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by

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JANUS KINASES AS TARGETS FOR SMALL MOLECULE INHIBITORS

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1 INTRODUCTION

1.1 PROTEIN KINASES

1.1.1 Protein phosphorylation

Proteins are in a constant state of flux, with their activity, sub-cellular localization, molecular interactions and stability being modified in response to external and internal signals. Many cellular processes, e.g. those involved in intracellular communication and coordination of complex functions, are controlled by protein phosphorylation. The phenomenon of protein phosphorylation is known for over 100 years by now: in 1906 the presence of a phosphate in the protein Vitellin was described (1) and in 1933 a phosphate group was found to be attached to a serine residue in Vitellin (2). Interestingly, it took another 20 years before the first enzymatic phosphorylation of proteins was reported (3).

1.1.2 Protein kinases

Eukaryotic protein phosphorylation is conducted mainly by a single superfamily of eukaryotic protein kinases, one of the largest of protein families, being encoded by 1.5-2.5% of all eukaryotic genes (4). 518 protein kinases have been identified. Interestingly, 48 of these proteins are called pseudokinases and lack one or more of the conserved amino acids in the kinase domain that are required for catalytic activity. Pseudokinases are therefore predicted to be enzymatically inactive. However, five pseudokinases in addition to their pseudokinase domains contain a fully functional kinase fold making them catalytically active (5). Furthermore, several pseudokinases were shown to interact with other kinases leading to their activation. For example, HER3 upon binding to its ligand forms a complex with the kinase HER2 stimulating its autophosphorylation and activation (6).

Protein kinases add the γ-phosphate group of ATP to specific amino acid residues on protein substrates. Within a protein, phosphorylation can occur on several amino acids such as serine, threonine, tyrosine or histidine. Phosphorylation on serine residues is the most common, followed by threonine residues. Tyrosine phosphorylation is relatively rare and was discovered by Hunter and colleagues in 1979 (7). Depending on the specificity of the substrate amino acids, protein kinases are known as serine/threonine

(Ser/Thr) kinases, tyrosine (Tyr) kinases, histidine kinases (8) or as dual specificity (Ser/Thr and Tyr) kinases (9).

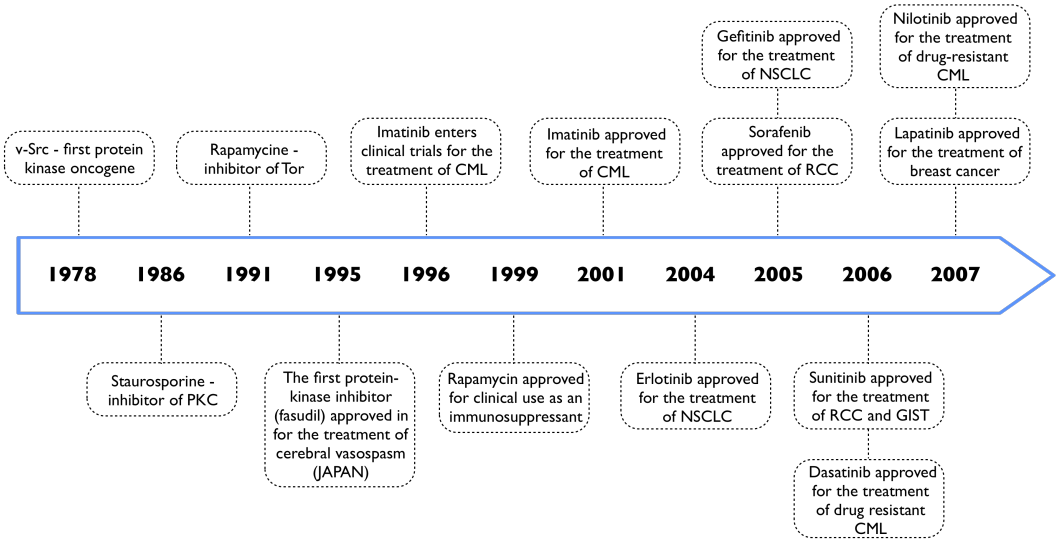

Figure 1

A timeline of key events in the development of protein kinase inhibitors.

Kinase activity is subjected to spatial and temporal regulation, often by more than one regulatory mechanism. Mutations and deregulations of protein kinases are often associated with cellular transformation and cancer and as a matter of fact the first discovered oncogene was the protein kinase v-Src (7, 10). Since the importance of kinase activities in many physiological processes was recognized, extensive efforts have been undertaken to develop kinase inhibitors (Fig. 1) for the treatment of a wide range of cancers.

Table 1

Drugs approved for clinical use in human cancer therapy.

DRUG	TRADEMARK	PRODUCER	TARGET
IMATINIB	GLEEVEC	Novartis	ABL, KIT, PDGFR
ERLOTINIB	TARCEVA	Roche	EGFR
GEFITINIB	IRESSA	AstraZeneca	EGFR
SORAFENIB	NEXAVAR	Bayer	KIT, VEGFR, PDGFR, FLT3, RAF
SUNITINIB	SUTENT	Pfizer	KIT, FLT3, PDGFR
DASATINIB	SPRYCEL	Bristol-Myers Squibb	ABL
LAPATINIB	TYKERB	GlaxoSmithKline	HER2, EGFR
NILOTINIB	TASIGNA	Novartis	ABL

Up to date, there are 8 kinase inhibitors that received a FDA approval for the clinical use in human cancer (Table 1). Many more are currently in the clinical trials. Interestingly, a first protein kinase inhibitor (Fusadil) was approved for clinical use in Japan to treat cerebral vasospasm.

1.1.3 Tyrosine protein kinases

The human genome contains 90 tyrosine kinase genes and 9 presumed tyrosine kinase pseudogenes (6).

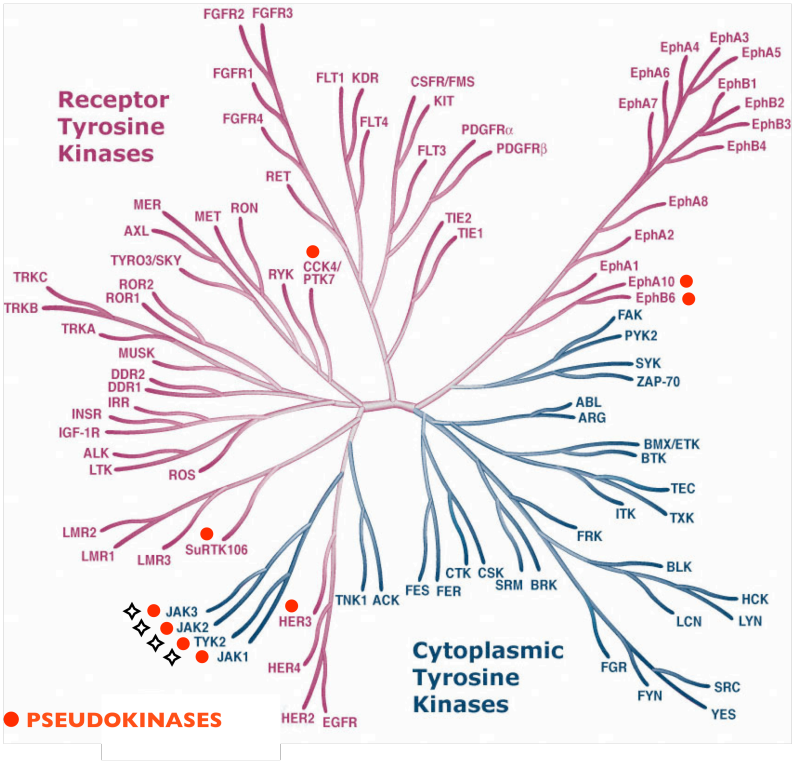

Figure 2

Dendrogram representing the tyrosine kinase family comprising 90 proteins (adapted from www.cellsignal.com). 58 of them are classified as receptor tyrosine kinases (magenta) and 32 as cytoplasmic tyrosine kinases (blue). Kinases that are classified as pseudokinases are marked by a red dot and those that contain in addition to the pseudokinase domain a kinase domain are marked with a white star.

There are two classes of tyrosine kinases (Fig. 2). Receptor tyrosine kinases (RTKs) are type I transmembrane proteins possessing an N-terminal extracellular domain, which can bind activating ligands, a single transmembrane domain and a C-terminal cytoplasmic domain that includes the catalytic domain. Cytoplasmic tyrosine kinases

lack the transmembrane domain and they act as the catalytic subunits for cytokine receptors that lack their own catalytic domain. Based on kinase domain sequences, the family of RTKs and cytoplasmic kinases can be grouped into 20 and 10 subfamilies, respectively (11).

