.000 x g and 4°C. The protein-containing supernatant was transferred into fresh tubes.

To determine protein concentration, 5 μ l of a 1:5 dilution of protein extract are mixed with 200 μ l Bradford solution (1:5 solution of Bradford stock solution, BioRad, Hercules, USA) in a 96-well plate. The resulting color reaction was photometrically measured in a microplate reader and protein concentrations calculated by using a

calibration curve consisting of bovine serum albumin (BSA) dilutions (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 µg/µl protein).

Protein extracts were diluted to a concentration of 2 µg/µl with sodium-dodecyl-sulfate (SDS)-loading buffer (composition see below) and ddH₂O, incubated at 95°C for 5 minutes, vortexed and loaded on gels.

SDS-polyacrylamide gel electrophoresis

Separation gels containing polyacrylamide (PAA) with a given percentage were generated to separate the protein of interest. The PAA gel was casted between the spaced glass plates of the gel casting equipment (Bio Rad, Hercules, USA) and overlaid with isopropanol, which was discarded after polymerization.

Separation gel (sufficient for 2 gels)

	12 % PAA
ddH ₂ O	4,42 ml
2 M Tris; pH 8,8	2,25 ml
30 % PAA / 1%PDA	5,2 ml

+ 50 µl 10% ammonium persulfate (APS) + 8 µl tetramethylethylenediamine (TEMED, Sigma)

The stacking gel and combs were applied and gel polymerized.

stacking gel (sufficient for 2 gels)

ddH ₂ O	3 ml
2M Tris; pH 6,8	0,5 ml
30 % PAA / 1%PDA	0,5 ml

+ 20 µl 10% APS / + 4 µl TEMED

Protein samples containing 30 µg protein were loaded and electrophoretically separated according to their size at 5 V/cm in a electrophoresis equipment (Bio Rad, Hercules, USA) filled up with electrophoresis buffer (composition see below). Proteins were blotted on a nitrocellulose membrane at 100 V for 1 hour in a blotting equipmment (Bio Rad, Hercules, USA). The blotting chamber contained blotting buffer (composition see below), a thermal pack and stirring staff to avoid destruction

of proteins due to developing heat. Membranes were stained with Ponceau solution to visualize proteins. Membranes were destained with ddH₂O before blocking in Tris-buffered saline-0,1% Tween (TBST)/3% BSA for 1 hour.

Membranes were incubated with 3 ml primary antibody solutions overnight at 4°C on a turntable.

Table 3: Primary antibodies used in the study

Protein	Weight	Dilution	Blocking reagent	Source	Company
pStat3	86, 79 kDa	1:1000	BSA or milk	rabbit	Cell Signaling (Beverly, USA)
Stat3	86, 79 kDa	1:1000	BSA or milk	rabbit	Cell Signaling
Actin	42 kDa	1:2000	TBST	rabbit	Sigma (St.Louis, USA)

Primary antibodies were collected for re-use and membranes were washed 3 times for 15 minutes with TBST before applying 3 ml secondary antibody solution for 1 hour at room temperature.

Table 4: Secondary antibody used in the study

	Dilution	Company
Peroxidase-conjugated rabbit IgG	1:10.000 in TBST	Vector Laboratories (Southfield, USA)

The secondary antibody was discarded and membranes were washed 3 times with TBST for 15 minutes.

For signal detection, membranes were incubated with luminol/coumarin working solution (composition see below) plus 3 µl/ml 3% H₂O₂ for 2 minutes, then put into a film cassette and developed.

For reprobing with primary antibodies, membranes were stripped in 3 ml stripping solution (composition see below) + 7 µl/ml β-mercaptoethanol for 30 minutes at 50°C. Membranes were extensively washed before proceeding with blocking and subsequent incubation with primary antibodies as described above.

3.3. Immunofluorescence

After washing cells twice with cold PBS for 5 minutes, Histofix 4% formaldehyde-solution (Roth Lactan, Graz, Austria) was added for 30 minutes at room temperature to fix cells. Formaldehyde-solution was to be handled in the extractor hood and discarded separately. After washing once with cold PBS for 5 minutes, NH_4Cl -solution (125 mg/ 50 ml ddH₂O) was added for 5 minutes to inactivate the formaldehyde-solution and glass slides were gripped in a cassette. To permeabilize the membranes, 0,05 % Triton (Roth Lactan, Graz, Austria) in PBS was added for 5 minutes and afterwards, slides were washed again with PBS for 5 minutes. Next, blocking solution consisting of 0,2% fish gelatine (Sigma, St. Louis, USA) in PBS was added for 30 minutes before 150 μl first antibody solution was applied for 1 hour.

Table 5: Primary antibody used in this study

protein	dilution	source	company
Smad2/3	1:100 in blocking solution	mouse	BD (Franklin Lakes, USA)

Slides were washed with PBS for 5 minutes. From here on all steps were performed in the dark. The secondary antibody mix was applied for 45 minutes and afterwards slides were washed three times with PBS and once with ddH₂O for 5 minutes each.

Table 6: Secondary antibody solution used in this study

reactivity	name	dilution	company
mouse IgG	anti-mouse-488-FITS	1:1000	Invitrogen, (Carlsbad, USA)
actin	Phalloidin-TexasRed	1:500	Invitrogen
nuclei	Top-pro	1:5000	Invitrogen

Slides were removed from the cassette, covered with moviol (Fluca, Seelze, Germany) and a glass top and stored in the dark at 4°C.

The confocal laser microscope TCS-SP (Leica, Heidelberg, Germany) was used for signal detection.

3.4. Buffers, Solutions and Equipment

RIPA-buffer: 50 mM Tris (pH 7.4)

150 mM NaCl

1 mM β -glycerophosphat (pH 7.2)

0,5 % DOC (Na-deoxycholate)

1 % Nonidet P-40

set pH 7.4, store at 4°C

+ Na_3VO_4 1mM (inhibition of membrane-bound enzymes)

+ NaF 1mM (phosphatase inhibitor)

+ Leupeptin 10 μ g/ml (protease inhibitor)

+ Aprotinin 10 μ g/ml (protease inhibitor)

+ PMSF 1mM (protease inhibitor)

storage at -20°C

5x SDS sample buffer: 250 mM Tris (pH 6.8)

10 % SDS

30 % glycerol

5 % β -mercaptoethanol

100 nM dithiotitol

some crumbs bromphenolblue

10xTris/glycine: 30g Tris

144g glycine

ddH₂O to 1liter

(RT)

Electrophoresis buffer: 25mM Tris

192mM glycine (100ml 10xTris/Glycine in 1 liter)

0,1 % SDS

(RT)

Blotting buffer: 25mM Tris

192mM glycine (100ml 10xTris/Glycine in 1 liter)

0,02 % SDS

15 % Methanol

(RT)

Luminol/Coumarin working solution: 200ml 0,1 M Tris (pH 8.8)

+ 500 µl p-coumarin acid (340 ng/26 ml DMSO)

+ 1ml luminol (2,26 g in 51 ml DMSO)

+ freshly 3% H₂O₂ 3 µl/ml

Stripping buffer: 20 nM Tris pH6.8

2 % SDS

(RT)

+ β-mercaptoethanol 7µl/ml

Primary antibodies: pStat3 (Tyr⁷⁰⁵) and Stat3 (Cell Signaling, Beverly, USA)

actin and p14^{ARF} (Sigma, St.Louis, USA)

smad2/3 (Invitrogen, Carlsbad, USA)

phalloidin-Texas Red (Invitrogen)

Top-pro-3 iodide (Invitrogen)

store at -20°C

Secondary antibodies: peroxidase-labelled rabbit IgG (Vector Laboratories,

Southfield, USA)

anti-mouse-488-FITS (Invitrogen)

store at 4°C

Equipment

Cell culture plates 96-well, 6-well, 6cm, 10 cm (CytoOne, Orlando, USA)

Incubator: HERAccl 150i (ThermoScientific, Waltham, USA)

Light microscope: Nikon TMS (Nikon,Tokyo, Japan)

Cell counter and analyser system (Schärfe System, Reutlingen, Germany)

refrigerated centrifuge Megafuge 1.0 R (Kendro Laboratory products, Langensebold, Germany)

microtiter carrier # 2704 (Heraeus instruments, Hanau, Germany)

tubes, 15-ml, 50-ml falcons (greiner bio one, Kremsmünster, Austria)

refrigerated centrifuge 5415R + rotor F45-24-11 (both eppendorf, Hamburg, Germany)

table centrifuge: Sigma 1-3 + rotor 12034 (both Sigma, St.Louis, USA)

Thermomixer compact (eppendorf)

Vortex Genie-2 (Scientific Industries, Bohemia, USA)

Expert Plus Microplate Reader (ASYS, Dornstadt, Germany)

Gel casting equipment, electrophoresis and blotting chamber (BioRad, Hercules, USA)

Turntable Duomax1030 (Heidolph, Schwabach, Germany)

Hypercassette (Amersham Life Science, Arlington Heights, USA)

BioMaxMS Intensifying screen (Kodak, Rochester, USA)

Hyperfilm ECL (GE Healthcare, Chalfont St Giles, UK)

Optimax 2010 X-ray film processor (PROTEC Medizintechnik, Oberstenfeld, Germany)

Histo-cassette rack (Thermo Scientific)

TCS-SP confocal microscope (Leica, Heidelberg, Germany)

Remark:

Parts of the results presented in the following, are published in Schneller D, Machat G, Sousek A, Proell V, van Zijl F, Zulehner G, Huber H, et al. p19(ARF) /p14(ARF) controls oncogenic functions of Stat3 in hepatocellular carcinoma. Hepatology 2011.

Contribution to this publication: ascertainment of the need for *de novo* RNA and protein synthesis for Stat3 phosphorylation irrespective of the expression of p14^{ARF}, identification of Jak1 as crucial mediator of Stat3 phosphorylation independently of p14^{ARF}, development of the model mechanism of nuclear U-Stat3/ARF interaction.

4. Results

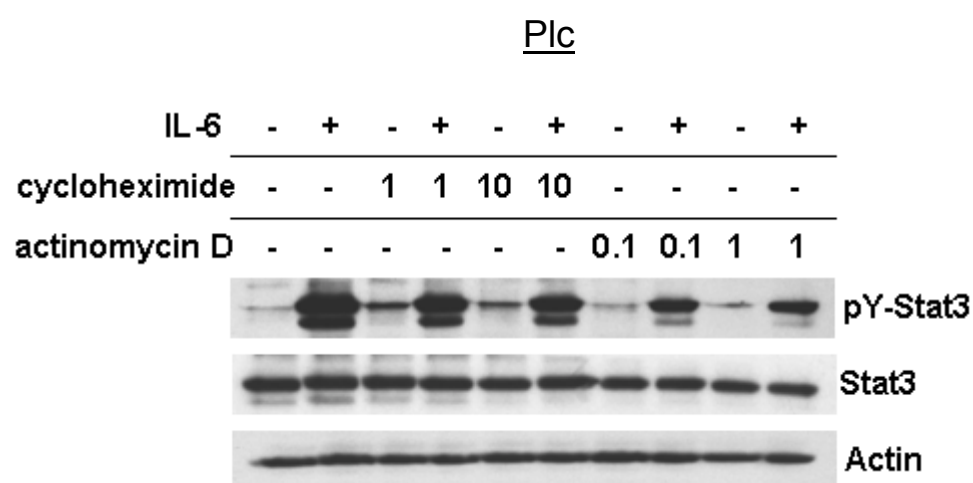
De novo synthesis of an upstream mediator is required for Stat3 phosphorylation independently of p14^{ARF}

To get insights into the regulation of Stat3 tyrosine 705 phosphorylation in the presence or absence of p14^{ARF} in the human hepatoma cell lines Plc and Hep3B we foremost addressed the question if *de novo* synthesis is required. Inhibition of transcription with actinomycin D as well as inhibition of translation with cycloheximide lead to a diminished level of tyrosine 705 phosphorylated Stat3 (pY-Stat3) upon stimulation with IL-6. Stat3 itself remained unaffected in Plc cells (Fig. 15A, B). Comparable results were obtained in Hep3B cells (Fig. 15C, D). Thus, *de novo* synthesis of an upstream mediator is required to forward the IL-6 signal by tyrosine 705 phosphorylation of Stat3. Since the loss of the pStat3 signal could be observed in Plc and Hep3B expressing or lacking p14^{ARF}, the requirement for *de novo* synthesis is not dependent on the p14^{ARF}-status in neither of them.

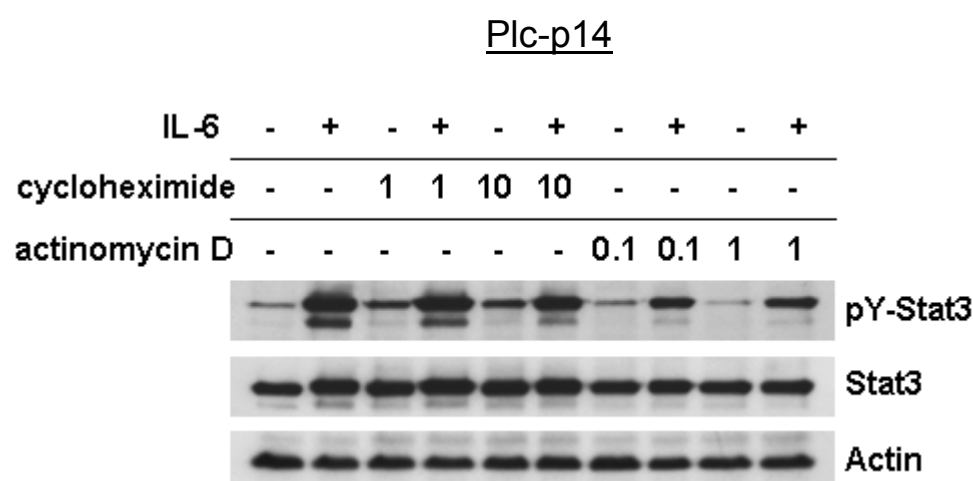
From these data we conclude that *de novo* synthesis of an upstream mediator is necessary for Stat3 705 tyrosine phosphorylation upon IL-6 treatment. However, this regulatory event is independent of p14^{ARF} expression.

Figure 15

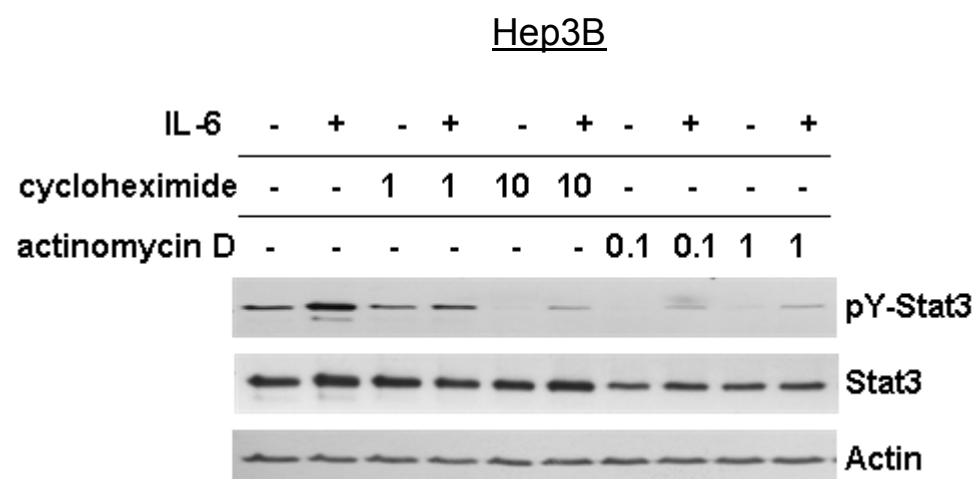
A



B



C



D

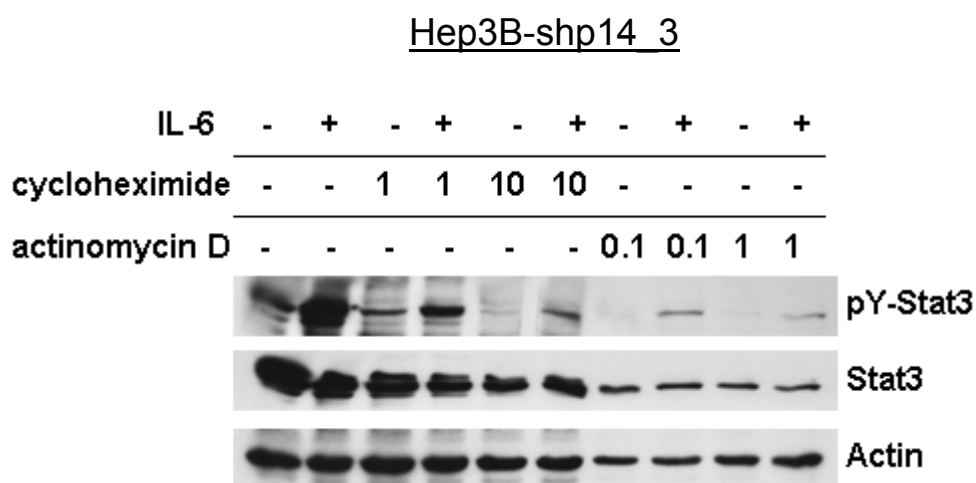


Figure 15: *De novo* synthesis is required for IL-6 induced Stat3 tyrosine phosphorylation.