Tyrosine kinases contain highly conserved catalytic domains similar to those in protein serine/threonine and dual specificity kinases but with unique sub-domain motifs clearly identifying the members as tyrosine kinases (12).

1.1.4 Activation mechanism of kinases

To date, numerous protein kinase structures have been solved in both active and inactive states (13, 14). Comparative studies of structures yielded insights in the mechanism of kinase activation, substrate recognition and ATP/inhibitor binding (15, 16). Modulation of kinase activity is vitally important and its malfunction leads to many diseases. Most kinases are multi-domain proteins and the regulation occurs at multiple levels to keep these enzymes in the non-active state. Thus, the activation mechanisms tend to be very different from one another on the level of the activation of whole protein. Kinase activity can be regulated by different mechanisms such as protein-protein interactions, alteration in subcellular localization within the cell, phosphorylation/dephosphorylation and many more competitive, non-competitive or allosteric mechanisms (17-20).

1.1.4.1 Regulation of the kinase domain

Phospho-transfer activity relies on the kinase domain, which consists of two lobes: a smaller N-terminal lobe and a larger C-terminal lobe (Fig. 3). The space between these two lobes is the catalytic cleft, which serves as docking site for ATP and the substrate. The kinase domain of protein kinases can adopt two fundamentally different states: the inactive “OFF” and active “ON” state. Upon activation all serine/threonine kinases and tyrosine kinases catalyze the same reaction of the transfer of the γ-phosphate from ATP to hydroxyl groups of serine, threonine or tyrosine. Therefore, the catalytically active conformation they adopt shows high structural similarity (19).

Transition from the inactive to the active state requires changes in the conformation of the kinase domain (Fig. 3).

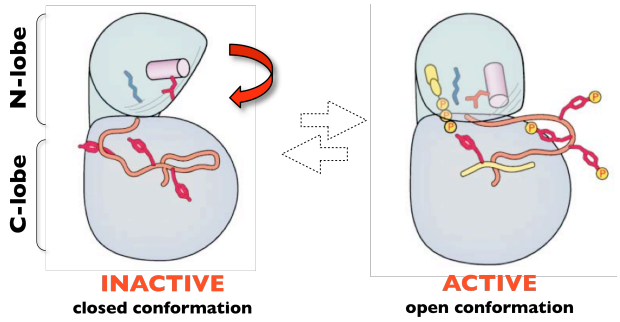

Figure 3

Schematized structures of the insulin receptor kinase (IRK) depicting the conformational transitions involved in the regulation of kinase activity. Key structural elements within the kinase domain are colored as follows: αC helix (purple) and activation loop (red). The red arrow indicates the direction of N-lobe rotation upon kinase activation. In the inactive state the IRK does not accommodate ATP. Upon binding of insulin to the IRK the conformational change is induced. Phosphorylation of the activation loop at tyrosine residues stabilizes the kinase in an active conformation what enables binding of ATP and substrate (yellow). Adapted from (17).

In the inactive state the kinase domain displays a closed conformation. Upon phosphorylation the activation loop moves away from the catalytic center and adopts a conformation that allows substrate binding and further catalysis. Additionally, the N-lobe undergoes a certain twist leading to the formation of an ion pair between the highly conserved glutamate residue localized in αC helix and the conserved lysine residue which binds the α and β phosphates of ATP. Then, the active kinase toggles between the open and closed conformation as it goes through the catalytic cycle (see also Fig. 5B).

1.1.4.2 Structural similarities in the activation mechanism of protein kinase domains

The first crystal structure to be solved was the one of cyclic AMP-dependent kinase PKA (protein kinase A) (21). The structure of PKA in complex with ATP and substrate was visualized by X-ray crystallography capturing the protein kinase in a conformation that is primed and ready for the phosphotransfer (21, 22). The comparison of the inactive and active status of many solved kinase structures has shown that the activated state of kinase domains is very conserved. In the following these common traits are presented using PKA as an example.

The N-terminal lobe of the protein kinase fold is composed of five β sheets and one α helix, which is called αC helix (Fig. 4A). The C-terminal lobe is predominantly helical. The transition from the "OFF" to "ON" state leads to the formation of the so-called hydrophobic spine that links together the N- and C-lobes of the kinase domain (Fig. 4A).

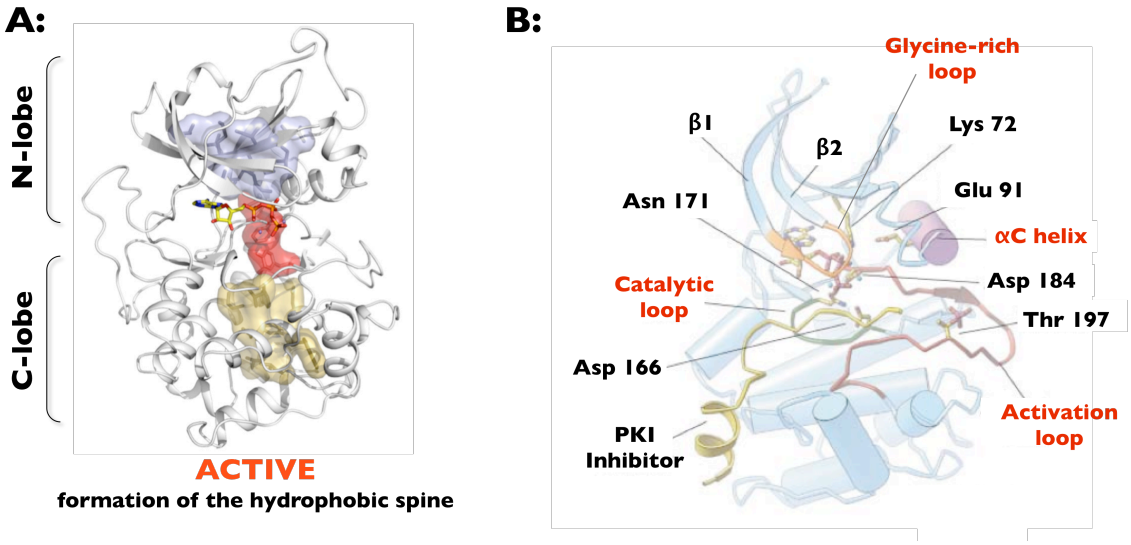

Figure 4

A: *Model structure of the serine/threonine kinase PKA where the hydrophobic interior of the catalytic core of the N-lobe (blue) and the C-lobe (yellow) is indicated. Upon kinase activation the hydrophobic spine is formed (red) that links the cores of two lobes. Adapted from (18).* ***B:*** *The ribbon representation of the crystal structure of the kinase domain of PKA. Key functional elements within the kinase domain are highlighted. A PKI inhibitor colored in yellow mimics the PKA substrate. Adapted from (17).*

The spine is formed only when the activation segment and the αC helix have reorganized. Under such conditions several residues building the spine become aligned what supports the active conformation of the kinase domain. Another proposed role for the spine is to provide a flexible hinge for the open/closed transition required for binding ATP and releasing ADP (23). There are several residues and loops important for interactions with ATP and substrate that are conserved among protein kinase families (Fig 4B). The ATP-binding pocket is deeply buried between these two lobes right beneath a highly conserved glycine-rich loop connecting the β1 and β2 strands. This loop contains a conserved glycine-rich sequence (GXGXϕG) where ϕ stands usually for a tyrosine or phenylalanine residue. The glycine residues make the loop very flexible in the absence of bound ATP. Upon kinase activation, the loop gets into close proximity of ATP and modulates the phosphoryl transfer step.