(A) Plc, (B) Plc-p14, (C) Hep3B and (D) Hep3B-shp14 cells were treated with actinomycine D or cycloheximide to block transcription or translation, respectively, for 24 hours at indicated concentrations ($\mu\text{g}/\mu\text{l}$). Cells were treated with 20 ng/ml IL-6 20 minutes prior to cell lysis. Western blots were probed with anti-pY-Stat3, and re-probed with an anti-Stat3. Actin was used as loading control.

Inhibition of Janus kinases (Jaks) abolishes phosphorylation of Stat3 upon IL-6 treatment

To get a more detailed insight into the mechanism of Stat3 phosphorylation in our cell lines, we addressed the question if Janus kinases are necessary to forward IL-6 signaling and if p14^{ARF} has an influence on that. There are four members of the Jak family, Jak1, Jak2, Jak3 and tyrosine kinase 2 (Tyk2), which are known to transduce signals from activated cytokine and growth factor receptors. Upon ligand-binding, Jaks get phosphorylated and in turn phosphorylate the receptor they are bound to. Thereby docking sites for SH2-domain containing proteins like Signal transducers and activators of transcription (Stats) are created at the receptor. Upon binding to these sites Stat3 gets phosphorylated at its tyrosine 705, dimerizes and translocates into the nucleus where it modulates transcription of its target genes (66). To analyze whether Jaks are necessary for this signal transduction, all four Jaks (pan-jak) were blocked and the levels of tyrosine phosphorylation of Stat3 upon IL-6 treatment evaluated (Fig. 16). This experiment was performed in Plc and Hep3B cells each expressing or lacking p14^{ARF} to determine the influence of p14^{ARF}.

Figure 16

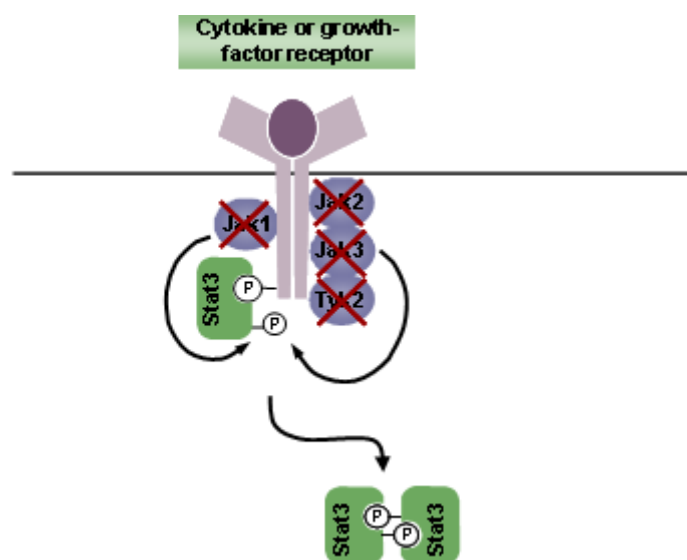
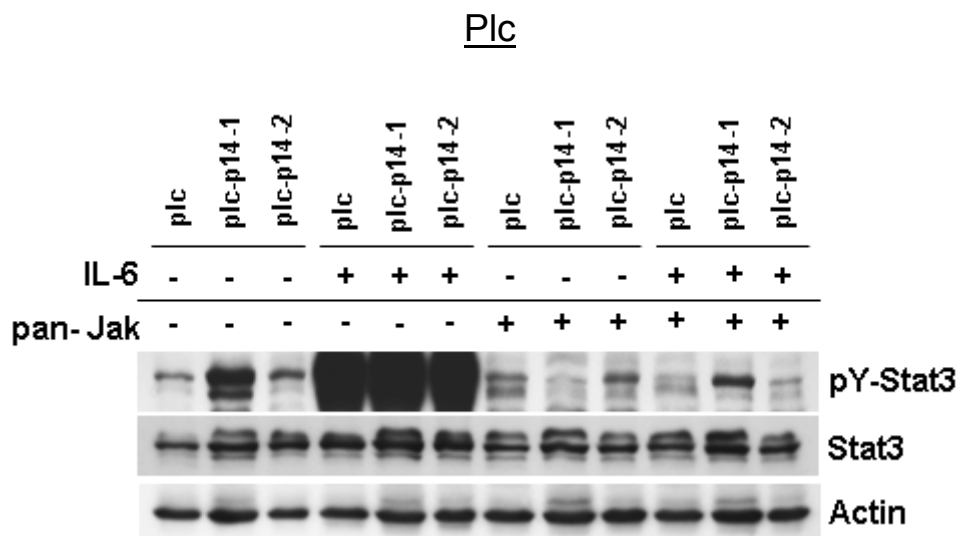


Figure 16: Model of pan-jak blocking. To determine the dependence of phosphorylation of Stat3 upon IL-6 on Janus kinases, all members of the Jak family of kinases were blocked with a pan-jak inhibitor and the effect of IL-6 on Stat3 phosphorylation was evaluated.

Figure 17:

A



B

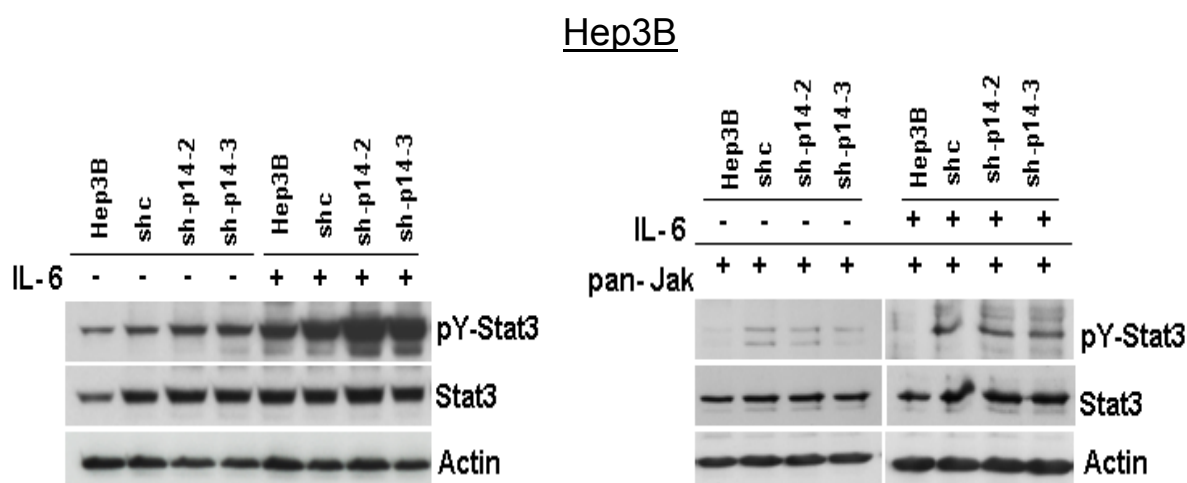


Figure 17: Jak activity is required for IL-6 induced phosphorylation of Stat3.

(A) Plc cells and two clones expressing p14^{ARF} (plc-p14_1 and plc-p14_2) as well as (B) Hep3B cells, sh-control (shc) and two clones not expressing p14^{ARF} (sh-p14_2 and sh-p14_3) were treated with either 20 ng/ml IL-6 alone for 20 minutes or pre-treated with a pan-jak inhibitor (10 ng/ml) for 1 hour. Protein extracts were probed with anti-phospho-Stat3 and re-probed with anti-Stat3 antibodies. Actin was used as a loading control.

Plc cells as well as the two clones expressing p14^{ARF} (plc-p14_1 and plc-p14_2) showed a tremendous rise in pStat3 levels upon treatment with IL-6, whereas this activation was completely lost after pre-treatment with a pan-jak-inhibitor. The basal phosphorylation was diminished as well (Fig. 17A). In the Hep3B cell lines expressing or lacking p14^{ARF}, the rise in pStat3 level was also impeded when cells were treated with the pan-jak inhibitor, and the basal level was almost lost (Fig. 17B). In both cell lines the presence or absence of p14^{ARF} showed no obvious impact on tyrosine 705 phosphorylation of Stat3.

These data show that Jaks are crucial for transducing IL-6 signals and Stat3 activation. They further show that p14^{ARF} has no influence on the requirement for Jaks to phosphorylate Stat3.

Selective blocking of Jak 2, Jak3 or Tyk2 cannot impede phosphorylation of Stat3 upon IL-6 treatment

We next analyzed which Jak is responsible for Stat3 tyrosine phosphorylation. Therefore, Jak activity was inhibited individually and the level of Stat3 tyrosine 705 phosphorylation (pY-Stat3) upon IL-6 treatment was monitored. To determine a putative influence of p14^{ARF}, all experiments were performed with Plc cells, two Plc clones expressing p14^{ARF} (plc-p14_1 and plc-p14_2), with Hep3B cells, Hep3B shc and two clones with a p14^{ARF}-knockdown (sh-p14_2 and sh-p14_3). Since there is no Jak1 inhibitor available, we started with blocking of Jak2 with the inhibitor AG490 before stimulation of cells with IL-6 (Fig. 18).

Figure 18

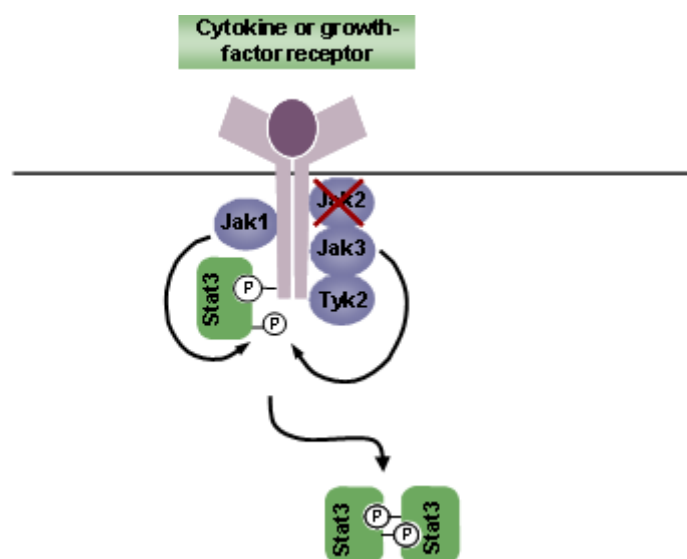
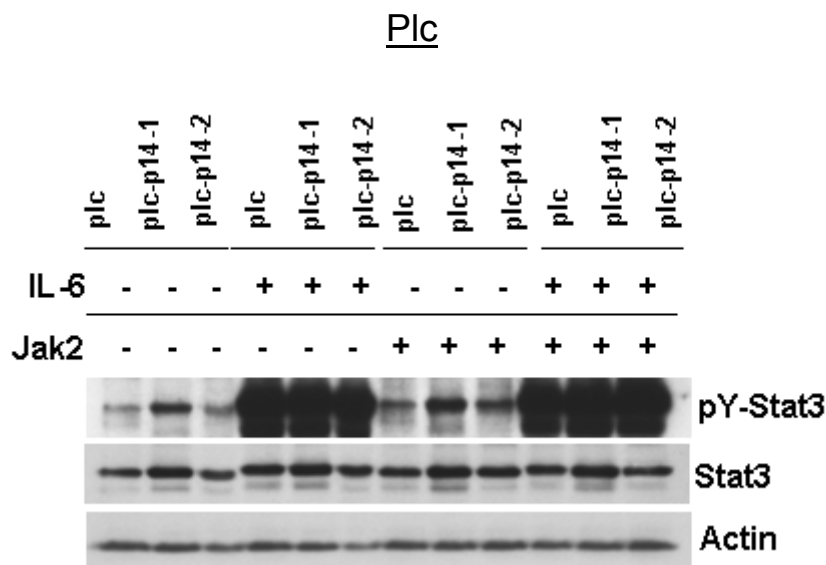
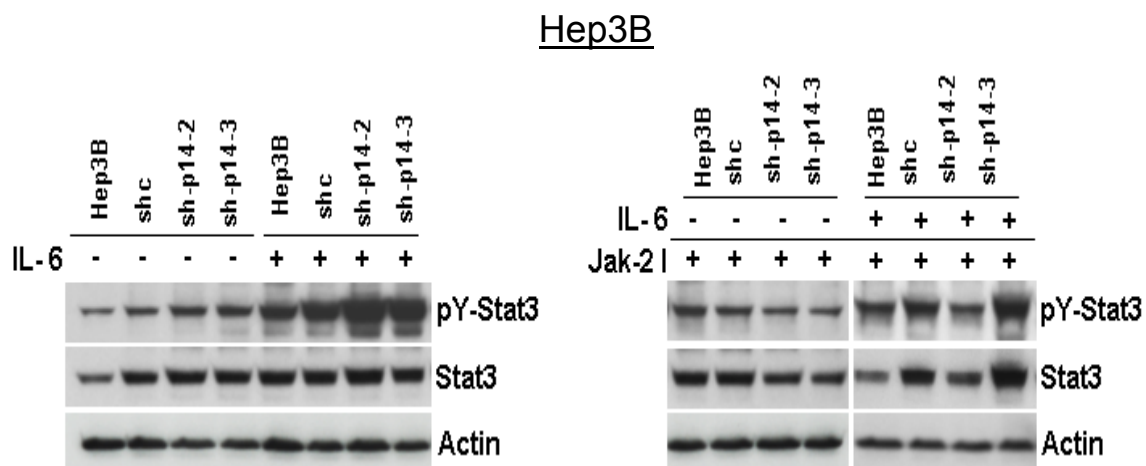


Figure 18: Model of Jak2 blocking. To determine the need for active Jak2 in IL-6 signal transduction by phosphorylation of Stat3, Jak2 activation was inhibited and phospho-Stat3 levels determined.

Figure 19**A****B****Figure 19: Jak2 inhibition cannot impede IL-6 induced Stat3 phosphorylation.**

(A) Plc cells and 2 clones expressing expressing p14^{ARF} (plc-p14_1 and plc-p14_2) as well as (B) Hep3B cells, sh-control (shc) and 2 clones not expressing p14^{ARF} (sh-p14_2 and sh-p14_3) were treated with either 20 ng/ml IL-6 alone for 20 minutes or pre-treated with 20 μ M of the Jak 2 inhibitor AG490 for 24 hours. Protein extracts were probed with anti-phospho-Stat3 and re-probed with anti-Stat3 antibody in Western blots. Actin was used as a loading control.

The inhibition of Janus kinase 2 had no diminishing effect on the phosphorylation of Stat3 upon IL-6 in neither Plc cells lacking nor those expressing p14^{ARF} (Fig. 19A). In Hep3B cell lines neither the basal level of pY-Stat3 nor the induction upon growth factor treatment could be averted by blocking the activity of Janus kinase 2 (Fig. 19B). Thus, these data confirm our observations in Plc cells.

Next, Jak3 was blocked with the Jak3 inhibitor VI and cells were stimulated with IL-6 (Fig. 20).

Figure 20

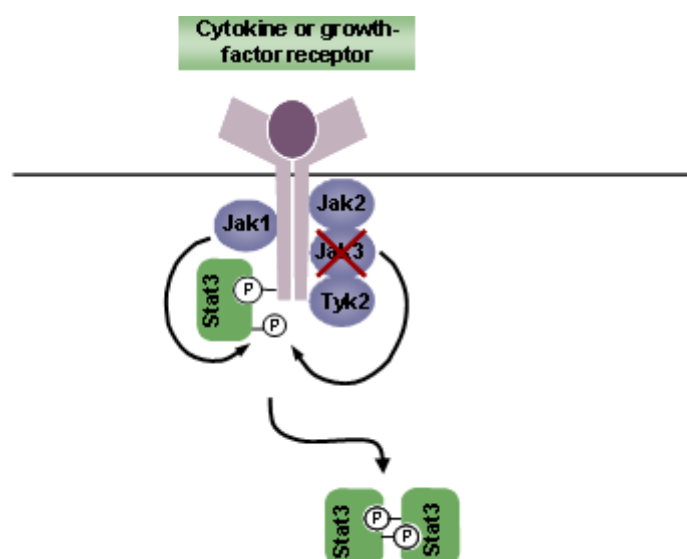


Figure 20: Model of Jak3 blocking. Jak3 activity was blocked with Jak3 inhibitor VI to determine if it is necessary for Stat3 tyrosine phosphorylation upon IL-6 treatment.

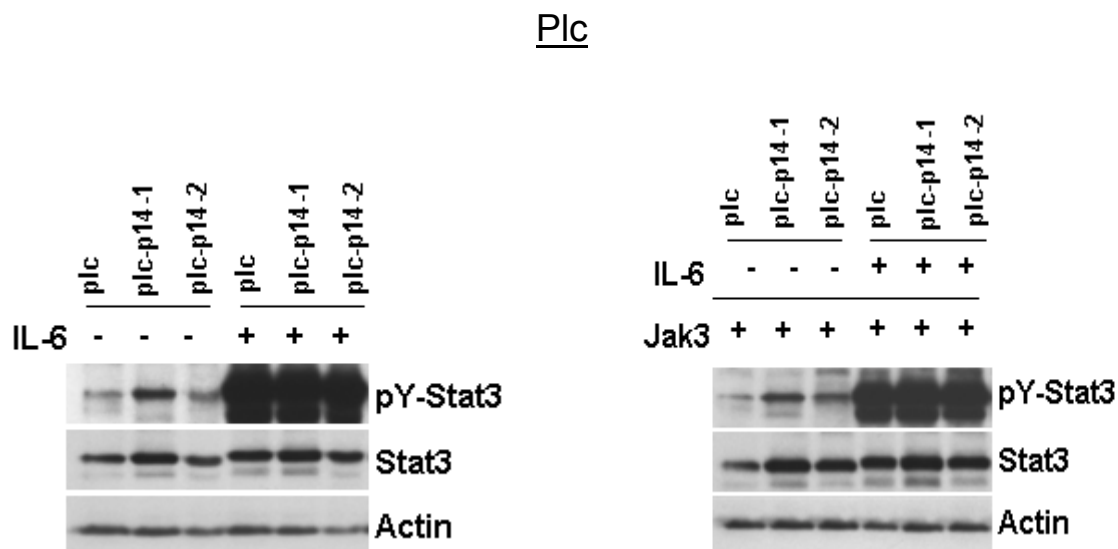
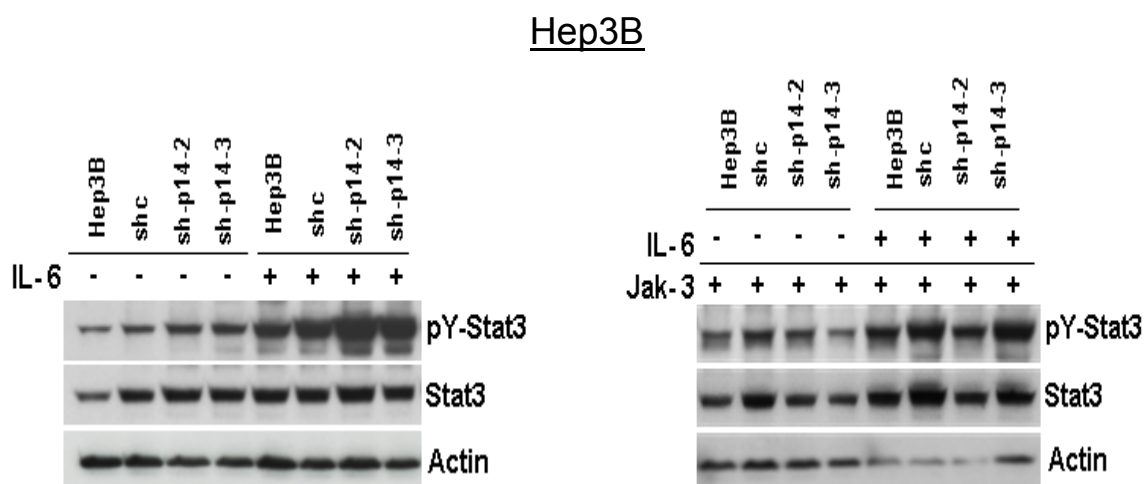
Figure 21**A****B**

Figure 21: Inhibition of Jak3 cannot impede IL-6 mediated pY-Stat3 phosphorylation. (A) Plc cells and 2 clones expressing p14^{ARF} (plc-p14_1 and plc-p14_2) as well as (B) Hep3B cells, sh-control (shc) and 2 clones not expressing p14^{ARF} (sh-p14_2 and sh-p14_3) were treated with either 20 ng/ml IL-6 alone for 20 minutes or pre-treated with 60nM of a Jak3 inhibitor (Jak3 inhibitor VI) for 24 hours. Protein extracts were probed in Western blots with anti-phospho-Stat3 and re-probed with anti-Stat3 antibodies. Actin was used as a loading control.

The treatment with a Jak3 inhibitor could not change the levels of Stat3 activation induced by IL-6 in neither of the cell lines (Fig. 21A, B). Thus, Jak3 is not necessary for IL-6 signal transduction, independently of p14^{ARF}.

To determine the influence of Tyk2, its activity was blocked with AG9 before treating cells with IL-6 and the levels of tyrosine phosphorylated Stat3 were evaluated (Fig. 22).

Figure 22

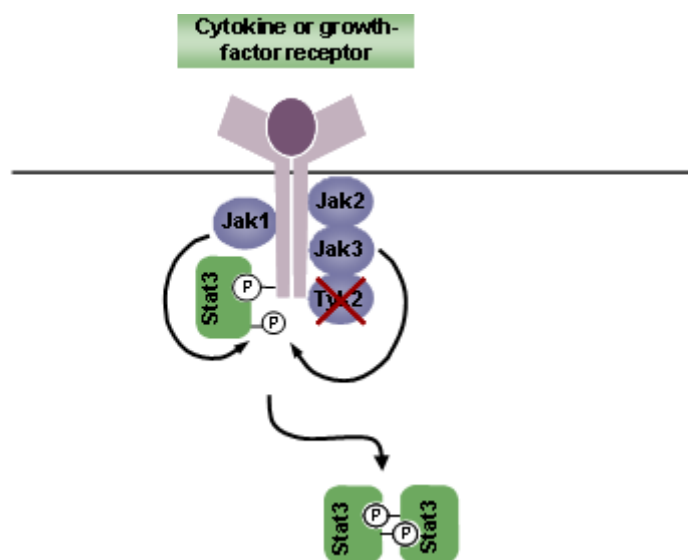
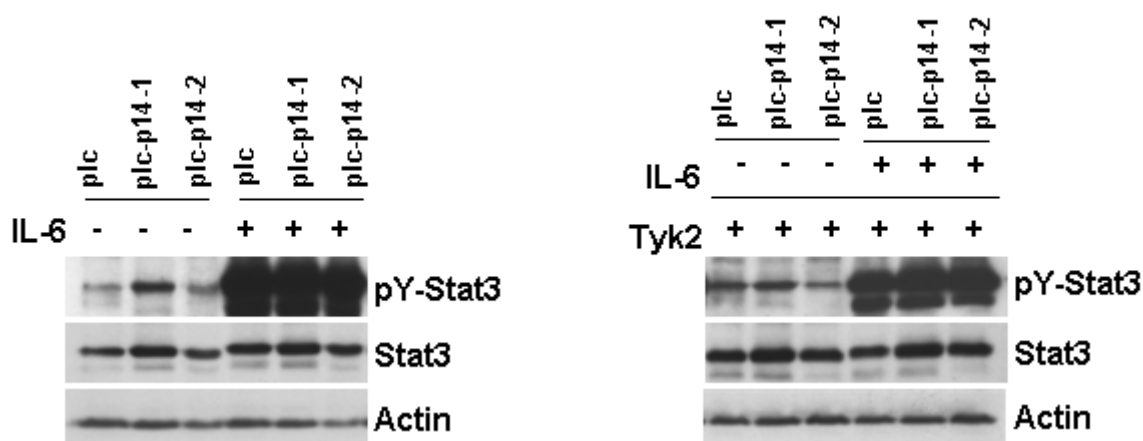


Figure 22: Model of Tyk2 blocking. Tyk2 was blocked with AG9 to determine its impact on IL-6 induced phosphorylation of Stat3.

Figure 23

A

Plc

B

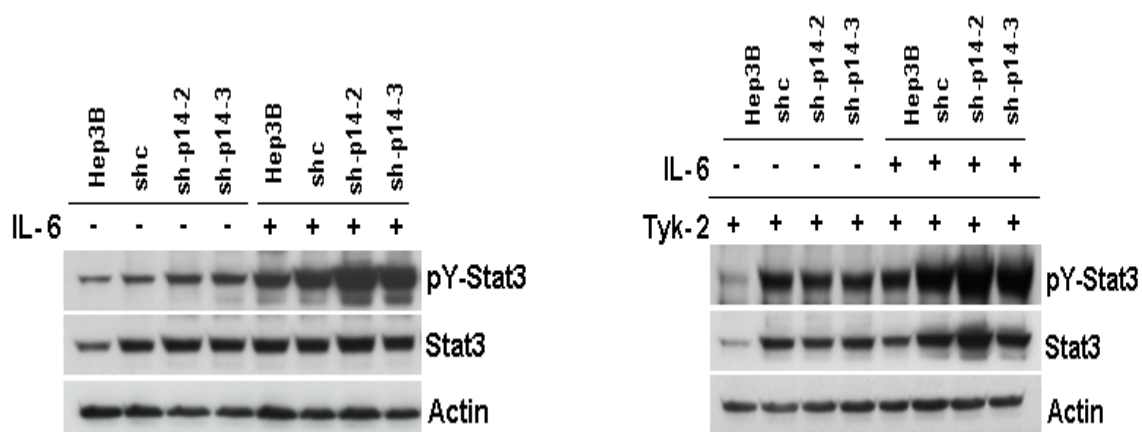
Hep3B

Figure 23: Tyk-2 inhibition cannot impede the phosphorylation of Stat3 upon IL-6 treatment. (A) Plc cells and 2 clones expressing p14^{ARF} (plc-p14_1 and plc-p14_2) as well as (B) Hep3B cells, sh-control (shc) and 2 clones not expressing p14^{ARF} (sh-p14_2 and sh-p14_3) were treated with either 20 ng/ml IL-6 alone for 20 minutes or pre-treated with 50μM of the Tyk2 inhibitor AG9 for 24 hours. Protein extracts were probed with anti-phospho-Stat3 and re-probed with anti-Stat3 antibody in Western blots. Actin was used as a loading control.

Blocking Tyk2 activity had no effect on the phosphorylation of Stat3 upon IL-6 treatment in none of the employed cell lines, and thus is not needed for signal transduction independently of the expression of p14^{ARF} (Fig. 23A,B).

Together, from these data we conclude that neither Jak2, nor Jak3 or Tyk2 are necessary for tyrosine 705 phosphorylation of Stat3 upon IL-6 treatment, independently of p14^{ARF}. Thus, Jak1 is considered the responsible kinase for the transduction of IL-6 signaling. These results further suggest that the Jak1-mediated phosphorylation of Stat3 upon IL-6 stimulation is independent of the expression of p14^{ARF}.

Blocking of Jak2, Jak3 and Tyk2 cannot impede phosphorylation of Stat3 upon IL-6 treatment

To exclude an accumulative effect of single inhibitors and thus proof the need for active Jak1, Plc and Hep3B cell lines each expressing or lacking p14^{ARF}, were pre-treated with Jak2, Jak3 and Tyk2 inhibitor and analyzed for p-Stat3 activation after IL-6 stimulation (Fig. 24).

Figure 24

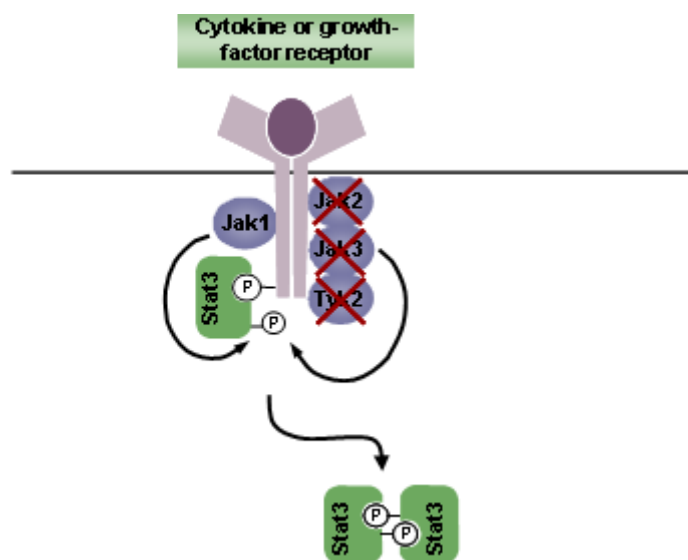


Figure 24: Model of combined Jak2, Jak3 and Tyk2 inhibition. Jak2, Jak3 and Tyk2 were inhibited with AG490, Jak3 inhibitor VI and AG9, respectively, to examine the requirement of Jak1 for Stat3 phosphorylation.