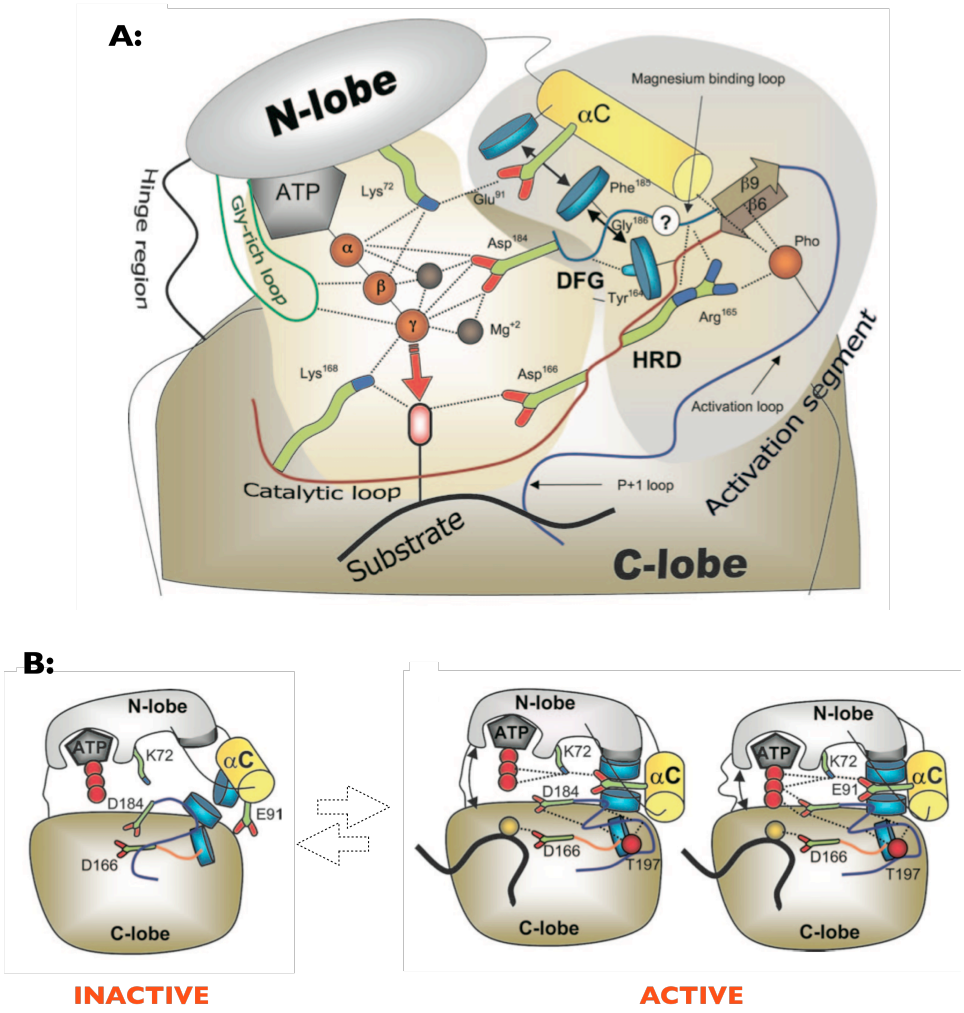

Figure 5

A: *Diagram of known interactions between the protein kinase catalytic core, ATP and the substrate peptide (PKA numbering is used). Hydrophobic interactions between the HRD motif, the DFG motif and the αC helix leading to the formation of the hydrophobic spine are shown by black arrows, while important polar interactions between the kinase residues and ATP or substrate are highlighted by dashed lines. The red arrow indicates catalyzed transfer of the γ phosphate of ATP to the hydroxyl group of a protein substrate. Adapted from (19)* ***B:*** *Schematic representation of protein kinase activation on the example of PKA. In the inactive state the hydrophobic spine is not stabilized and the αC helix with E91 has not undergone rearrangement. Upon activation the threonine 197 residue in the A-loop becomes phosphorylated. The A-loop is translocated as to interact with R165 and is stabilized in this position. The N-lobe of the kinase domain is twisted bringing the αC helix into close proximity of the ATP-binding pocket what results in formation of a polar contact between Lys 72 and Glu 91. Furthermore, the hydrophobic spine is formed (blue disks) which stabilizes the kinase domain in the active state. During the catalytic cycle the kinase performs motions going through open (left) and closed (right) conformations. Adapted from (19).*

One of the most important residues for the transition from the inactive state to the active state is the lysine residue (Lys72) positioned in the β3 strand of the N-terminal part of the kinase domain. Lys72 directly interacts with ATP by binding to its α and β

phosphates and positioning ATP properly for the catalysis. Moreover, in the active state Lys72 makes a polar contact with the conserved glutamic acid residue Glu91, which is localized in the αC helix (Fig. 5A and 5B). The altering of the conformation of the αC helix is an important step in the modulation of the kinase activity. The Lys72-Glu91 interaction couples the conformation of the αC helix to nucleotide binding.

One of the most important motifs for catalysis is the universally conserved Asp-Phe-Gly (DFG) motif, where an aspartate (Asp184) residue contacts the phosphates of ATP. Asp184 together with another residue Asn171 positioned in the catalytic loop are required for the binding of two divalent cations involved in coordinating ATP. The most conserved motif throughout the protein kinase family is composed of the histidine, arginine and aspartate residues (His-Arg-Asp, HRD). The Asp166 residue of HRD triad is responsible for the correct orientation of the hydroxyl group of the peptide substrate that becomes phosphorylated. Furthermore, most protein kinases require phosphorylation of the activation loop and the role of HRD Arg165 is to support the proper configuration of the activation segment by coordinating the phosphate of the phosphorylated residue in the A-loop (Thr197 in PKA). This stabilizes the PKA kinase domain in the active conformation. Interestingly, this arginine is conserved only in the eukaryotes, and usually kinases lacking the HRD arginine are not phosphorylated in the activation loop (13).

1.2 KINASES AS INHIBITOR TARGETS

Since protein kinases are directly involved in many diseases, including cancer and inflammation, they have become one of the most important classes for drug development (24, 25). The approval of imatinib (Gleevec) for chronic myeloid leukemia (CML), gefitinib (Iressa) and erlotinib (Tarceva) for non-small lung cancer (NSCLC) has provided proof-of-principle that small molecule kinase inhibitors can be effective drugs. Since the vast majority of these compounds target the kinase ATP site, which is highly conserved within the protein kinase family, they might target more than only one single kinase. However, even though they target many signaling pathways, multikinase inhibitors have been approved for the use in patients with cancer (see Table 1) and they are well tolerated with minor side effects (26). In contrast to clinical utility, the specificity of an inhibitor is of critical importance for its utility in research.

1.2.1 The chemical genetics approach

The common mechanism of action of the majority of kinase inhibitors relies on the competition of the inhibitor and ATP for binding to the catalytic cleft of a target kinase. The importance of a given kinase can be assessed by inhibitor-based suppression of its activity and monitoring the response of the biologic system. However, due to possible unspecific actions of an inhibitor on kinase-mediated signaling cascades, it is not possible to directly attribute the biological effects of the inhibitor to its effects on the investigated kinase. To circumvent the specificity problem associated with conventional small-molecule inhibitors of kinases, a chemical genetics approach has been developed (27-29). The approach exploits a conserved, large hydrophobic residue in the kinase active site (termed the “gatekeeper”), which makes direct contact with the N^6 group of ATP. In the case of Janus kinases the gatekeeper corresponds to a methionine residue localized within the β5-sheet (Fig. 6). When this residue is mutated from the naturally occurring bulky residue to glycine or alanine, a pocket not found in any wild-type kinase is created within the kinase’s active site (27).

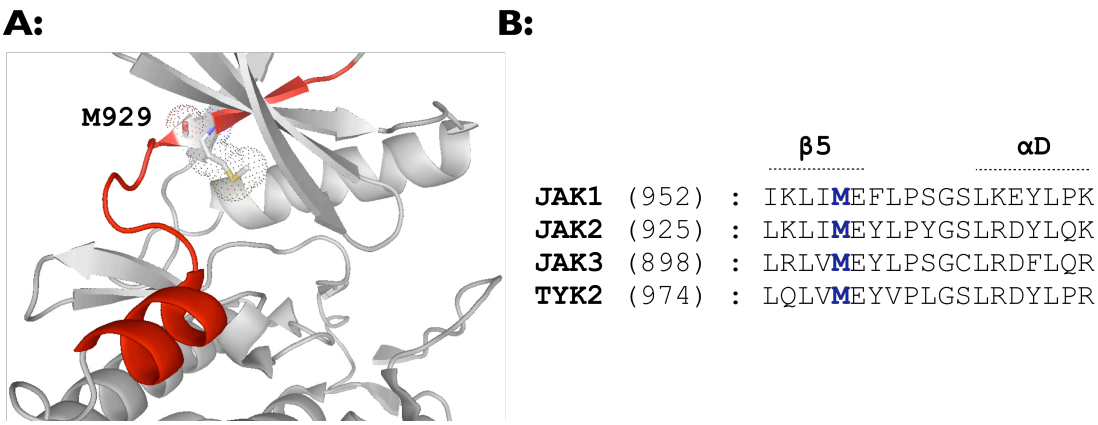

Figure 6

A: *View of the catalytic cleft of the Jak2 kinase domain where the space occupied by the atoms constituting Met929 is represented as dotted spheres and the region from the β5-sheet to the αD helix (also shown in the sequence alignment in B) is highlighted by a colored backbone.* ***B:*** *Sequence alignments of the four Jaks with the methionine at the gatekeeper position highlighted in blue.*