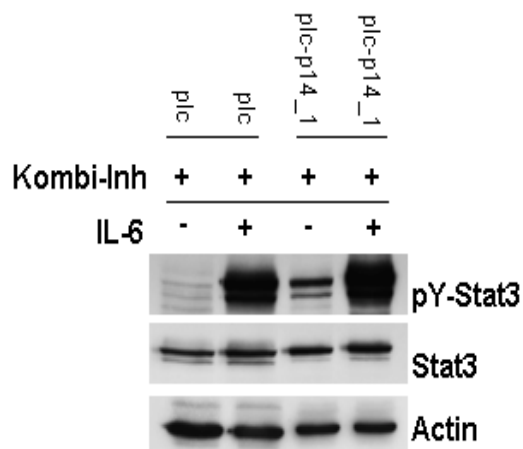
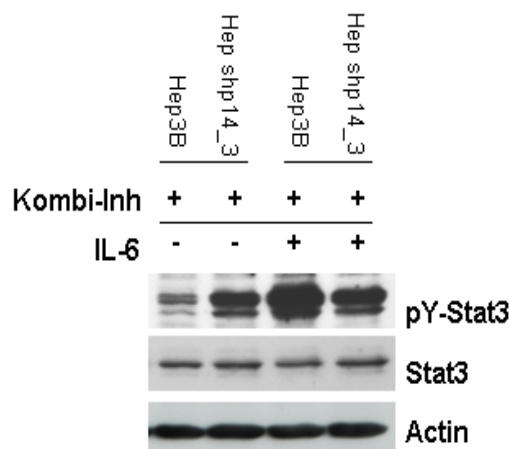
Figure 25**A****B**

Figure 25: Jak2, Jak3 and Tyk2 inhibition together fails to reduce IL-6 induced phosphorylation of Stat3. (A) Plc and plc-p14₋₁ cells as well as (B) Hep3B and Hep3B sh p14₋₃ cells were treated with 20 μ M Jak2 inhibitor AG490, 60nM Jak3 inhibitor VI and 50 μ M Tyk2 inhibitor AG9 for 24 hours and supplied or not with 20 ng/ml IL-6 20 minutes before preparation cell lysis. Protein extracts were probed in Western blots with anti-phospho-Stat3 and re-probed with anti-Stat3 antibody. Actin was used as a loading control.

According to the settings where each kinase was inhibited alone, the elevation of pY-Stat3 upon IL-6 could not be hindered by combined inhibition of Jak2, Jak3 and Tyk2. Again, this was independent of p14^{ARF} expression in both employed cell lines (Fig 25).

Taken together, we assume that Jak1 is the kinase crucial for tyrosine 705 phosphorylation of Stat3 in Plc and Hep3B hepatoma cell lines. Further, our data show that Stat3 phosphorylation is independent of the expression of p14^{ARF} *in vitro*.

Stat3 is a mediator for anti-proliferative effects of TGF β 1

We addressed the role of TGF- β 1 in the proliferation of Stat3-deficient immortalized hepatocytes and those transformed with oncogenic Ha-Ras. Immortalized MIM cells, those lacking Stat3 (MIM-Stat3 Δ hc -1 and MIM-Stat3 Δ hc -2) or Stat3-deficient hepatocytes with reintroduced wild type Stat3 (MIM-Stat3 Δ hc -2-wtStat3) were cultivated and administrated with TGF- β 1.

Figure 26

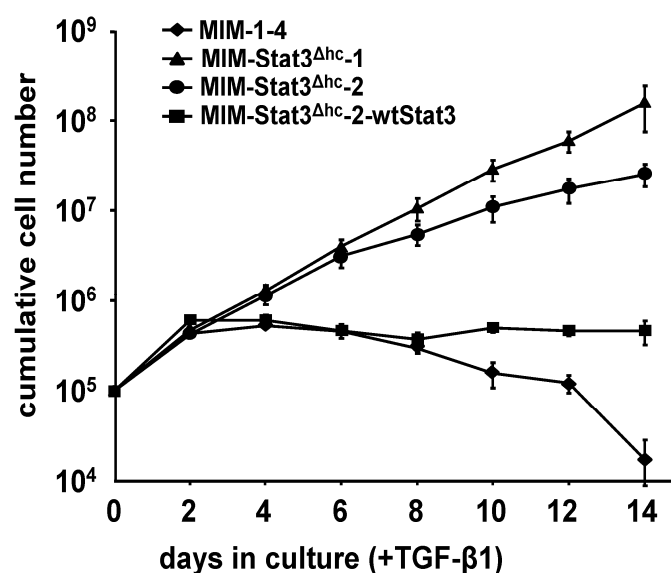


Figure 26: Lack of Stat3 prevents anti-proliferative effects of TGF- β 1. Proliferation kinetics of MIM cells, MIM-Stat3 Δ hc -1 and MIM-Stat3 Δ hc -2 cells lacking Stat3 and MIM-Stat3 Δ hc -2-wtStat3 with reintroduced wild-type Stat3 exposed to TGF- β 1 (1 ng/ml). These data are provided by Doris Schneller (PhD thesis, 2010).

While TGF- β 1 acted anti-proliferative on MIM cells as expected, MIM cells lacking Stat3 (MIM-Stat3 Δ hc-1 and MIM-Stat3 Δ hc-2) were insensitive to these effects (Fig. 26). Reintroduction of wtStat3 (MIM-Stat3 Δ hc-2-wtStat3) rescued the phenotype and cells became sensitive to TGF- β 1-mediated growth arrest again. These results suggest that Stat3 is an important mediator of TGF- β 1 effects.

To further address the influence of oncogenic Ras, the Ras-transformed cell lines MIM-R and MIM-R-Stat3^{Δhc}-2, where Stat3 has been knocked-out, were cultured in the presence or absence of TGF-β1 and proliferation kinetics were performed.

Figure 27

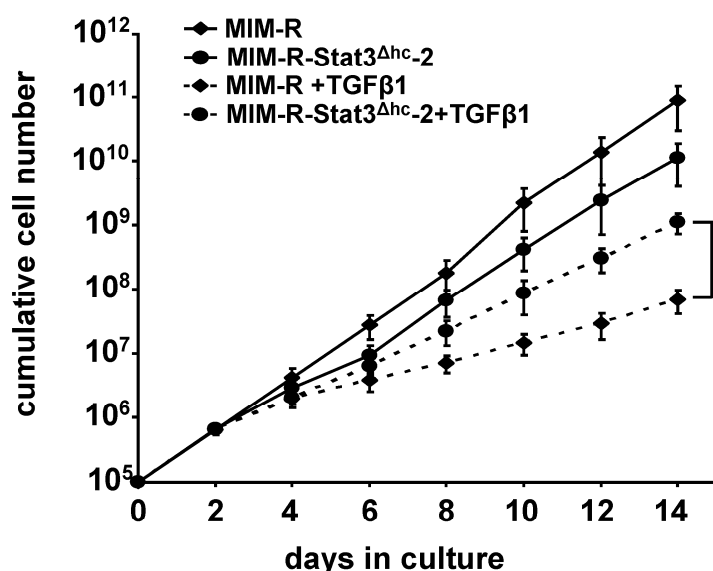


Figure 27: Lack of Stat3 induces insensitivity to TGF-β1. Proliferation kinetics of Ras-transformed MIM cells lacking or expressing Stat3 (MIM-R and MIM-R-Stat3^{Δhc}-2, respectively) and each treated with TGFβ1 (1 ng/ml) (MIM-R+TGFβ1 and MIM-R-Stat3^{Δhc}-2+TGFβ1). These data are provided by Doris Schneller (PhD thesis, 2010).

MIM-R cells expressing or lacking Stat3 showed no significantly different proliferation kinetics (Fig. 27). TGF-β1 treatment of MIM-R cells lead to strongly reduced proliferation compared to MIM-R cells not exposed to TGF-β1. According to the data from MIM cells lines, MIM-R cells without Stat3 (MIM-R-Stat3^{Δhc}-2) were less sensitive to TGF-β1 and their proliferation was significantly higher. Indeed, their proliferation did not vary significantly from MIM-R-Stat3^{Δhc}-2 not treated with TGF-β1. These results indicate a crucial role of Stat3 in the execution of TGF-β1 induced anti-proliferative effects.

Stat3 affects the abundance but not the translocation of Smad2/3 upon TGF- β 1 treatment

Since Stat3 seems to be an important mediator of anti-proliferative TGF- β 1 effects, the question arose whether the nuclear translocation of Smad2/3 is affected by the loss of Stat3. To address this question, MIM cells and Ras-transformed MIM cells (MIM-R) as well as those with a Stat3 knock-out (MIM-Stat3 Δ^{hc} -2 and MIM-R-Stat3 Δ^{hc} -2) and a reintroduced wild type Stat3 (MIM-Stat3 Δ^{hc} -2-wtStat3 and MIM-R-Stat3 Δ^{hc} -2-wtStat3) were treated with TGF- β 1 and Smad2/3 localization was determined by immunofluorescence microscopy.

Figure 28

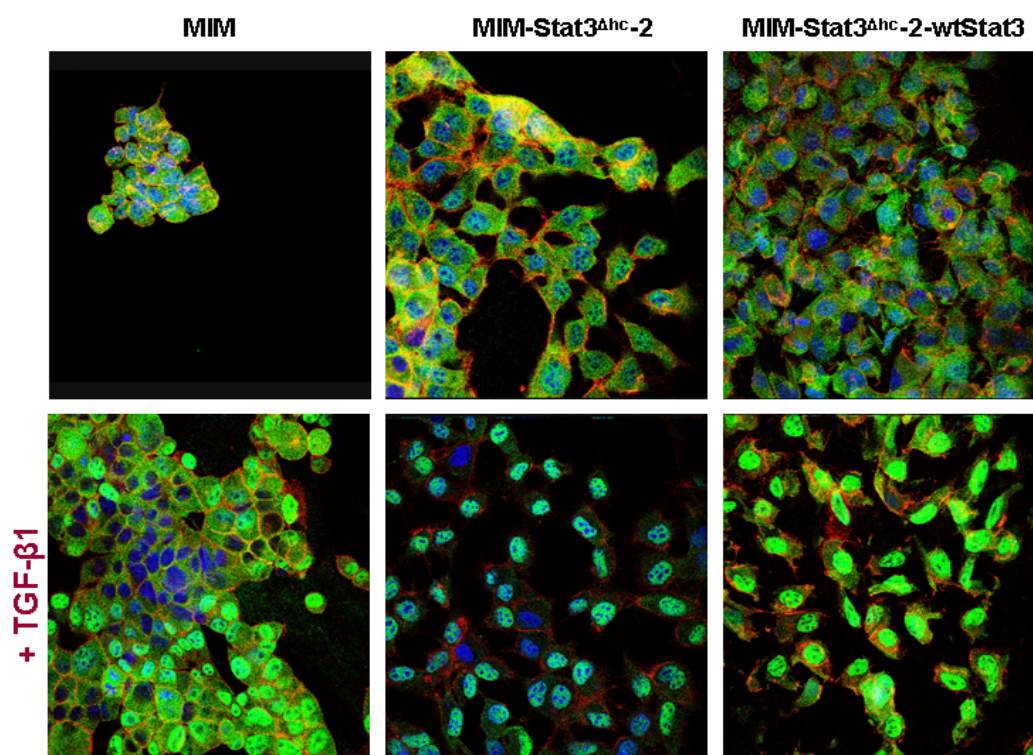


Figure 28: Lack of Stat3 entails less nuclear Smad2/3 upon TGF- β 1 treatment. MIM cells, MIM cells with a Stat3 knock-out (MIM-Stat3 Δ^{hc} -2) and a reintroduced Stat3 wild type construct (MIM-Stat3 Δ^{hc} -2-wtStat3) were treated with 2,5 ng/ml TGF- β 1 for 1 hour. Untreated control cells (upper panel) and TGF- β 1-treated cells (lower panel) were probed with a Smad2/3 antibody (green), To-Pro3 to localize nuclei (blue) and Phalloidin for actin-staining (red).

MIM, MIM-Stat3^{Δhc}-2 and MIM-Stat3^{Δhc}-2-wtStat3 did not differ in neither amount nor localization of Smad2/3 in the untreated condition. There was a comparable basal level of the protein localized in the cytoplasm (Fig. 28, upper panel). Upon TGF-β1 treatment, Smad2/3 localized and accumulated in the nucleus in MIM cells, which was also observed in MIM-Stat3^{Δhc}-2 lacking Stat3 but with a clearly less amount of Smad2/3. When Stat3 was reintroduced (MIM-Stat3^{Δhc}-2-wtStat3), the levels of nuclear Smad2/3 were enhanced again (Fig. 28, lower panel). Thus, we conclude that Stat3 is rather involved in Smad2/3 expression than localization upon TGF-β1 treatment in MIM cells.

Figure 29

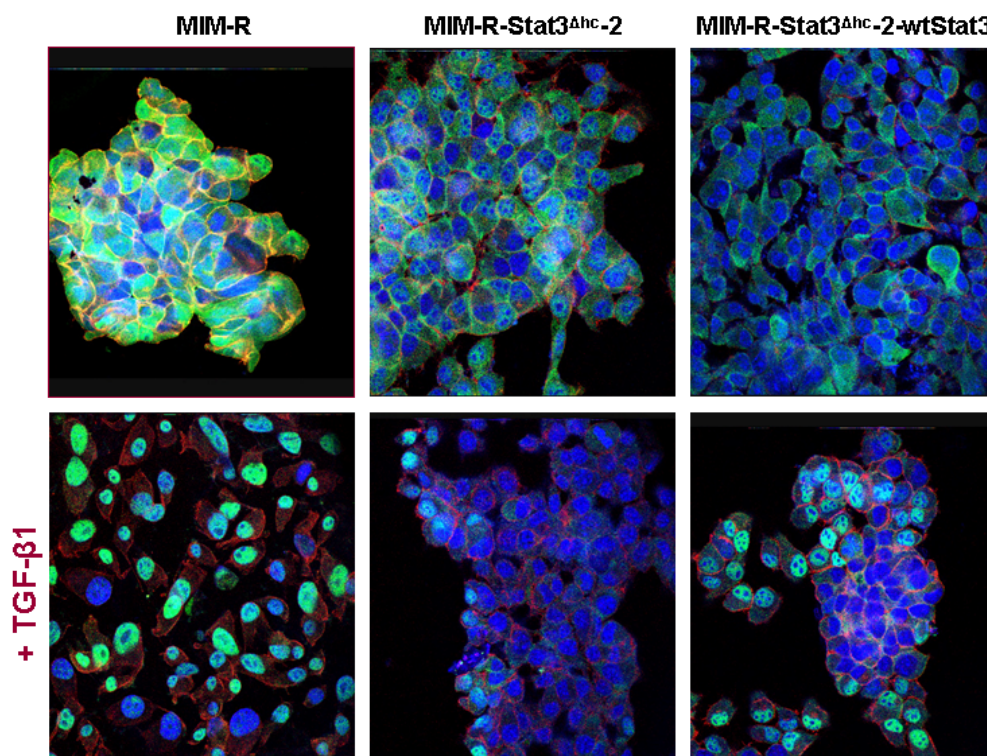


Figure 29: Lack of Stat3 results in less Smad2/3 expression. MIM-R cells, MIM-R cells with a Stat3 knock-out (MIM-R-Stat3^{Δhc}-2) and a reintroduced Stat3 wild type construct (MIM-R-Stat3^{Δhc}-2-wtStat3) were treated with 2,5 ng/ml TGF-β1 for 1 hour. Untreated control cells (upper panel) and TGF-β1-treated cells (lower panel) were probed with a Smad2/3 antibody (green), To-Pro3 to localize nuclei (blue) and Phalloidin for actin-staining (red).

MIM-R cells lacking Stat3 (MIM-R-Stat3^{Δhc}-2) showed a less amount of cytoplasmic Smad2/3 than MIM-R cells expressing Stat3. This could not be rescued by the reintroduction of a Stat3 wild type construct (MIM-R-Stat3^{Δhc}-2-wtStat3) (Fig. 29, upper panel). Upon TGF-β1 treatment, Smad2/3 localized and accumulated in the nucleus in all three cell types but in clearly different amounts. While in MIM-R cells nuclear signals could be observed, in MIM-R-Stat3^{Δhc}-2 only very little Smad2/3 was detected in the nuclei and this only at the borders of the cell layer. When Stat3 was reintroduced (MIM-R-Stat3^{Δhc}-2-wtStat3), more Smad2/3 was found in the nuclei again, but not as much as in the initial condition. Again, Smad2/3 positive cells were those at the borders (Fig. 29, lower panel).

These results show that Stat3 might play a role in the expression of Smad2/3 also in Ras-transformed MIM-R cells, but not in the translocation upon TGF-β1 treatment. Interestingly, the reintroduction of Stat3 in MIM-R cells could not rescue the phenotype in the same extend as in MIM cells.

Taken together, our results suggest that Stat3 plays a crucial role as a mediator of TGF-β1-induced effects in both, untransformed and Ras-transformed hepatocytes. Lack of Stat3 caused insensitivity to the anti-proliferative effects of TGF-β1 which might be linked to the level of nuclear Smad2/3 upon TGF-β1 treatment.

5. Discussion

Recent findings revealed that Stat3, so far considered as an oncogene, could also act in a tumor-suppressive manner dependent on the expression of p14^{ARF}. In the presence of p14^{ARF} it exerts its well-known tumor-promoting properties while in the absence of p14^{ARF} it was found to act tumor-suppressive in *in vivo* models. (145) Mouse hepatocytes expressing p14^{ARF} and harbouring a transcriptionally constitutive active variant of Stat3 resulted in augmented tumors when injected subcutaneously into SCID mice, while mouse hepatocytes lacking p14^{ARF} and harbouring also this construct entailed smaller tumors than the respective controls. When a U-Stat3 construct which cannot be phosphorylated due to a Y705F mutation was introduced into the p14^{ARF}-lacking cells, a tremendous rise in tumor growth could be observed, indicating a tumor-suppressive role of activated Stat3 in a p14^{ARF}-deficient background. The human HCC cell line Hep3B where p14^{ARF} had been knocked down showed no change in tumor growth but the phosphorylation of Stat3 was lost in tumor tissue indicating the circumvention of Stat3's tumor-suppressing effects without p14^{ARF} (145).

To achieve mechanistic understanding of these processes we were interested whether p14^{ARF} has an influence on the activation of Stat3. First, we addressed the question if *de novo* RNA and/or protein synthesis is necessary for Stat3 tyrosine phosphorylation and if this is influenced by p14^{ARF}. Inhibition of transcription or translation showed very clearly that *de novo* RNA and protein synthesis are required for phosphorylation of Stat3 upon growth factor stimulation with IL-6 in cell lines expressing or lacking p14^{ARF} (Fig. 15A-D). Thus, the requirement for *de novo* synthesis is independent of p14^{ARF}. Interestingly, in Plc cell lines the levels of Stat3 remain unaffected by inhibition of *de novo* synthesis, while in Hep3B cell lines the inhibition of transcription reduces Stat3. This indicates a possibly different stability of Stat3 between these cell types, which could be due to different deregulation of physiological mechanisms. Nevertheless, also in Hep3B cells there remains sufficient Stat3 to be phosphorylated. Thus, the absence of a molecule triggering phosphorylation is thought to be responsible for the attenuation of pY-Stat3 levels. This crucial mediator of phosphorylation is likely a tyrosine kinase positively regulating pStat3 levels but could also be another element of the Stat3 signaling network. Actually, it could concern a component interfering with one of the negative

regulators of Stat3 which are responsible for fine-tuning the extent and duration of Stat3 phosphorylation. This affected enzyme could imaginably be a tyrosine phosphatase like Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1), which directly interacts and dephosphorylates Jaks, or protein tyrosine phosphatase receptor-T (PTPRT), which specifically dephosphorylates Stat3 and can act as tumor suppressor (66, 154). Also a member of the family of suppressors of cytokine signaling (SOCS) could be affected. Their degradation is regulated tightly and if some required molecule was no longer expressed and thus SOCS were no longer negatively regulated Jaks would get deactivated and Stat3 could no longer be phosphorylated (155). However, the lack of an inhibitory component could lead to unlimited action of these pY-Stat3 suppressors resulting in diminished pY-Stat3 levels.

The kinases coming into consideration first for forwarding the IL-6 signal and phosphorylating Stat3 are the members of the Janus family of kinases Jak1, Jak2, Jak3 and Tyk2 (38). Jak3 was long thought to be exclusively expressed in the hematopoietic system, but it was found to be mutated in breast and gastric carcinomas and to contribute to EGF-signalling in a breast cancer cell line (45, 49). Thus, we investigated the requirement of each and addressed the question whether the expression or absence of p14^{ARF} has an impact on their function in activating Stat3. When we pharmacologically blocked the activity of all Jaks, Stat3 phosphorylation upon IL-6 was hindered in all employed cell lines. In both cell lines, Plc and Hep3B also the basic level of pY-Stat3 was diminished by pan-jak inhibition (Fig. 17A,B). These effects are independent of the expression of p14^{ARF}. These results indicate that in fact Janus kinases are responsible for the tyrosine 705 phosphorylation of Stat3 and that they are independent of p14^{ARF} *in vitro*.

Further investigations which Jaks contribute to Stat3 phosphorylation revealed that neither Jak2, nor Jak3 or Tyk2 are crucial in this process, since IL-6 treatment entailed enhanced pY-Stat3 levels despite inhibition of the respective kinase (Fig. 19, 21, 23). Notably, again we observed comparable results in all employed cell lines irrespective of p14^{ARF} expression. Since the inhibition of Jak1, Jak2, Jak3 and Tyk2 together negatively influenced pY-Stat3 levels basically and upon treatment with IL-6, but the inhibition of Jak2, Jak3 or Tyk2 alone did not, we concluded that Jak1 is responsible for the phosphorylation of Stat3 in hepatocytes, irrespective of p14^{ARF}. To exclude a collaborative effect of Jak2, Jak3 and Tyk2 and to underscore the

importance of Jak1, we inhibited Jak2, Jak3 and Tyk2 together. According to the data observed until then, this combined inhibition could not impede Stat3 phosphorylation upon IL-6 treatment irrespective of p14^{ARF} (Fig. 25). This corroborates our supposition that Jak2, Jak3 and Tyk2 are negligible for tyrosine phosphorylation of Stat3. Considering all obtained data we ascribe Jak1 the responsibility for Stat3 tyrosine705 phosphorylation in hepatocellular carcinoma cell lines expressing or lacking p14^{ARF} (Fig. 30).

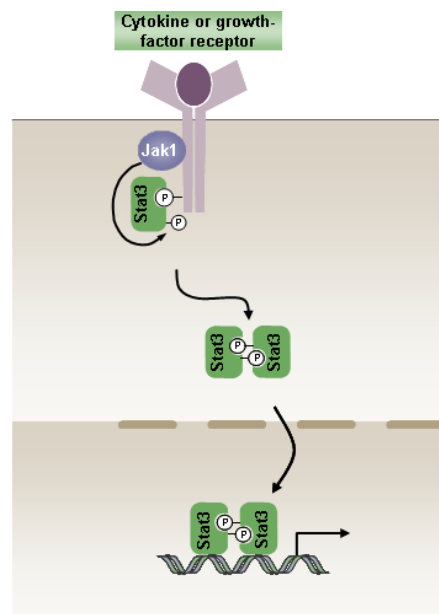


Figure 30: Jak1 forwards IL-6 signalling by phosphorylation of Stat3.

To proof this assumption the selective inhibition of Janus kinase 1 would be necessary. Since there is no pharmacological Jak1 inhibitor available, the use of a small interfering (si) or small hairpin (sh) RNA targeted against Jak1 on a cellular level or a Jak1 knock-out could be performed. Since the lack of Jak1 is not embryonically lethal the deletion of Jak1 in mice is possible and has been done already (156). However, the lack of Stat3 is embryonically lethal and so the suspicion arises that in a Jak1-deficient mouse other Jaks would fill in for the missing Jak1 in order to phosphorylate Stat3 (156). Generally, it would be interesting if and under which conditions/ circumstances Stat3 would be activated by the other members of Jaks in hepatocytes. To confirm that the *de novo* synthesis of Jak1 is required for Stat3 phosphorylation, the purified kinase could be introduced in transcription- or translation-blocked cells by microinjection. The introduced kinase should then rescue the phenotype and pY-Stat3 levels would be unaffected. Unfortunately, there are

major technical limitations to this method, namely that only single cells can be injected. Thus, an inducible construct harbouring Jak1 would be useful. Since transcription and translation are required to express the exogenous kinase the question asked above could not be addressed, but still, we should observe major differences in pY-Stat3 levels, while the hyperactivation of the other family members should not have the same impact. Another possibility to emphasize the mechanistic role of Jak1 in Stat3 activation would be to correlate active Stat3 with active Jak1. Unfortunately, the available phospho-Jak1 antibodies did not work properly in our cell lines. Still, we consider Jak1 as the crucial kinase for Stat3 tyrosine phosphorylation in HCC cell lines and, importantly, found that p14^{ARF} had no influence on Stat3 activation *in vitro*. Thus, we state that the impact of p14^{ARF} conveying tumorigenic properties to Stat3 *in vivo* is located downstream of Stat3 phosphorylation.

If p14^{ARF} is expressed *in vivo*, Stat3 gets phosphorylated by Jak1, dimerizes, translocates into the nucleus and performs its oncogenic action as do introduced transcriptionally constitutive active (ca) Stat3 constructs. Also unphosphorylated Stat3 (U-Stat) is able to enter the nucleus but has no impact on tumor development in this genetic background (57) (Fig. 31A). In the case that p14^{ARF} is missing, the situation changes dramatically. Ca-Stat3 expression leads to diminished tumor growth, thus Stat3 acts anti-oncogenic. Concomitant, pY-Stat3 levels are decreased in tumor tissue to circumvent further anti-oncogenic effects. U-Stat3 again enters the nucleus, and this time without p14^{ARF} this results in strongly enhanced tumor growth. Therefore, we assume that U-Stat3 interacts with a so far unidentified factor, termed ARF-X, to drive gene expression (Fig. 31B). This factor is thought to be occupied by p14^{ARF} under physiological conditions, since p14^{ARF} is known to interact with a variety of partners, including transcription factors (Fig. 31) (95). Thus, finding ARF-X would be of high priority.

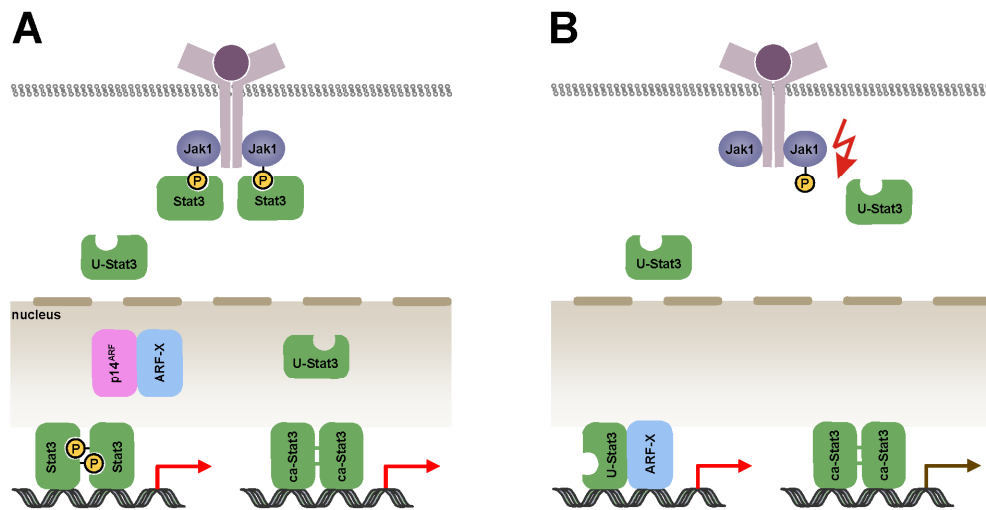


Figure 31: Model of pro- and antioncogenic actions of Stat3 depending on p14^{ARF}.

(A) When p14^{ARF} is expressed, Stat3 gets phosphorylated by Jak1 and performs its pro-oncogenic action. ARF-X is occupied by p14^{ARF} and thus, U-Stat3 remains transcriptionally inactive. (B) In the absence of p14^{ARF}, phosphorylation of Stat3 is circumvented and U-Stat3 drives oncogenic gene expression in concert with ARF-X. Ca-Stat3 dimers turn on the expression of genes with anti-oncogenic effects.

To ascertain if in fact the inhibition of Jak1 is responsible for the reduced pY-Stat3 levels in tumor tissues lacking p14^{ARF} these tissues need to be probed with a phospho-Jak1 antibody. To find the putative U-Stat3 binding partner ARF-X co-immun-precipitation of these two proteins could be performed by fishing for U-Stat3 in a p14^{ARF} – negative background. Though, a strong binding between them is a precondition for this approach and we do not know about the strength of this interaction.

Stat3 was found to hinder transformation in the absence of tumor suppressors like PTEN or p53 in glioblastoma or rodent fibroblasts, respectively, and thus to act as tumor suppressor in these genetic backgrounds (90, 91). In our case, we can exclude that the tumor suppressive effect of Stat3 is ascribed to the lack of p53, since on the one hand the used p14^{ARF}^{-/-} cell lines show an active p53 response and on the other Hep3B cells harbour a p53 mutation, but in both a tumor suppressive effect of Stat3 in the absence of p14^{ARF} was observed (157, 158). Since Hep3B cells express PTEN, the tumor suppressive effect of Stat3 can not be rooted in its lack (159, 160). The finding that caStat3 leads to diminished tumor growth when p14^{ARF} is lacking,

raises the question which different set of genes mediates this effect and how Stat3 is able to turn on their transcription. It would be of interest how Stat3 is directed towards these promoters and if there are putative interaction partners that do so. Despite a different set of genes, the different outcome in this setting could also be rooted in different effects of the transcribed target genes, potentially involving the tumor-microenvironment. For example, the constitutive activation of the Stat3 target and oncogene *myc* can indirectly promote senescence and apoptosis in lymphoma mouse models with involvement of tumor-surrounding non-malignant cells which secrete cytokines in response to the constantly activated *myc* (55, 161). A similar non-cell-autonomous tumor suppression mechanism could be imagined in the case of constitutively activated Stat3, which somehow only takes effect if p14^{ARF} is missing.

Moreover, p14^{ARF} influences the levels of pY-Stat3 *in vivo* but not *in vitro* and obviously Stat3 activation is affected via a still unknown feed-back loop if it turns out to act anti-oncogenic. This, together with the fact that the absence of p14^{ARF} converts the pro-oncogenic properties of Stat3 into anti-oncogenic ones guides the considerations about mechanistic sequences towards a putative participation of the tumor-microenvironment in these processes. Non-malignant cells that are recruited to the tumor and act out a variety of supportive functions like growth factor secretion could be involved in those mechanisms (1). To figure out which cells are involved and bring to light the underlying mechanisms of tumor-stroma interactions contributing to the switch of Stat3 being pro-or anti-oncogenic would be a surely challenging but worthwhile purpose.

TGF- β 1 stimulates tumor progression at later stages of tumor development but has tumor-suppressive effects in early tumorigenesis (106). During early stages TGF- β 1 inhibits cell growth and induces apoptosis. (106) Since TGF- β 1 can execute different cellular programs during the course of tumorigenesis, it is of highest priority to understand the underlying mechanisms and which players are involved in either mode of TGF- β 1 action. We were particularly interested in a putative cooperation of Stat3 and TGF- β 1 in tumorigenesis, namely the control of cell cycle progression on the one hand, and survival and apoptosis on the other. MIM hepatocytes as well as Ras-transformed MIM cells (MIM-Ras, MIM-R) were employed.

When MIM cells were exposed to TGF- β 1, it exerted its anti-proliferative effect as expected. Interestingly, cells lacking Stat3 (MIM-Stat3 Δ^{hc} -1 and MIM-Stat3 Δ^{hc} -2) were insensitive to growth-inhibition and induction of apoptosis by TGF- β 1. To prove the crucial importance of Stat3, a wild-type construct was introduced in the Stat3 Δ^{hc} cells and indeed, the phenotype was partially rescued. The cells did not restore proliferation arrest without an increase in proliferation kinetics (Fig. 12). Thus, we considered Stat3 an important mediator of TGF- β 1-mediated effects, namely TGF- β 1-induced cell death and cell cycle arrest. This opened the question whether Stat3 affects the canonical TGF- β 1 signalling cascade.