The re-engineered kinase becomes *analogue-sensitive* and can be potently and uniquely targeted by inhibitors that contain a bulky substituent that can occupy this enlarged ATP-binding pocket (30). Likewise, this enlarged ATP-binding pocket allows such mutants to use unnatural N^6-modified ATP analogues that are not accepted by any wild-type kinases, what enables the labeling of specific kinase substrates (Fig. 7) (31, 32). So far two different substrate identification methods have been reported. The first one relies on the radioactive labeling of the direct substrates by the γ-phosphate group (γ-^{32}P) of an ATP analogue that can be used only by the analogue-sensitive kinase (33). Very recently, a non-radioactive method for substrate identification has been proposed. In this case, the analogue-sensitive kinase uses an N^6-alkylated ATPγS analogue what leads to thiophosphorylation of its substrates. All other kinases will instead transfer phosphate groups to their substrates. Thiophosphorylated substrates can be enriched by a thio-phosphate ester-specific antibody (31) or by a thiopropyl Sepharose (34) and identified by mass spectrometry.

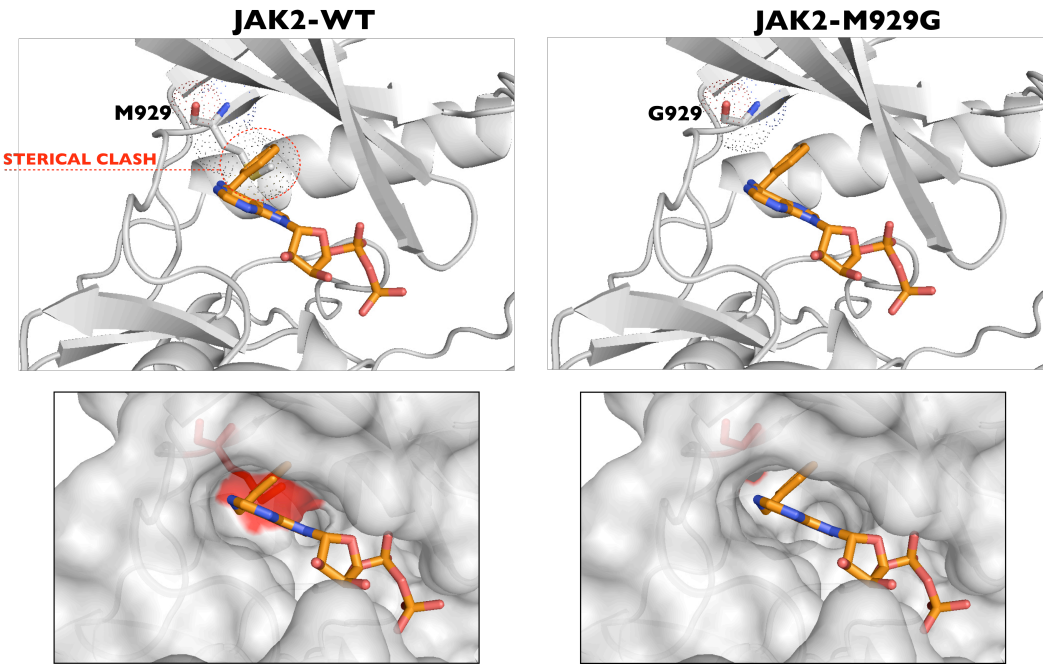

Figure 7

(Upper part) View of the catalytic cleft of the Jak2 kinase domain with the bulky N^6-benzyl-ADP molecule highlighted in orange. (Lower part) Ribbon and surface representation of the Jak2 kinase domain with the bulky ADP molecule. Mutation of methionine at the gatekeeper position in the Jak2 kinase domain to the smaller residue glycine creates a new pocket that can be uniquely accessed by the "bulky" analogue molecule. The presence of the benzyl group in the analogue molecule precludes the binding to the pocket of the wild-type kinase. The sterical clash between the methionine gatekeeper residue of wild-type Jak2 and the analogue molecule are highlighted in red in the left panels. In the case of the analogue-sensitive mutant no sterical clash occurs (right panels). The space occupied by the gatekeeper residue is represented as dotted spheres.

1.2.2 Possible intolerance of a kinase to the gatekeeper residue mutation

Since all protein kinases contain the conserved gatekeeper residue, the chemical genetics approach is in principle applicable to the whole kinome. Bcr-Abl, PI3-K, Erk2, Src, Jnk2 and Zap70 represent only few examples of kinases that were subjected to this approach and rendered analogue-sensitive (32, 35-38, 45). Despite the broad applicability, a considerable number of kinases does not tolerate the mutation of the gatekeeper residue to glycine or alanine, and is thus not amenable to the chemical genetics approach (36, 39, 40). These intolerant kinases have a severe loss in catalytic activity and/or cellular function upon introduction of the space-creating gatekeeper mutation. To solve the problem of severe loss in activity by some analogue-sensitive mutants, a structure-guided mutational approach has been developed (41). It has been

suggested that the space-creating gatekeeper mutation can destabilize the N-terminal lobe of the kinase domain which contains numerous residues important for ATP binding and catalysis. To minimize the perturbation of the structure and function of the analogue-sensitive kinase, conservative suppressor mutations have been designed (such as C→V, S→T, and L→I). This mutational strategy should stabilize the upper lobe of the kinase domain, and thus rescue the loss of activity due to the gatekeeper mutations. Noteworthy, in addition to constituting a minimal perturbation, second-site suppressor mutations should not negatively affect the ability of the engineered kinase to accept unnatural ATP analogues or inhibitors.

However, even in cases in which the gatekeeper mutation resulted in an inactive kinase, valuable results could be obtained, e.g. for IRE1. Binding of the inhibitor analogue 1NM-PP1 by this transmembrane kinase which is involved in the unfolded protein response (UPR) resulted in an activated conformation of the kinase domain, which in turn activated the RNase domain adjacent to the kinase domain resulting in activation of the XBP1-dependent ER-stress response (42).

1.2.3 Elucidation of kinase-mediated effects

The function of a given enzyme in cells can be probed by two dominant approaches: genetics and pharmacology. In many cases, a small molecule and a genetic mutation can perturb a protein's activity in different ways, leading to different conclusions about the protein's biological function. There are many examples where different phenotypes emerge depending on whether a protein is targeted by a small molecule inhibitor or a knockout of the gene (43). Gene knockout approaches have provided important insights into the roles of specific proteins. However, some knockout mice may not be viable at all, or "secondary" adaptations such as cellular compensation may occur: e.g. the Jnk2 knockout caused a compensatory increase in Jnk1 activity and c-Jun expression. On the other hand, suppression of Jnk2 activity by a small molecule inhibitor resulted in blockade of c-Jun phosphorylation and suppression of cellular proliferation (44).

Kinase inhibitors are all too often unspecific and even the few potent compounds may have more than one target. Only 5% of the human kinome is currently targeted by potent inhibitors and there is no way to comprehensively identify the cellular targets of a small molecule inhibitor.

Chemical genetics represents a good alternative approach for investigation of specific kinase-mediated effects. A clear advantage of chemical genetics is the fact that the analogue-sensitive kinase can be reversibly inhibited, and more importantly, this specific inhibition can be controlled over time, which is not possible if the whole protein expression is regulated or if kinase-inactive mutants are used (46). Since the importance of protein kinases as a major class of drug targets has been recognized, generation of "fingerprints" of transcriptional changes associated with selective kinase inhibition would be of great use. A comparison of the effects of a drug candidate inhibitor with data obtained with a corresponding analogue-sensitive variant, thereby providing the "fingerprint", would identify off-targets effects elicited by the tested compound (47). What makes the chemical genetics approach even more valuable with respect to pharmacologic target validation is the fact that the mutated alleles of analogue-sensitive kinases have been successfully introduced into mouse models (48).

1.3 JANUS KINASES

1.3.1 Discovery

In the early 1990s four members of the family of Janus kinases were identified (49-52). This family comprises Jak1, Jak2, Jak3 and Tyk2. While Jak1, Jak2 and Tyk2 are ubiquitously expressed, Jak3 is confined mainly to cells of the hematopoietic system (53, 54).