In MIM-Ras cells, TGF- β 1 treatment does not lead to cell death, but entails significantly slower proliferation. In these cells the lack of Stat3 had no influence on proliferation, since untreated cells without Stat3 showed no significantly different kinetics from untreated cells expressing Stat3. Stat3-deficient cells seem to be resistant to TGF- β 1 mediated effects, since in these cells and untreated Stat3 Δ^{hc} cells no significantly different cumulative cell numbers were observed. Stat3-deficient cells treated with TGF- β 1 were clearly less sensitive to its growth hindering effects than cells expressing Stat3, since there was a significant difference in their proliferation kinetics. Upon oncogenic Ras transformation, Stat3 seems to be important for the reduction of proliferation kinetics upon TGF- β 1 treatment. Again the question arose where Stat3 influences the signal transduction cascade following the exposure to TGF- β 1.

Interestingly, TGF- β 1 treatment induced apoptosis in untransformed MIM cells, but lead to cell cycle arrest in Ras-transformed MIM cells. In both cell lines the lack of Stat3 induced insensitivity to TGF- β 1 (Fig.12, 13). These findings indicate that (i) MIM-R cells gained the ability to evade apoptosis by Ras transformation, and (ii) the

forwarding of the TGF- β 1-signal relies on Stat3 irrespective of Ras. To complete these observations it would be necessary to show the effect of a reintroduced wt-Stat3 construct on proliferation kinetics of MIM-R cells treated with TGF- β 1.

In a next step, we addressed the question how Stat3 affects the TGF- β 1 signalling cascade. Since Smad signalling is the canonical signal transduction pathway of TGF- β 1, we focussed on receptor-mediated Smad2/3 translocation into the nucleus and investigated if Stat3 is required (103).

In untreated MIM cells, a cytoplasmic distribution of Smad2/3 in comparable amounts was observed irrespective of the presence or absence of Stat3 (Fig. 14, upper panel). Upon TGF- β 1 treatment, MIM cells exposed strong signals in their nuclei, but surprisingly only at the periphery of collected cells, while a complete lack in cells surrounded by others was observed. Without Stat3, translocation into the nucleus was still induced, but the amount of Smad2/3 was found to be clearly reduced. Upon reintroduction of a wt-Stat3 construct we observed a dramatically elevated amount of Smad2/3, most of it located in nuclei (Fig. 14, lower panel). Thus, the lack of Stat3 did not hinder translocation, but seemed to negatively influence the abundance of Smad2/3 in MIM cells. The assumption that Stat3 modulates Smad2/3 transcription was supported by rise of Smad2/3 levels upon Stat3 wild type introduction.

When we looked for a putative change in the translocation capacity of Smad2/3 upon TGF- β 1 treatment in MIM-Ras cells, we found less Smad2/3 expressed when Stat3 was lacking even in untreated MIM-R cells. Further, the introduction of a wt-Stat3 construct could not totally rescue that phenotype (Fig. 15, upper panel). Upon exposure to TGF- β 1, all Smad2/3 was translocated into the nuclei in wt-MIM-R cells. In cells lacking Stat3 we could detect only a small amount of Smad2/3, which was still translocated into the nuclei, but only at the borders of the cell layer. Reintroduction of wt-Stat3 increased the amount of Smad2/3, but not as much as in MIM cells. Again, translocation was observed at the borders of the cell layer (Fig. 15, lower panel). Taken together, also for Ras-transformed cells we exclude the requirement of Stat3 for Smad2/3 nuclear translocation. Stat3 seems to have an impact on the expression of Smad2/3, especially upon treatment with TGF- β 1, but also in untreated cells. The question remains, whether TGF- β 1 directly forces Stat3 to activate the transcription of Smad2 or Smad3 or if there exists a feed-back loop that allows Stat3 to modulate transcription only in the case of reduced Smad levels or possibly after sustained

TGF- β 1 signalling. Since Smads are not among the known Stat3 transcriptional targets it is unclear how it influences the Smad level. It is possible, that Stat3 affects a transcriptional mediator required for Smad-expression.

Besides Smad signalling, TGF- β 1 can also activate the MAP kinase pathway (112). Since there is evidence that Stat3 can be serine phosphorylated by MAP kinases the finding that Stat3 is required for the execution of TGF- β 1-induced effects could be rooted in this non-canonical signalling of TGF- β 1 (67). Further, TGF- β -activated kinase 1 (TAK1) binds to Stat3 and triggers Ser727 phosphorylation (55). Serine phosphorylation can entail different effects, i.e. it can enhance or repress transcriptional activity (67). Since the binding of Stat1 to CBP/p300 was decreased upon loss of serine727 phosphorylation, also Stat3 could require Ser727 phosphorylation to bind CBP/p300, form complexes with Smad and modulate TGF- β -Smad-mediated transcription (162).

By focusing on Smad2/3 distribution in cell layers, we found that expression and translocation of Smad2/3 was stronger if not exclusively at the periphery of cell collections. This observation fits together with findings that migration and invasion of tumor cells can be enhanced by TGF- β 1 treatment (137). Furthermore, at the invading edges of carcinomas TGF- β 1-signalling was reported to be changed to the Smad2/3 linker phosphorylated isoform which was found to be more invasive and proliferative (130). Therefore, not only the change of phosphorylation sites but also the amount of expressed Smad2/3 contributes to the increase of malignancy and mobility of cells at the leading edges of tumors. In further experiments, the phosphoisotype of the expressed Smad2/3 should be investigated. Since the linker-phosphorylated Smad3 isoform (pSmad3L) was associated with enhanced proliferative and invasive potential, we would expect to find mainly this isotype at the cell layer borders (123).

The levels of Smad2/3 in wild type cells are enhanced upon TGF- β 1 treatment. Thus, we assume that TGF- β 1 enhances Smad2/3 expression. Even without Stat3 there was some Smad2/3 detected in TGF- β 1-treated cells, but clearly less (Fig. 14, 15, lower panels). An expression analysis of Stat3 in MIM-stat3 Δ hc-wt-stat3 and MIM-R-stat3 Δ hc-wt-Stat3 cells by Western blot analysis was performed before the experiments. Since Stat3 is expressed in both cell lines, the question arises where its different influence and capacity to rescue the phenotype upon reintroduction is based

on. One consideration would be that Ras-transformed cells are less sensitive to any influences at all and more autonomous in their growth due to their transformation (1). Furthermore, the crosstalk with the MAK kinase pathway could account for enhanced TGF- β 1 tolerance of Ras-transformed cells. On the one hand, the activation of Erk/MAP kinase pathway by Ras can result in the inhibition of TGF- β 1-induced growth arrest but on the other, Ras and TGF- β 1 can act synergistically, since MAP kinase signalling is a non-canonical TGF- β signalling pathway (106, 121). So, since Ras and TGF- β 1 converge in the activation of the same pathway, the constitutively active Ras could render the cells insensitive to treatment with TGF- β 1.

One pathway of inducing proliferation arrest upon TGF- β 1 is the Smad-mediated release of c-myc from the promoters of the cyclin-dependent kinase (CDK) inhibitors p21^{WAF1} and p15^{Ink4B} (113). Since c-myc is a prominent transcriptional target of Stat3 we gave consideration into the possibility that this could be the crucial point of interference (66). The lack of Stat3 could entail a smaller amount of available c-myc, and thus the repression of the CDK inhibitors would be less. Consequently, we should observe diminished cell proliferation in Stat3-lacking cells. Since this was not the case, we exclude the role of Stat3 as transcription factor inducing the expression of c-myc as crucial for TGF- β 1-induced anti-proliferative effects.

There are a few transcriptional targets known to be shared by Stat3 and TGF- β Smad2/3. Among them are VEGF, MMP-2 and MMP-9 (66, 136, 137). These proteins affect the tumor-microenvironment. Smad2/3 binds to Smad-binding elements on DNA and Stat3 can induce the expression of genes containing SBEs in their promoters (111, 163). Possibly, there are other targets that need the concerted action of Stat3 and Smad2/3 to forward TGF- β -induced anti-proliferative and apoptotic effects. Bone morphogenic protein, which is a protein of the TGF- β family, activates Smad-Stat3 complexes to induce gene expression of ID1 (164). Smad1 and Stat3 both bind to the p300 cofactor to induce the expression of a set of genes leading to astrocyte differentiation (165). Smad1/p300 complex binds to Stat1/3 on glial-specific promoters (166). These findings strongly support this hypothesis.

Since the induction of apoptosis upon TGF- β was reported to be mediated via death-associated protein kinase (DAPK) in a hepatoma cell line, and via signalling factor GADD45b in hepatocytes, it would be of interest if the activity of these proteins is affected by the lack of Stat3 in our model system (108).

Src activation is a Smad-independent pathway of TGF- β signal transduction (106). Stat3 is a target of src tyrosine kinase, and this could be the reason why the lack of Stat3 impairs the anti-mitogenic effects of TGF- β (15). To prove this assumption it would be necessary to show that the proliferation-inhibitory and apoptotic effects of TGF- β rely largely on src kinase activity. This could be achieved if elevated levels of activated src were found upon TGF- β treatment and if the outcome of cell fate was changed in src kinase inactive of missing conditions.

In summary, we conclude that Stat3 is necessary for TGF- β 1-induced transcription of Smad2/3 but not its nuclear translocation, and the lack of Stat3 impairs TGF- β 1-mediated negative effects on survival and proliferation in untransformed and Ras-transformed hepatocytes. However, the inhibition of Stat3 would be harmful for patients since the tumor-suppressive effect of TGF- β 1 at early stages of tumor development gets blunted.

6. References

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646-674.
2. Parkin DM. Global cancer statistics in the year 2000. *Lancet Oncol* 2001;2:533-543.
3. Severi T, van Malenstein H, Verslype C, van Pelt JF. Tumor initiation and progression in hepatocellular carcinoma: risk factors, classification, and therapeutic targets. *Acta Pharmacol Sin* 2010;31:1409-1420.
4. Tischoff I, Tannapfe A. DNA methylation in hepatocellular carcinoma. *World J Gastroenterol* 2008;14:1741-1748.
5. Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. *Nat Genet* 2002;31:339-346.
6. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat Rev Cancer* 2009;9:798-809.
7. Iliopoulos D, Hirsch HA, Struhl K. An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. *Cell* 2009;139:693-706.
8. Silver JS, Hunter CA. gp130 at the nexus of inflammation, autoimmunity, and cancer. *J Leukoc Biol* 2010;88:1145-1156.
9. Kawai H, Suda T, Aoyagi Y, Isokawa O, Mita Y, Waguri N, Kuroiwa T, et al. Quantitative evaluation of genomic instability as a possible predictor for development of hepatocellular carcinoma: comparison of loss of heterozygosity and replication error. *Hepatology* 2000;31:1246-1250.
10. Maggioni M, Coggi G, Cassani B, Bianchi P, Romagnoli S, Mandelli A, Borzio M, et al. Molecular changes in hepatocellular dysplastic nodules on microdissected liver biopsies. *Hepatology* 2000;32:942-946.
11. Kondo Y, Kanai Y, Sakamoto M, Mizokami M, Ueda R, Hirohashi S. Genetic instability and aberrant DNA methylation in chronic hepatitis and cirrhosis--A comprehensive study of loss of heterozygosity and microsatellite instability at 39 loci and DNA hypermethylation on 8 CpG islands in microdissected specimens from patients with hepatocellular carcinoma. *Hepatology* 2000;32:970-979.
12. Saito Y, Kanai Y, Sakamoto M, Saito H, Ishii H, Hirohashi S. Expression of mRNA for DNA methyltransferases and methyl-CpG-binding proteins and DNA methylation status on CpG islands and pericentromeric satellite regions during human hepatocarcinogenesis. *Hepatology* 2001;33:561-568.
13. Huynh H. Molecularly targeted therapy in hepatocellular carcinoma. *Biochem Pharmacol* 2010;80:550-560.
14. Aaronson DS, Horvath CM. A road map for those who don't know JAK-STAT. *Science* 2002;296:1653-1655.
15. Buettner R, Mora LB, Jove R. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. *Clin Cancer Res* 2002;8:945-954.
16. Steelman LS, Chappell WH, Abrams SL, Kempf RC, Long J, Laidler P, Mijatovic S, et al. Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways in controlling growth and sensitivity to therapy-implications for cancer and aging. *Aging (Albany NY)* 2011;3:192-222.
17. Zhang YY, Xia HH. Novel therapeutic approaches for hepatocellular carcinoma: fact and fiction. *World J Gastroenterol* 2008;14:1641-1642.
18. Harada K, Shiota G, Kawasaki H. Transforming growth factor-alpha and epidermal growth factor receptor in chronic liver disease and hepatocellular carcinoma. *Liver* 1999;19:318-325.
19. Dewilde S, Vercelli A, Chiarle R, Poli V. Of alphas and betas: distinct and overlapping functions of STAT3 isoforms. *Front Biosci* 2008;13:6501-6514.
20. Takahashi-Tezuka M, Yoshida Y, Fukada T, Ohtani T, Yamanaka Y, Nishida K, Nakajima K, et al. Gab1 acts as an adapter molecule linking the cytokine receptor gp130 to ERK mitogen-activated protein kinase. *Mol Cell Biol* 1998;18:4109-4117.
21. Culig Z. Cytokine disbalance in common human cancers. *Biochim Biophys Acta* 2011;1813:308-314.
22. Levy DE, Darnell JE, Jr. Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol* 2002;3:651-662.
23. Rebouissou S, Amessou M, Couchy G, Poussin K, Imbeaud S, Pilati C, Izard T, et al. Frequent in-frame somatic deletions activate gp130 in inflammatory hepatocellular tumours. *Nature* 2009;457:200-204.
24. Fizazi K. The role of Src in prostate cancer. *Ann Oncol* 2007;18:1765-1773.
25. Giambartolomei S, Covone F, Levrero M, Balsano C. Sustained activation of the Raf/MEK/Erk pathway in response to EGF in stable cell lines expressing the Hepatitis C Virus (HCV) core protein. *Oncogene* 2001;20:2606-2610.

26. Yoshida T, Hanada T, Tokuhisa T, Kosai K, Sata M, Kohara M, Yoshimura A. Activation of STAT3 by the hepatitis C virus core protein leads to cellular transformation. *J Exp Med* 2002;196:641-653.
27. Yun C, Cho H, Kim SJ, Lee JH, Park SY, Chan GK. Mitotic aberration coupled with centrosome amplification is induced by hepatitis B virus X oncoprotein via the Ras-mitogen-activated protein/extracellular signal-regulated kinase-mitogen-activated protein pathway. *Mol Cancer Res* 2004;2:159-169.
28. Schmitz KJ, Wohlschlaeger J, Lang H, Sotiropoulos GC, Malago M, Steveling K, Reis H, et al. Activation of the ERK and AKT signalling pathway predicts poor prognosis in hepatocellular carcinoma and ERK activation in cancer tissue is associated with hepatitis C virus infection. *J Hepatol* 2008;48:83-90.
29. Shaw RJ, Cantley LC. Ras, PI(3)K and mTOR signalling controls tumour cell growth. *Nature* 2006;441:424-430.
30. Calvisi DF, Ladu S, Conner EA, Seo D, Hsieh JT, Factor VM, Thorgeirsson SS. Inactivation of Ras GTPase-activating proteins promotes unrestrained activity of wild-type Ras in human liver cancer. *J Hepatol* 2011;54:311-319.
31. Calvisi DF, Ladu S, Gorden A, Farina M, Conner EA, Lee JS, Factor VM, et al. Ubiquitous activation of Ras and Jak/Stat pathways in human HCC. *Gastroenterology* 2006;130:1117-1128.
32. Hwang YH, Choi JY, Kim S, Chung ES, Kim T, Koh SS, Lee B, et al. Over-expression of c-raf-1 proto-oncogene in liver cirrhosis and hepatocellular carcinoma. *Hepatol Res* 2004;29:113-121.
33. Benn J, Schneider RJ. Hepatitis B virus HBx protein activates Ras-GTP complex formation and establishes a Ras, Raf, MAP kinase signaling cascade. *Proc Natl Acad Sci U S A* 1994;91:10350-10354.
34. Bernardi R, Guernah I, Jin D, Grisendi S, Alimonti A, Teruya-Feldstein J, Cordon-Cardo C, et al. PML inhibits HIF-1alpha translation and neoangiogenesis through repression of mTOR. *Nature* 2006;442:779-785.
35. Martelli AM, Evangelisti C, Chiarini F, Grimaldi C, Cappellini A, Ognibene A, McCubrey JA. The emerging role of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin signaling network in normal myelopoiesis and leukemogenesis. *Biochim Biophys Acta* 2010;1803:991-1002.
36. Horie Y, Suzuki A, Kataoka E, Sasaki T, Hamada K, Sasaki J, Mizuno K, et al. Hepatocyte-specific Pten deficiency results in steatohepatitis and hepatocellular carcinomas. *J Clin Invest* 2004;113:1774-1783.
37. Kisseleva T, Bhattacharya S, Braunstein J, Schindler CW. Signaling through the JAK/STAT pathway, recent advances and future challenges. *Gene* 2002;285:1-24.
38. Verma A, Kambhampati S, Parmar S, Plataniias LC. Jak family of kinases in cancer. *Cancer Metastasis Rev* 2003;22:423-434.
39. O'Shea JJ, Gadina M, Schreiber RD. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. *Cell* 2002;109 Suppl:S121-131.
40. O'Sullivan LA, Liongue C, Lewis RS, Stephenson SE, Ward AC. Cytokine receptor signaling through the Jak-Stat-Socs pathway in disease. *Mol Immunol* 2007;44:2497-2506.
41. O'Shea JJ, Kanno Y, Chen X, Levy DE. Cell signaling. Stat acetylation--a key facet of cytokine signaling? *Science* 2005;307:217-218.
42. Notarangelo LD, Mella P, Jones A, de Saint Basile G, Savoldi G, Cranston T, Vihinen M, et al. Mutations in severe combined immune deficiency (SCID) due to JAK3 deficiency. *Hum Mutat* 2001;18:255-263.
43. Martin J, Dufour JF. Tumor suppressor and hepatocellular carcinoma. *World J Gastroenterol* 2008;14:1720-1733.
44. Hornakova T, Springuel L, Devreux J, Dusa A, Constantinescu SN, Knoops L, Renaud JC. Oncogenic JAK1 and JAK2-activating mutations resistant to ATP-competitive inhibitors. *Haematologica* 2011;96:845-853.
45. Jeong EG, Kim MS, Nam HK, Min CK, Lee S, Chung YJ, Yoo NJ, et al. Somatic mutations of JAK1 and JAK3 in acute leukemias and solid cancers. *Clin Cancer Res* 2008;14:3716-3721.
46. Cornejo MG, Boggon TJ, Mercher T. JAK3: a two-faced player in hematological disorders. *Int J Biochem Cell Biol* 2009;41:2376-2379.
47. Lin Q, Lai R, Chirieac LR, Li C, Thomazy VA, Grammatikakis I, Rassidakis GZ, et al. Constitutive activation of JAK3/STAT3 in colon carcinoma tumors and cell lines: inhibition of JAK3/STAT3 signaling induces apoptosis and cell cycle arrest of colon carcinoma cells. *Am J Pathol* 2005;167:969-980.
48. Wen R, Wang D, McKay C, Bunting KD, Marine JC, Vanin EF, Zambetti GP, et al. Jak3 selectively regulates Bax and Bcl-2 expression to promote T-cell development. *Mol Cell Biol* 2001;21:678-689.

49. Kim S, Choi JH, Lim HI, Lee SK, Kim WW, Cho S, Kim JS, et al. EGF-induced MMP-9 expression is mediated by the JAK3/ERK pathway, but not by the JAK3/STAT-3 pathway in a SKBR3 breast cancer cell line. *Cell Signal* 2009;21:892-898.
50. Frost RA, Nystrom GJ, Lang CH. Regulation of IGF-I mRNA and signal transducers and activators of transcription-3 and -5 (Stat-3 and -5) by GH in C2C12 myoblasts. *Endocrinology* 2002;143:492-503.
51. Bromberg J, Darnell JE, Jr. The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* 2000;19:2468-2473.
52. Takeda K, Noguchi K, Shi W, Tanaka T, Matsumoto M, Yoshida N, Kishimoto T, et al. Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proc Natl Acad Sci U S A* 1997;94:3801-3804.
53. Yu H, Jove R. The STATs of cancer--new molecular targets come of age. *Nat Rev Cancer* 2004;4:97-105.
54. Horvath CM. STAT proteins and transcriptional responses to extracellular signals. *Trends Biochem Sci* 2000;25:496-502.
55. Aggarwal BB, Kunnumakkara AB, Harikumar KB, Gupta SR, Tharakan ST, Koca C, Dey S, et al. Signal transducer and activator of transcription-3, inflammation, and cancer: how intimate is the relationship? *Ann N Y Acad Sci* 2009;1171:59-76.
56. Garcia R, Yu CL, Hudnall A, Catlett R, Nelson KL, Smithgall T, Fujita DJ, et al. Constitutive activation of Stat3 in fibroblasts transformed by diverse oncoproteins and in breast carcinoma cells. *Cell Growth Differ* 1997;8:1267-1276.
57. Yang J, Stark GR. Roles of unphosphorylated STATs in signaling. *Cell Res* 2008;18:443-451.
58. Brown S, Zeidler MP. Unphosphorylated STATs go nuclear. *Curr Opin Genet Dev* 2008;18:455-460.
59. Paulson M, Pisharody S, Pan L, Guadagno S, Mui AL, Levy DE. Stat protein transactivation domains recruit p300/CBP through widely divergent sequences. *J Biol Chem* 1999;274:25343-25349.
60. Zhang X, Wrzeszczynska MH, Horvath CM, Darnell JE, Jr. Interacting regions in Stat3 and c-Jun that participate in cooperative transcriptional activation. *Mol Cell Biol* 1999;19:7138-7146.
61. Decker T, Kovarik P. Serine phosphorylation of STATs. *Oncogene* 2000;19:2628-2637.
62. Darnell JE, Jr. STATs and gene regulation. *Science* 1997;277:1630-1635.
63. Caldenhoven E, van Dijk TB, Solari R, Armstrong J, Raaijmakers JA, Lammers JW, Koenderman L, et al. STAT3beta, a splice variant of transcription factor STAT3, is a dominant negative regulator of transcription. *J Biol Chem* 1996;271:13221-13227.
64. Maritano D, Sugrue ML, Tininini S, Dewilde S, Strobl B, Fu X, Murray-Tait V, et al. The STAT3 isoforms alpha and beta have unique and specific functions. *Nat Immunol* 2004;5:401-409.
65. Fukuzawa M, Araki T, Adrian I, Williams JG. Tyrosine phosphorylation-independent nuclear translocation of a dictyostelium STAT in response to DIF signaling. *Mol Cell* 2001;7:779-788.
66. Groner B, Lucks P, Borghouts C. The function of Stat3 in tumor cells and their microenvironment. *Semin Cell Dev Biol* 2008;19:341-350.
67. Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis. *Oncogene* 2000;19:2474-2488.
68. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE, Jr. Stat3 as an oncogene. *Cell* 1999;98:295-303.
69. He G, Karin M. NF-kappaB and STAT3 - key players in liver inflammation and cancer. *Cell Res* 2011;21:159-168.
70. Li WC, Ye SL, Sun RX, Liu YK, Tang ZY, Kim Y, Karras JG, et al. Inhibition of growth and metastasis of human hepatocellular carcinoma by antisense oligonucleotide targeting signal transducer and activator of transcription 3. *Clin Cancer Res* 2006;12:7140-7148.
71. Zhang CH, Xu GL, Jia WD, Li JS, Ma JL, Ren WH, Ge YS, et al. Activation of STAT3 Signal Pathway Correlates with Twist and E-Cadherin Expression in Hepatocellular Carcinoma and Their Clinical Significance. *J Surg Res* 2010.
72. Ram PT, Horvath CM, Iyengar R. Stat3-mediated transformation of NIH-3T3 cells by the constitutively active Q205L Galphao protein. *Science* 2000;287:142-144.
73. Bromberg JF, Horvath CM, Besser D, Lathem WW, Darnell JE, Jr. Stat3 activation is required for cellular transformation by v-src. *Mol Cell Biol* 1998;18:2553-2558.
74. Yu CL, Meyer DJ, Campbell GS, Larner AC, Carter-Su C, Schwartz J, Jove R. Enhanced DNA-binding activity of a Stat3-related protein in cells transformed by the Src oncoprotein. *Science* 1995;269:81-83.
75. Yang SF, Wang SN, Wu CF, Yeh YT, Chai CY, Chunag SC, Sheen MC, et al. Altered p-STAT3 (tyr705) expression is associated with histological grading and intratumour microvessel density in hepatocellular carcinoma. *J Clin Pathol* 2007;60:642-648.

76. Starr R, Willson TA, Viney EM, Murray LJ, Rayner JR, Jenkins BJ, Gonda TJ, et al. A family of cytokine-inducible inhibitors of signalling. *Nature* 1997;387:917-921.
77. Endo TA, Masuhara M, Yokouchi M, Suzuki R, Sakamoto H, Mitsui K, Matsumoto A, et al. A new protein containing an SH2 domain that inhibits JAK kinases. *Nature* 1997;387:921-924.
78. Shuai K, Liu B. Regulation of JAK-STAT signalling in the immune system. *Nat Rev Immunol* 2003;3:900-911.
79. Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signalling and immune regulation. *Nat Rev Immunol* 2007;7:454-465.
80. Niwa Y, Kanda H, Shikauchi Y, Saiura A, Matsubara K, Kitagawa T, Yamamoto J, et al. Methylation silencing of SOCS-3 promotes cell growth and migration by enhancing JAK/STAT and FAK signalings in human hepatocellular carcinoma. *Oncogene* 2005;24:6406-6417.
81. Okochi O, Hibi K, Sakai M, Inoue S, Takeda S, Kaneko T, Nakao A. Methylation-mediated silencing of SOCS-1 gene in hepatocellular carcinoma derived from cirrhosis. *Clin Cancer Res* 2003;9:5295-5298.
82. Yoshida T, Ogata H, Kamio M, Joo A, Shiraishi H, Tokunaga Y, Sata M, et al. SOCS1 is a suppressor of liver fibrosis and hepatitis-induced carcinogenesis. *J Exp Med* 2004;199:1701-1707.
83. Irie-Sasaki J, Sasaki T, Matsumoto W, Opavsky A, Cheng M, Welstead G, Griffiths E, et al. CD45 is a JAK phosphatase and negatively regulates cytokine receptor signalling. *Nature* 2001;409:349-354.
84. Chung CD, Liao J, Liu B, Rao X, Jay P, Berta P, Shuai K. Specific inhibition of Stat3 signal transduction by PIAS3. *Science* 1997;278:1803-1805.
85. Jackson PK. A new RING for SUMO: wrestling transcriptional responses into nuclear bodies with PIAS family E3 SUMO ligases. *Genes Dev* 2001;15:3053-3058.
86. Myers MP, Andersen JN, Cheng A, Tremblay ML, Horvath CM, Parisien JP, Salmeen A, et al. TYK2 and JAK2 are substrates of protein-tyrosine phosphatase 1B. *J Biol Chem* 2001;276:47771-47774.
87. Chen KF, Tai WT, Liu TH, Huang HP, Lin YC, Shiau CW, Li PK, et al. Sorafenib overcomes TRAIL resistance of hepatocellular carcinoma cells through the inhibition of STAT3. *Clin Cancer Res* 2010;16:5189-5199.
88. Lau CK, Yang ZF, Lam SP, Lam CT, Ngai P, Tam KH, Poon RT, et al. Inhibition of Stat3 activity by YC-1 enhances chemo-sensitivity in hepatocellular carcinoma. *Cancer Biol Ther* 2007;6:1900-1907.
89. Lin L, Amin R, Gallicano GI, Glasgow E, Jogunoori W, Jessup JM, Zasloff M, et al. The STAT3 inhibitor NSC 74859 is effective in hepatocellular cancers with disrupted TGF-beta signaling. *Oncogene* 2009;28:961-972.
90. de la Iglesia N, Konopka G, Puram SV, Chan JA, Bachoo RM, You MJ, Levy DE, et al. Identification of a PTEN-regulated STAT3 brain tumor suppressor pathway. *Genes Dev* 2008;22:449-462.
91. Ecker A, Simma O, Hoelbl A, Kenner L, Beug H, Moriggl R, Sexl V. The dark and the bright side of Stat3: proto-oncogene and tumor-suppressor. *Front Biosci* 2009;14:2944-2958.
92. Musteanu M, Blaas L, Mair M, Schleder M, Bilban M, Tauber S, Esterbauer H, et al. Stat3 is a negative regulator of intestinal tumor progression in Apc(Min) mice. *Gastroenterology* 2010;138:1003-1011 e1001-1005.
93. Sherr CJ. The INK4a/ARF network in tumour suppression. *Nat Rev Mol Cell Biol* 2001;2:731-737.
94. Kim WY, Sharpless NE. The regulation of INK4/ARF in cancer and aging. *Cell* 2006;127:265-275.
95. Pollice A, Vivo M, La Mantia G. The promiscuity of ARF interactions with the proteasome. *FEBS Lett* 2008;582:3257-3262.
96. Sherr CJ. Divorcing ARF and p53: an unsettled case. *Nat Rev Cancer* 2006;6:663-673.
97. Tannapfel A, Busse C, Weinans L, Benicke M, Katalinic A, Geissler F, Hauss J, et al. INK4a-ARF alterations and p53 mutations in hepatocellular carcinomas. *Oncogene* 2001;20:7104-7109.
98. Weber JD, Jeffers JR, Rehg JE, Randle DH, Lozano G, Roussel MF, Sherr CJ, et al. p53-independent functions of the p19(ARF) tumor suppressor. *Genes Dev* 2000;14:2358-2365.
99. Qi Y, Gregory MA, Li Z, Brousal JP, West K, Hann SR. p19ARF directly and differentially controls the functions of c-Myc independently of p53. *Nature* 2004;431:712-717.
100. Anzola M, Cuevas N, Lopez-Martinez M, Saiz A, Burgos JJ, Martinez de Pancorboa M. P14ARF gene alterations in human hepatocellular carcinoma. *Eur J Gastroenterol Hepatol* 2004;16:19-26.