1.3.2 Structure and function of Jaks

Out of the 518 protein kinases encoded by the human genome, 48 family members contain pseudokinase domains (5). Combination of a pseudokinase domain and a kinase domain directly adjacent in the same protein was found only in five pseudokinases. These are the four members of Janus kinase family and the serine/threonine kinase GCN2. This unique feature clearly distinguishes these five kinases from other members of the protein kinase family. This is, however, the only similarity between the Jaks and GCN2. As for the Jaks, the pseudokinase domain in GCN2 is thought to keep the kinase domain inactive in the absence of stimulus (6). In addition to the pseudokinase and kinase domain Jaks contain two more structural domains, namely the SH2 and FERM domain (Fig. 8).

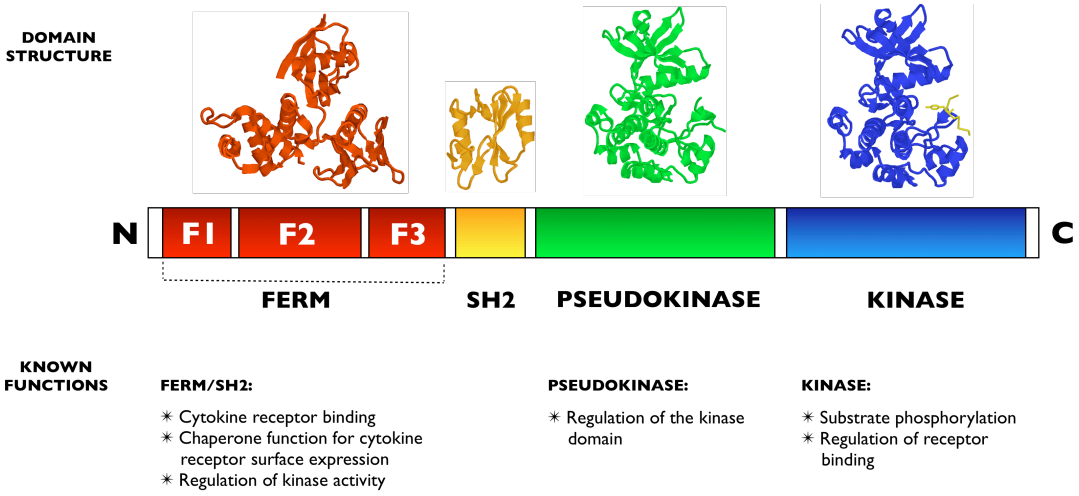

Figure 8

Structural organization of the Janus kinases and known functions of the different domains. Model structures of the FERM, SH2 and pseudokinase domains of Jak1 as well as the solved crystal structure of the kinase domain of Jak2 (PDB entry code: 2B7A) are represented. Adapted from (55).

Sequence similarities between Jak family members had led after their discovery to the description of seven Jak homology (JH) domains (50). The JH3 to JH7 regions are better described as FERM and SH2 domain, while the JH1 and JH2 domains represent the kinase and pseudokinase domains, respectively (50, 56).

1.3.2.1 The FERM domain: receptor binding and localization

The N-terminal part of the Janus kinases contains a FERM domain (FERM stands for: band four point one (4.1) protein, ezrin, radixin, and moesin) (56). The FERM domain is mainly involved in constitutive binding of Jaks to the cytokine receptors (57-60), which lack an own kinase domain. The association of Jak with the cytokine receptor results in a tight complex that mimics (in many aspects) the RTKs. Published X-ray structures of FERM domains show that these domains consist of three separate subdomains arranged in a clover shape. The N-terminal F1 subdomain has a ubiquitin-like β-grasp fold, the F2 subdomain contains many α-helices and shows structural similarity to the acetyl-CoA-binding protein and the F3 subdomain has a PH-domain (pleckstrin homology) fold (61-65).

Jaks associate with cytokine receptors at the membrane-proximal region containing the so-called box1/2 region. Due to the constitutive binding of Jaks to cytokine receptors, these tyrosine kinases are mostly localized to the plasma membrane (Fig. 9), although it has been reported that e.g. Tyk2 can be found also in the nucleus, if it is expressed to higher level (66). The significance of nuclear Jaks is not clear, given that all functional features of Jak proteins point to their involvement in events occurring at the cell surface. Recently, however, Jak2 was reported to directly phosphorylate histone H3 in the nucleus (67). Further studies involving e.g. the preparation of highly purified membrane-free nuclear fractions will have to verify a potential presence of Jak2 in the nucleus. It will be also interesting to address the possible involvement of other kinases mediating histone tyrosine phosphorylation.

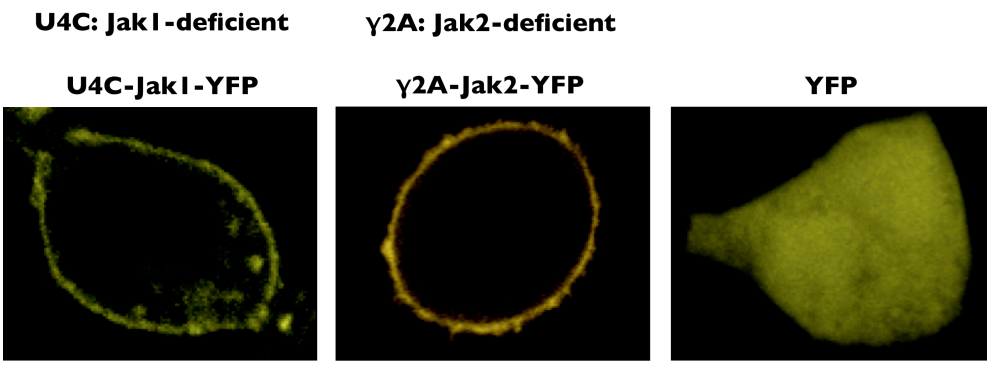

Figure 9

U4C cells (Jak1-deficient) were transfected with Jak1-YFP (Yellow Fluorescent Protein) and γ2A cells (Jak2-deficient) with Jak2-YFP or YFP. The YFP-associated fluorescence was monitored in living cells by confocal microscopy showing exclusive membrane localization of Jaks. Adapted from (68).

1.3.2.2 The unconventional SH2 domain

The FERM domain is followed by a predicted SH2 domain, which does not seem to fulfill a classical SH2 domain function, i.e. the binding to specific motifs containing phosphorylated tyrosine residues (69). SH2 domains contain about 100 amino acids and consist of two α-helices and a central antiparallel β-sheet. A conserved arginine residue at position βB5 in SH2 domains contacts the phosphate group of a binding phosphotyrosine motif. Accordingly, this residue was found to be present in 99.8% of SH2 domain sequences, emphasizing the strict requirement for this amino acid at this position. Interestingly, this arginine is not equally well conserved in Jaks and it is found only in 80% of all Jak SH2 sequences. A functional study of Jak1 SH2 domain revealed that mutation of arginine (R466) to lysine had no effect on neither the sub-cellular localization of Jak1 nor its binding to the cytokine receptor or signaling capacity (70). Noteworthy, mutation of the corresponding arginine in human Jak2 (R426) had no effect on STAT1 activation upon IFN treatment (71). However, in truncation mutants and SH2 domain swapping mutants, the SH2 domain of Jak1 was shown to be structurally important for binding to OSMR and consequently for efficient OSMR surface expression (70). In contrast, the SH2 domain of Jak1 was not necessary to bind the gp130 receptor chain (72). Binding of Jak2 and Tyk2 mutants to EpoR and interferon-α receptor (IFNαR1), respectively, was also not affected by the lack of SH2 domain, although the SH2 domain was required for the upregulation of receptor surface expression (73, 74).

1.3.2.3 The pseudokinase domain

The Jak pseudokinase domain is localized between the SH2 domain and the kinase domain. Pseudokinase domains of Jaks lack conserved residues critical for kinase activity (6) what renders them inactive (50, 75). The loss of function of the pseudokinase domain is thought to be mainly due to the lack of the aspartate residue within the HRD motif necessary for correct ATP orientation. Moreover, two tyrosine residues in the activation loop whose phosphorylation stabilizes the activation loop of a kinase in the active conformation are missing in pseudokinase domains. The Jaks' pseudokinase domain interacts with the kinase domain and it is assumed to prevent basal activity of the kinase domain (76-78). The assumption of an inhibitory role of the pseudokinase domain is largely supported by the identification of many mutations in this domain that render the Jaks hyperactive (79). However, structural data would be necessary to reveal how exactly the pseudokinase domain interacts with the kinase domain.

1.3.2.4 The kinase domain

The kinase activity is exerted by the C-terminal kinase domain comprising approximately 300 amino acids. All protein kinases share the bilobal kinase fold (Figs. 4, 10) and Jaks contain all the critical functional regions of kinases which have been described above (see Fig. 5A).

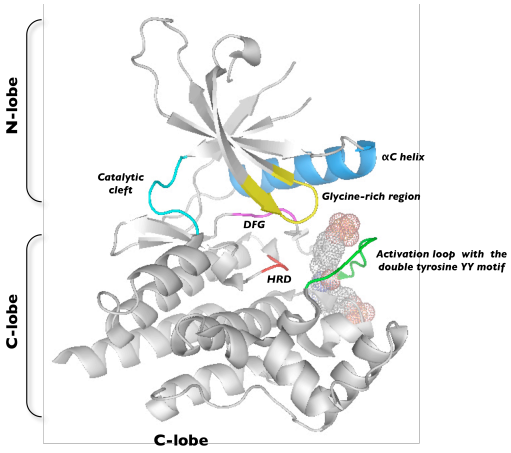

Figure 10

The solved crystal structure of the kinase domain of Jak2 (PDB entry code: 2B7A) where the following regions are highlighted: αC helix (blue), glycine-rich region (yellow), catalytic cleft (cyan), the HRD motif (red), the DFG motif (magenta) and the activation loop (green) where the phosphotyrosine residues are represented as dotted spheres. The N-terminal and C-terminal lobes are indicated. Pymol was used for molecular modeling and graphical representation (The PyMOL Molecular Graphics System, DeLano Scientific, San Carlos, CA, USA).

The two tyrosine residues in the activation loop that become phosphorylated upon kinase activation have been investigated by a mutational analysis of Jaks. It has been shown that the first tyrosine residue from the double tyrosine motif (Y1007 in Jak2, Y1033 in Jak1) (80, 81) if mutated to phenylalanine renders the kinase inactive (Table 2). Additionally, substitution of the aspartates in the extremely conserved HRD motif by asparagine leads to inactive Jaks (Table 2). Jak1 and Jak2 mutants bearing a lysine residue substitution at position 908 and 882, respectively, showed a similar phenotype (82).

Interestingly, according to sequences of murine Jak1 (reference number NP_666257) and human Jak1 (reference number NP_002218) that are available in the database of NCBI, the double tyrosine motif is located at position 1033/1034 and 1034/1035, respectively. This is not consistent with the numbering which is currently in use by e.g. biotech companies producing antibodies against phosphorylated tyrosines of Jak1, here these two tyrosine residues are supposedly placed at position 1022/1023.

Table 2

Amino acids sequences of selected loops and motifs important for the catalytic activity of the kinase domain of human and murine Jak1 as well as Jak2 are shown. The glycine-rich loop (GxGxxG), the ATP-binding loop (AVKxL), the HRD, DFG and double tyrosine motifs are shown. Positions of highlighted residues and functional consequences of their substitutions are indicated.

MOTIF	JAK1 SEQUENCE	MURINE	HUMAN	MUTATION	PHENOTYPE	REFERENCE
GxGxxG	GEGHFG	G881	G882	-	-	-
AVKxL	AVKSL	K907	K908	K-E	Kinase-inactive	82
HRD	HRD	D1002	D1003	D-N	Kinase-inactive	Kaczor *et al.* *
DFG	DFG	D1020	D1021	D-S	Kinase-inactive	82
YY	YY	Y1033/1034	Y1034/1035	Y1033-F	Kinase-inactive	81
				Y1034-F	Kinase-active	81

MOTIF	JAK2 SEQUENCE	MURINE	HUMAN	MUTATION	PHENOTYPE	REFERENCE
GxGxxG	GKGNFG	G856	G856	-	-	-
AVKxL	AVKKL	K882	K882	K-E	Kinase-inactive	82
HRD	HRD	D976	D976	D-N	Kinase-inactive	Kaczor *et al.* *
DFG	DFG	D994	D994	-	-	-
YY	YY	Y1007/1008	Y1007/1008	Y1007-F	Kinase-inactive	80
				Y1008-F	Kinase-active	80

* “Jak1 and Jak2 in IFN signaling: a chemical genetics approach to dissect their relative contributions” – see Results section *page 47*

1.3.3 Activation mechanism of Jaks

Under normal conditions, Jaks are kept in their inactive state until receptor activation occurs leading to conformational changes of the receptor and receptor-bound Jaks. Janus kinases associate with the membrane-proximal region of cytokine receptors but due to the lack of crystallographic data, the mechanism leading to the initiation of the activation of Jaks remains unclear.

1.3.3.1 The cytokine receptor/FERM domain interaction

Janus kinases are constitutively bound to cytokine receptors and localized to the plasma membrane by receptor binding. Among receptors, there is little sequence homology except short stretches called box1 and box2, which serve as docking sites for Jaks. Within the relatively long stretches of the membrane-proximal region, quite a number of residues were shown to be essential for Jak/receptor interaction (55). Interestingly, for several cytokine receptors (gp130, EpoR), residues were identified, which do not impair binding but which affect Jak activation (83, 84). This shows that the Jak/receptor contacts determine a conformation, which is crucial for normal cytokine-dependent Jak activation.

Proper cytokine receptor function must depend on communication between the extracellular and intracellular domains that are connected by a rigid α-helical transmembrane domain. Upon stimulation, conformational changes of the extracellular domains of the receptor are transmitted to the cytosolic domain activating Jaks. There is growing evidence that the helical structure of the transmembrane regions can extend into the intracellular as well as extracellular region as became evident by mutagenesis studies involving insertions, deletions of amino acids of the transmembrane helix and by cysteine-scanning mutagenesis (83, 86, 87). The Jak/receptor interaction interface in the extension of the transmembrane helix plays a role in correct positioning of Jaks so that ligand-induced receptor conformational change can lead to their mutual activation.

Jaks bind to cytokine receptors via the FERM domain (60, 72). In addition to receptor binding, however, it has been proposed that the FERM domain also plays a role in the overall organization of the kinase structure and its subsequent activation. By a mutagenesis study it was shown that the integrity of the F1 and F2 subdomains is crucial for Jaks receptor binding (60, 88). Furthermore, the FERM mutations in Jak1 can influence the phosphorylation status of the kinase and its ability to become properly

activated to promote downstream signaling events upon cytokine stimulation. Moreover, naturally occurring mutations in the FERM domain of Jak3 have been identified in patients suffering from SCID (89). These mutants were characterized by reduced but not complete lack of receptor association. Regardless of that residual receptor binding, neither kinase activity nor downstream STAT5 activity was observed.

1.3.3.2 The pseudokinase/kinase domain interaction

Another level of regulation of Jak activity involves the pseudokinase domain, which according to the accepted theory negatively regulates activity of the kinase domain. Theoretical models of the Jak2 structure suggested that the kinase domain and the pseudokinase domain are facing each other and the activation loop is buried in this interface (90, 91). Phosphorylation of the activation loop is thought to prevent this pseudokinase/kinase interaction and therefore releases inhibition. The well-known V617F mutation, which renders Jak2 constitutively active, was shown to be transferable to Jak1 and Tyk2 (92), i.e. the homologous mutations in Jak1 (V658F) and Tyk2 (V678F) also lead to hyperactive kinases. Interestingly, the conserved valine residue in Jak1, Jak2 and Tyk2 is replaced by methionine in Jak3 and the mutation of this residue to phenylalanine does not activate the enzyme and that indicates a different regulatory mechanism occurring for Jak3.

The enzymatic activity of Jaks relies on the kinase domain. Similarly to the other tyrosine kinases, maximal stimulation of Jak activity is attained upon the phosphorylation of tyrosine residues positioned in the activation loop. The double tyrosine motif is found in all members of the Jak family and its phosphorylation has been well documented. The role of these residues has been assessed through the study of mutants bearing phenylalanine substitutions. Maximal Jak activity relies on the phosphorylation of the first tyrosine, while the role of the second tyrosine residue seems to be different among Jaks, e.g. the Y1008 residue in Jak2 does not seem to be important for its activity while Y981 in Jak3 acts as a negative regulatory site (80, 93).