101. Edamoto Y, Hara A, Biernat W, Terracciano L, Cathomas G, Riehle HM, Matsuda M, et al. Alterations of RB1, p53 and Wnt pathways in hepatocellular carcinomas associated with hepatitis C, hepatitis B and alcoholic liver cirrhosis. *Int J Cancer* 2003;106:334-341.
102. Akhurst RJ, Derynck R. TGF-beta signaling in cancer--a double-edged sword. *Trends Cell Biol* 2001;11:S44-51.
103. Meulmeester E, Ten Dijke P. The dynamic roles of TGF-beta in cancer. *J Pathol* 2011;223:205-218.
104. Flavell RA, Sanjabi S, Wrzesinski SH, Licona-Limon P. The polarization of immune cells in the tumour environment by TGFbeta. *Nat Rev Immunol* 2010;10:554-567.
105. Verrecchia F, Mauviel A. Transforming growth factor-beta and fibrosis. *World J Gastroenterol* 2007;13:3056-3062.
106. Heldin CH, Moustakas A. Role of Smads in TGFbeta signaling. *Cell Tissue Res* 2011.
107. Chacko BM, Qin BY, Tiwari A, Shi G, Lam S, Hayward LJ, De Caestecker M, et al. Structural basis of heteromeric smad protein assembly in TGF-beta signaling. *Mol Cell* 2004;15:813-823.
108. Massague J, Seoane J, Wotton D. Smad transcription factors. *Genes Dev* 2005;19:2783-2810.
109. Wu JW, Hu M, Chai J, Seoane J, Huse M, Li C, Rigotti DJ, et al. Crystal structure of a phosphorylated Smad2. Recognition of phosphoserine by the MH2 domain and insights on Smad function in TGF-beta signaling. *Mol Cell* 2001;8:1277-1289.
110. Ikushima H, Miyazono K. TGFbeta signalling: a complex web in cancer progression. *Nat Rev Cancer* 2010;10:415-424.
111. Shi Y, Wang YF, Jayaraman L, Yang H, Massague J, Pavletich NP. Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-beta signaling. *Cell* 1998;94:585-594.
112. Massague J. TGFbeta in Cancer. *Cell* 2008;134:215-230.
113. Derynck R, Akhurst RJ, Balmain A. TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet* 2001;29:117-129.
114. Wong C, Rougier-Chapman EM, Frederick JP, Datto MB, Liberati NT, Li JM, Wang XF. Smad3-Smad4 and AP-1 complexes synergize in transcriptional activation of the c-Jun promoter by transforming growth factor beta. *Mol Cell Biol* 1999;19:1821-1830.
115. Zhang Y, Feng XH, Derynck R. Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-beta-induced transcription. *Nature* 1998;394:909-913.
116. Siegel PM, Massague J. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat Rev Cancer* 2003;3:807-821.
117. Kang Y, Chen CR, Massague J. A self-enabling TGFbeta response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. *Mol Cell* 2003;11:915-926.
118. Azar R, Alard A, Susini C, Bousquet C, Pyronnet S. 4E-BP1 is a target of Smad4 essential for TGFbeta-mediated inhibition of cell proliferation. *EMBO J* 2009;28:3514-3522.
119. Seoane J, Le HV, Shen L, Anderson SA, Massague J. Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. *Cell* 2004;117:211-223.
120. Kang JS, Alliston T, Delston R, Derynck R. Repression of Runx2 function by TGF-beta through recruitment of class II histone deacetylases by Smad3. *EMBO J* 2005;24:2543-2555.
121. Daly AC, Vizan P, Hill CS. Smad3 protein levels are modulated by Ras activity and during the cell cycle to dictate transforming growth factor-beta responses. *J Biol Chem* 2010;285:6489-6497.
122. Kretschmar M, Doody J, Massague J. Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1. *Nature* 1997;389:618-622.
123. Matsuzaki K, Kitano C, Murata M, Sekimoto G, Yoshida K, Uemura Y, Seki T, et al. Smad2 and Smad3 phosphorylated at both linker and COOH-terminal regions transmit malignant TGF-beta signal in later stages of human colorectal cancer. *Cancer Res* 2009;69:5321-5330.
124. Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, et al. The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. *Cell* 1997;89:1165-1173.
125. Kavsak P, Rasmussen RK, Causing CG, Bonni S, Zhu H, Thomsen GH, Wrana JL. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. *Mol Cell* 2000;6:1365-1375.
126. Kim RH, Wang D, Tsang M, Martin J, Huff C, de Caestecker MP, Parks WT, et al. A novel smad nuclear interacting protein, SNIP1, suppresses p300-dependent TGF-beta signal transduction. *Genes Dev* 2000;14:1605-1616.
127. Benus GF, Wierenga AT, de Gorter DJ, Schuringa JJ, van Bennekum AM, Drenth-Diephuis L, Vellenga E, et al. Inhibition of the transforming growth factor beta (TGFbeta) pathway by interleukin-

- 1beta is mediated through TGFbeta-activated kinase 1 phosphorylation of SMAD3. *Mol Biol Cell* 2005;16:3501-3510.
128. Ho J, Cocolakis E, Dumas VM, Posner BI, Laporte SA, Lebrun JJ. The G protein-coupled receptor kinase-2 is a TGFbeta-inducible antagonist of TGFbeta signal transduction. *EMBO J* 2005;24:3247-3258.
 129. Matsuura I, Denissova NG, Wang G, He D, Long J, Liu F. Cyclin-dependent kinases regulate the antiproliferative function of Smads. *Nature* 2004;430:226-231.
 130. Matsuzaki K. Smad phosphoisoform signaling specificity: the right place at the right time. *Carcinogenesis* 2011.
 131. Wicks SJ, Lui S, Abdel-Wahab N, Mason RM, Chantry A. Inactivation of smad-transforming growth factor beta signaling by Ca(2+)-calmodulin-dependent protein kinase II. *Mol Cell Biol* 2000;20:8103-8111.
 132. Grusch M, Petz M, Metzner T, Ozturk D, Schneller D, Mikulits W. The crosstalk of RAS with the TGF-beta family during carcinoma progression and its implications for targeted cancer therapy. *Curr Cancer Drug Targets* 2010;10:849-857.
 133. Kretschmar M, Doody J, Timokhina I, Massague J. A mechanism of repression of TGFbeta/Smad signaling by oncogenic Ras. *Genes Dev* 1999;13:804-816.
 134. Roelen BA, Cohen OS, Raychowdhury MK, Chadee DN, Zhang Y, Kyriakis JM, Alessandrini AA, et al. Phosphorylation of threonine 276 in Smad4 is involved in transforming growth factor-beta-induced nuclear accumulation. *Am J Physiol Cell Physiol* 2003;285:C823-830.
 135. Levy L, Hill CS. Alterations in components of the TGF-beta superfamily signaling pathways in human cancer. *Cytokine Growth Factor Rev* 2006;17:41-58.
 136. Pertovaara L, Kaipainen A, Mustonen T, Orpina A, Ferrara N, Saksela O, Alitalo K. Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. *J Biol Chem* 1994;269:6271-6274.
 137. Welch DR, Fabra A, Nakajima M. Transforming growth factor beta stimulates mammary adenocarcinoma cell invasion and metastatic potential. *Proc Natl Acad Sci U S A* 1990;87:7678-7682.
 138. Gotzmann J, Mikula M, Eger A, Schulte-Hermann R, Foisner R, Beug H, Mikulits W. Molecular aspects of epithelial cell plasticity: implications for local tumor invasion and metastasis. *Mutat Res* 2004;566:9-20.
 139. van Zijl F, Mall S, Machat G, Pirker C, Zeillinger R, Weinhaeusel A, Bilban M, et al. A human model of epithelial to mesenchymal transition to monitor drug efficacy in hepatocellular carcinoma progression. *Mol Cancer Ther* 2011;10:850-860.
 140. Smith AP, Verrecchia A, Faga G, Doni M, Perna D, Martinato F, Guccione E, et al. A positive role for Myc in TGFbeta-induced Snail transcription and epithelial-to-mesenchymal transition. *Oncogene* 2009;28:422-430.
 141. Vincent T, Neve EP, Johnson JR, Kukalev A, Rojo F, Albanell J, Pietras K, et al. A SNAIL1-SMAD3/4 transcriptional repressor complex promotes TGF-beta mediated epithelial-mesenchymal transition. *Nat Cell Biol* 2009;11:943-950.
 142. Paschos KA, Majeed AW, Bird NC. Role of Kupffer cells in the outgrowth of colorectal cancer liver metastases. *Hepatol Res* 2010;40:83-94.
 143. Acun T, Oztas E, Yagci T, Yakicier MC. SIP1 is downregulated in hepatocellular carcinoma by promoter hypermethylation. *BMC Cancer* 2011;11:223.
 144. Murata M, Matsuzaki K, Yoshida K, Sekimoto G, Tahashi Y, Mori S, Uemura Y, et al. Hepatitis B virus X protein shifts human hepatic transforming growth factor (TGF)-beta signaling from tumor suppression to oncogenesis in early chronic hepatitis B. *Hepatology* 2009;49:1203-1217.
 145. Schneller D, Machat G, Sousek A, Proell V, van Zijl F, Zulehner G, Huber H, et al. p19(ARF)/p14(ARF) controls oncogenic functions of Stat3 in hepatocellular carcinoma. *Hepatology* 2011.
 146. Albritton LM, Kim JW, Tseng L, Cunningham JM. Envelope-binding domain in the cationic amino acid transporter determines the host range of ecotropic murine retroviruses. *J Virol* 1993;67:2091-2096.
 147. Kamijo T, Zindy F, Roussel MF, Quelle DE, Downing JR, Ashmun RA, Grosveld G, et al. Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. *Cell* 1997;91:649-659.
 148. Mikula M, Fuchs E, Huber H, Beug H, Schulte-Hermann R, Mikulits W. Immortalized p19ARF null hepatocytes restore liver injury and generate hepatic progenitors after transplantation. *Hepatology* 2004;39:628-634.
 149. Fischer AN, Herrera B, Mikula M, Proell V, Fuchs E, Gotzmann J, Schulte-Hermann R, et al. Integration of Ras subeffector signaling in TGF-beta mediated late stage hepatocarcinogenesis. *Carcinogenesis* 2005;26:931-942.

150. Mair M, Zollner G, Schneller D, Musteanu M, Fickert P, Gumhold J, Schuster C, et al. Signal transducer and activator of transcription 3 protects from liver injury and fibrosis in a mouse model of sclerosing cholangitis. *Gastroenterology* 2010;138:2499-2508.
151. Gotzmann J, Huber H, Thallinger C, Wolschek M, Jansen B, Schulte-Hermann R, Beug H, et al. Hepatocytes convert to a fibroblastoid phenotype through the cooperation of TGF-beta1 and Ha-Ras: steps towards invasiveness. *J Cell Sci* 2002;115:1189-1202.
152. Mikula M, Fuchs E, Huber H, Beug H, Schulte-Hermann R, Mikulits W. Immortalized p19ARF null hepatocytes restore liver injury and generate hepatic progenitors after transplantation. *Hepatology* 2004;39:628-634.
153. Gotzmann J, Huber H, Thallinger C, Wolschek M, Jansen B, Schulte-Hermann R, Beug H, et al. Hepatocytes convert to a fibroblastoid phenotype through the cooperation of TGF-beta1 and Ha-Ras: steps towards invasiveness. *J Cell Sci* 2002;115:1189-1202.
154. Scott A, Wang Z. Tumour suppressor function of protein tyrosine phosphatase receptor-T. *Biosci Rep* 2011;31:303-307.
155. Croker BA, Kiu H, Nicholson SE. SOCS regulation of the JAK/STAT signalling pathway. *Semin Cell Dev Biol* 2008;19:414-422.
156. Murray PJ. The JAK-STAT signaling pathway: input and output integration. *J Immunol* 2007;178:2623-2629.
157. Fischer AN, Fuchs E, Mikula M, Huber H, Beug H, Mikulits W. PDGF essentially links TGF-beta signaling to nuclear beta-catenin accumulation in hepatocellular carcinoma progression. *Oncogene* 2007;26:3395-3405.
158. Lin Y, Shi CY, Li B, Soo BH, Mohammed-Ali S, Wee A, Oon CJ, et al. Tumour suppressor p53 and Rb genes in human hepatocellular carcinoma. *Ann Acad Med Singapore* 1996;25:22-30.
159. Cao LQ, Chen XL, Wang Q, Huang XH, Zhen MC, Zhang LJ, Li W, et al. Upregulation of PTEN involved in rosiglitazone-induced apoptosis in human hepatocellular carcinoma cells. *Acta Pharmacol Sin* 2007;28:879-887.
160. Xie L, Qin WX, Li JJ, He XH, Shu HQ, Yao GF, Wan DF, et al. cDNA expression array analysis of gene expression in human hepatocarcinoma Hep3B cells induced by BNIP1-1. *Acta Biochim Biophys Sin (Shanghai)* 2005;37:618-624.
161. Reimann M, Schmitt CA, Lee S. Non-cell-autonomous tumor suppression: oncogene-provoked apoptosis promotes tumor cell senescence via stromal crosstalk. *J Mol Med (Berl)* 2011;89:869-875.
162. Brierley MM, Fish EN. Stats: multifaceted regulators of transcription. *J Interferon Cytokine Res* 2005;25:733-744.
163. Katoh M. Transcriptional mechanisms of WNT5A based on NF-kappaB, Hedgehog, TGFbeta, and Notch signaling cascades. *Int J Mol Med* 2009;23:763-769.
164. Ying QL, Nichols J, Chambers I, Smith A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* 2003;115:281-292.
165. Nakashima K, Yanagisawa M, Arakawa H, Kimura N, Hisatsune T, Kawabata M, Miyazono K, et al. Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300. *Science* 1999;284:479-482.
166. Sun Y, Nadal-Vicens M, Misono S, Lin MZ, Zubiaga A, Hua X, Fan G, et al. Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. *Cell* 2001;104:365-376.

7. Abbreviations

APS, ammonium persulfate
 ARF, p14^{ARF} and p19^{ARF}
 BAD, Bcl-2 associated death
 BMP, bone morphogenic protein
 BSA, bovine serum albumin
 ca, constitutively active
 CBP, Creb binding protein
 CDK, cyclin-dependent kinase
 DAPK, death associated protein kinase
 DMEM, Dulbecco's Modified Eagle's Medium
 DNMT, DNA-methyltransferase
 Erk, extracellular signal-regulated kinase
 FCS, fetal calf serum
 HCC, human hepatocellular carcinoma
 HDM2, human double minute 2
 HGF, hepatocyte growth factor
 HIF1 α , hypoxia-induced factor 1- α
 I-Smads, inhibitory Smads
 ID, inhibitor of differentiation
 IFN, interferon
 IGF-II, insulin-like growth factor
 IL, interleukin
 Jak, Janus kinase
 MAPK, mitogen-activated protein kinase
 MDM2, murine double minute 2
 MEF, mouse embryonic fibroblasts
 MH, Mad homology domain
 MMP, matrix metalloprotease
 mTOR, mammalian target of rapamycin
 NMP, nucleophosmin
 PAA, polyacrylic acid
 Pan-jak, Jak1 – 3 and Tyk2
 PBS, phosphate buffered saline
 PI3K, phosphatidylinositol-3-kinase
 Plc, Plc/prf/5

Plc-p14, Plc/prf/5-p14^{ARF}
 pSmad3L, linker phosphorylated Smad3
 PTEN, phosphatase and tensin homologue deleted on chromosome ten
 PTPRT, protein tyrosine phosphatase receptor-T
 pY-Stat3, tyrosine 705 phosphorylated Stat3
 R-Smads, receptor-associated Smads
 Raf, Ras-activated factor
 RIPA buffer, Radioimmunoprecipitation assay buffer
 RPMI, RPMI-1640 cell culture medium
 RT, room temperature
 SBE, Smad binding element
 SCID, severe combined immunodeficiency
 SDS, sodium-dodecyl-sulfate
 Ser, serine
 sh, small hairpin
 SH2, Src homology 2
 shc, small hairpin control
 SHIP, SH2 domain-containing inositol 5'phosphatases
 SHP-1, Src homology 2 domain-containing protein tyrosine phosphatase 1
 si, small interfering
 SOCS, suppressor of cytokine signalling
 Stat3, Signal transducer and activator of transcription 3
 TAK1, TGF- β -activated-kinase 1
 TBST, Tris-buffered saline-0,1% Tween
 TEMED, tetramethylethylenediamine
 TGF- β 1, transforming growth factor β 1
 TGF- α , transforming-growth factor- α
 Tyk, Tyrosine kinase
 Tyr, tyrosine
 T β R, TGF- β receptor type
 U-Stat, unphosphorylated Stat
 wt, wild type

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9. Curriculum vitae

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