1.3.3.3 Model of activation of Jaks

The FERM, pseudokinase and kinase domains represent interdependent modules, which in fact need to interact with each other to ensure the proper regulation of the

protein. The interaction of the FERM domain with the receptor additionally contributes to the regulation of the catalytic activity of Jaks.

Constitutively associated with the intracellular region of cytokine receptors Jaks are kept in an inactive state. After the receptor binds its respective ligand (a cytokine or a growth factor), it undergoes a conformational change reorganizing the position of its extracellular and intracellular domains (Fig. 11). Rearrangement of the receptor transforms the two Jaks into an active state, at which they can bind ATP. Additionally, they are brought close enough to phosphorylate each other leading to stabilization of the kinase domain in its active conformation enabling further transduction of the intracellular signal.

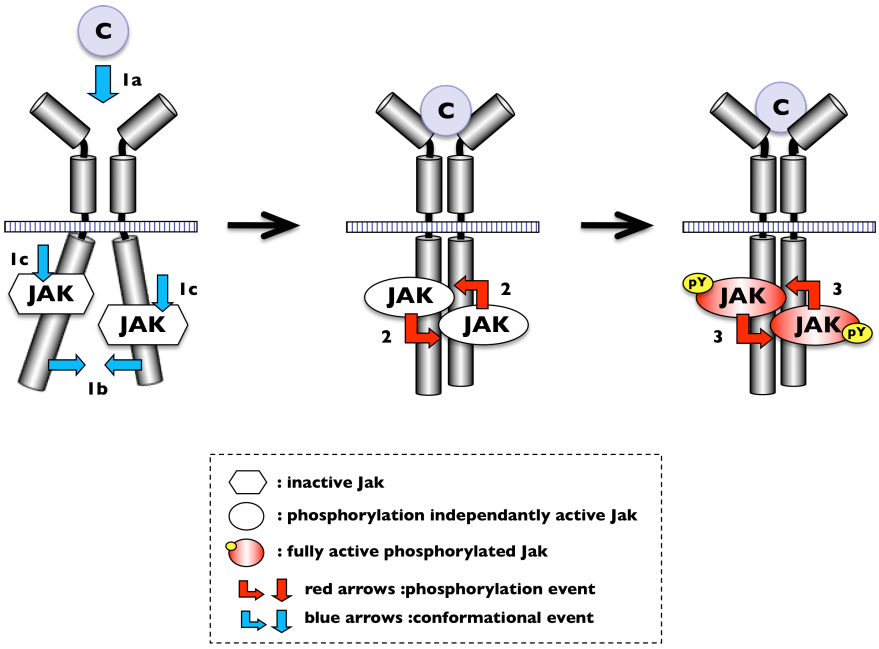

Figure 11

Model of activation of Jaks. Cytokine binding (1a) triggers a conformational change of the receptor, which is transduced to the intracellular domains (1b). The rearrangement of the receptor leads to the juxtaposition of the two Jak molecules and induces a conformational change of the Jaks (1c). Changes in the conformation of the receptor activate Jaks so that they can phosphorylate each other (2), leading to full activation. In the presence of cellular phosphatases dephosphorylating Jaks, fully activated Jaks are sustained by continuous phosphorylation of their tyrosine residues (3).

1.4 CYTOKINE-MEDIATED ACTIVATION OF JANUS KINASES

1.4.1 Jak-dependent cytokine signal transduction

Shortly after the discovery of Jaks, their essential function in cytokine signaling was established in a series of experiments using mutagenized cell lines that were resistant to the effects of interferons (IFNs). By transfecting the cells with expression vectors encoding different Jaks that were initially not expressed in those cells, the signaling could be fully reconstituted. Specifically, IFNα/β signaling was found to require Jak1 and Tyk2, whereas IFNγ needed Jak1 and Jak2 (85, 94, 95). To date, knockout mice of all four Jaks exist and their phenotypic analysis yielded valuable information for the understanding of their physiological role. These mice all show phenotypes that are linked to cytokine signaling deficiencies (Table 3) (54).

Table 3

Phenotype of Janus kinase gene-targeted mice.

GENE	PHENOTYPE
JAK1	Perinatal lethality caused by failure of cytokine signaling in neurogenesis
JAK2	Embryonically lethal due to failure in erythropoiesis
JAK3	SCID caused by failure of cytokine signaling from γc-containing receptors
TYK2	Increased susceptibility to pathogens caused by impaired responses to IFN α/β and IL-12

Nowadays, it is clear that a wide array of cytokines and growth factors activate Jaks thereby stimulating cell proliferation, differentiation, cell migration and survival. Cytokines first bind to their respective receptors to initiate the signaling cascade. With respect to Jaks, these cytokine receptor systems are restrictive concerning the usage of one Jak or Jak combinations, thereby, together with the differential activation of the various signaling proteins, determining the signaling characteristics. For instance, receptors required for hematopoietic cell development (EpoR, TpoR) use only Jak2, common γ-chain receptors transducing signals of e.g. IL-2, IL-4 or IL-7 use Jak1 and Jak3, while type II cytokine receptors use only combinations of Jak1 and Tyk2 (IFN α/β receptor) or Jak1 and Jak2 (IFNγ receptor) (79).

According to the accepted model, upon cytokine-induced receptor aggregation Jaks auto-activate and transphosphorylate themselves. Subsequently, the cytoplasmic tail of

the receptor containing several tyrosine residues becomes phosphorylated by Jaks and that provides docking sites for SH2 domain-containing signaling molecules (Fig. 12).

One class of SH2 domain-containing proteins, the STATs, latent cytoplasmic transcription factors, is particularly important for Jak-mediated signal transduction. Once recruited to the receptor complex, they can be phosphorylated by Jaks what further propagates the signal. Additionally, adaptor proteins such as Shc or the p85 subunit of PI3K can associate with certain phosphorylated tyrosines of the receptor leading to the activation of MAP kinase and PI3-K/Akt pathways (96).

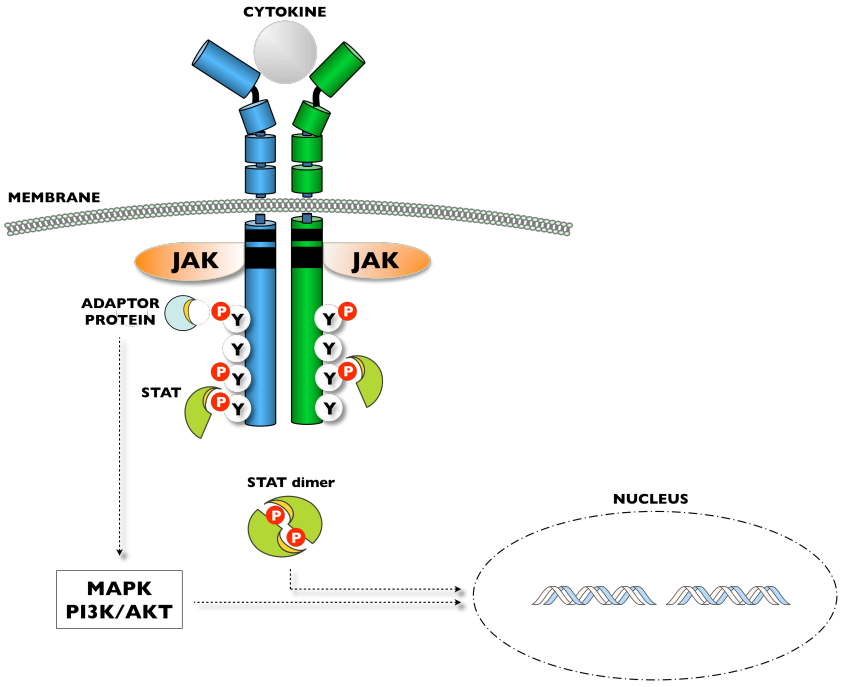

Figure 12

Activation of the Jak/STAT pathway by cytokine binding to its receptor. Cytokine-induced receptor aggregation brings Jaks into close proximity what results in their phosphorylation and activation. Tyrosine residues of the receptor become phosphorylated by Jaks, providing docking sites for STATs and other adaptor proteins.

The STAT proteins are thought to be recruited from a cytoplasmic pool and have been shown to interact with specific phosphotyrosine motifs within the activated receptors (97, 98). The receptor-bound STATs are then phosphorylated by Jaks on a conserved tyrosine residue and the SH2 domains mediate the dimerization through reciprocal phosphotyrosine/SH2 domain interactions. STAT dimers are then translocated to the nucleus where they bind to specific sequences in the genome and activate gene

expression. Seven mammalian STATs have been identified: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. Through generation of gene-targeted mice, the biological functions of STAT family proteins could be revealed. Knockout mice of each STAT displayed impaired cytokine-mediated functions (Table 4) (54, 99).

Table 4

Phenotype of STAT gene-targeted mice.

GENE	PHENOTYPE
STAT1	Viable and fertile, defective IFNα/β and IFNγ signaling, increased tumorigenicity
STAT2	Viable and fertile, defective IFNα/β signaling
STAT3	Embryonically lethal
STAT4	Viable and fertile, defective IL-12-driven Th1 differentiation, increased susceptibility to intracellular pathogens
STAT5a	Viable and fertile, defective in prolactin functions and mammary gland development
STAT5b	Viable and fertile, defective in sexually dimorphic growth
STAT5a/b	Viable, female infertility, defective mammary gland development, reduced body mass, defective T cell proliferation
STAT6	Viable and fertile, defective IL-4-driven Th2 differentiation, increased susceptibility to helminthic infestation

1.4.2 IFNγ-mediated signal transduction

In the year 1957 Isaacs and Lindenmann discovered a substance called interferon (IFN) that had protective properties on cells against viral infections (100, 101). IFNs represent proteins that are associated with host defense and homeostasis. They are secreted from cells in response to a variety of stimuli and can be organized into three classes: type I (α, β, ω and τ), type II (γ) and type III (λ). This chapter will focus on IFNγ.

IFNγ is secreted by activated immune cells (mainly T and NK cells) (102). Conventional IFNγ signaling follows the binding of IFNγ to its cell surface receptors IFNγR1 and IFNγR2 thereby leading to oligomerization of the receptor and activation of Jak1 and Jak2 (103). Jak-mediated phosphorylation of tyrosine Y440 of human IFNγR1 (104) provides a binding site for STAT1, which is phosphorylated by Jaks on tyrosine Y701. Interestingly, cells bearing the IFNγR1 mutant where Y440 was mutated to F still sustain a substantial IFNγ response (105).

In addition to Y701, STAT1 becomes phosphorylated in response to IFNγ on serine S727, which has been reported to have role in regulation of transcriptional activity (106).

The molecular mechanism underlying the IFNγ-mediated S727 phosphorylation is not completely understood. The reported candidate kinases and mechanisms responsible for S727 phosphorylation are very diverse (107-112). Nevertheless, *in vivo* studies clearly showed the importance of the S727 modification; mice expressing the STAT1 S727A mutant exhibit a strong reduction in IFNγ-mediated target gene expression (113). The phosphorylated STAT1 forms a homodimer and translocates to the nucleus in an importin-α5-dependent manner (114, 115) where it initiates transcription of genes containing a gamma activated sequence (GAS) (116) in their promotor region. Most STAT1-mediated transcription is entirely dependent on STAT1 tyrosine phosphorylation. However, there is some evidence that STAT1 can initiate transcription of a subset of genes independently of tyrosine phosphorylation (117, 118). Activated and phosphorylated STAT1 has been described to interact with other transcription cofactors. Among others, CREB-binding protein (CBP/p300), mini-chromosome maintenance protein 5 (MCM5) and breast cancer susceptibility gene 1 (BRCA1) have been identified as STAT1 interacting proteins modulating its transcriptional activity (119-121). CBP/p300 has histone acetyl transferase (HAT) activity and functions as co-activator, while MCM5 and BRCA1 associate with serine-phosphorylated STAT1 enhancing its transactivation. The physiological relevance of IFNγ-dependent signaling through the Jak/STAT pathway has been further established by generating and characterizing mice with a targeted disruption of the STAT1 gene (122, 123). STAT1-null mice revealed no defects in development; however, they lacked many classical responses to IFNs (α, β, γ) and thus were very susceptible to microbial and viral infections.

In addition to the Jak/STAT pathway, IFNγ was shown to activate several other signal transducing proteins, including kinases like proline-rich tyrosine kinase Pyk2, the Src-family kinase Fyn or MAP kinases. Except tyrosine and serine/threonine kinases, the adaptor proteins c-Cbl, CrkL and CrkII were reported to participate in generation of growth inhibitory effects of IFNs on primary hematopoietic progenitors (124).

Experiments with STAT1-null cells confirmed the existence of STAT1-independent pathways activated in response to IFNγ. Surprisingly, IFNγ exerts suppressive effects on the growth of wild-type cells but it enhances the proliferation of STAT1-null cells (125, 126). Genes, whose expression is not induced by IFNγ in wild-type cells, were transiently activated in STAT1-null cells (126). Furthermore, although STAT1 is crucial for efficient antiviral responses, STAT1-null mice were more resistant to some viruses like murine cytomegalovirus (MCMV) and Sindbis virus than mice lacking IFN receptors (125). Most

cytokines are known to activate multiple STATs. In wild type cells, IFNγ dominantly activates STAT1, but a residual STAT3 phosphorylation is also observed. In absence of STAT1 though, STAT3 activation is strongly enhanced compared to wild type cells (127), what has a stimulatory effect on the expression of genes that have a STAT3-binding site in their promotor sequences (SOCS3, GADD45 or CEBPB) (128, 129). A similar example is the switch of IL-6 signaling, which occurs mainly via STAT3, to a dominant STAT1 activation in STAT3-deficient cells (130). It causes the downstream induction of genes, whose expression would be normally induced by IFNγ.

It would therefore be of interest to compare STAT1-knockout with STAT1 dominant-negative systems e.g. STAT1-Y701F, to discriminate between effects dependent on DNA binding of phosphorylated STAT1 from effects maybe mediated by opportunistic modules taking advantage of the absence of STAT1.

1.4.3 Negative regulation of Jak-mediated cytokine signaling

Sustained and/or excessive action of cytokines can be harmful to organisms. Accordingly, several mechanisms have been reported to modulate cytokine signaling to prevent this overreaction to cytokine.

Proteolytic shedding of some cytokine receptors is known to control the action of cytokines (131). Proteosomal and lysosomal degradation have been shown to regulate a large set of signaling molecules and cytokine receptors (132-136). However, the key regulators of Jak/STAT signaling include suppressor of cytokine signaling (SOCS) proteins, protein inhibitors of activated STAT (PIAS) and protein tyrosine phosphatases (PTP).

1.4.3.1 SOCS proteins

The SOCS family comprising 8 members (CIS, SOCS1-SOCS7) is the most thoroughly studied family of regulatory proteins. Some of the SOCS proteins inhibit cytokine signaling through distinct mechanisms: SOCS1 mainly binds directly to tyrosine phosphorylated Jaks (137). SOCS3 binds to activated cytokine receptors and also directly to Jaks (138) while CIS competes with STATs for binding to the receptor docking sites (139). Noteworthy, only SOCS1 and SOCS3 can inhibit Jak tyrosine kinase activity because they both have the kinase inhibitory region (KIR) in their N-terminal domain. Moreover, all SOCS proteins contain a SOCS box that binds components of an ubiquitin E3 ligase complex which targets signaling proteins for degradation (140, 141). The importance of SOCS proteins in modulating cytokine responses was shown e.g. by the generation of several knockout mouse models (Table 5) (54).

Table 5

Phenotype of SOCS gene-targeted mice.

GENE	PHENOTYPE
SOCS1	Viable but perinatal lethality due to severe IFNγ-dependent inflammatory diseases
SOCS2	Viable, gigantism owing to unopposed signaling to GH and IGF-1
SOCS3	Embryonically lethal due to placental defects and hematopoietic defects
SOCS5	No phenotype
CIS	No phenotype

1.4.3.2 Phosphatases

Tyrosine dephosphorylation of cytokine receptors and associated kinases is carried out by protein tyrosine phosphatases. SHP1 and SHP2 are SH2-domain-containing phosphatases (142) that can bind directly to phosphorylated tyrosine motifs of the activated cytokine receptors and negatively affect Jak activity (143-145). Moreover, the SHP2 phosphatase can act as a scaffolding protein, leading to the recruitment of other adaptor proteins such as Grb2 and Sos and thereby contributing to the cytokine signaling (146). Three other members of the PTP family, CD45, PTP1B and TCPTP have also been reported to directly dephosphorylate Jaks (147-149) and STATs (150, 151).

1.4.3.3 PIAS

DNA binding of activated STATs in the nucleus can be negatively regulated by PIAS. The PIAS family consists of PIAS1, PIAS3, PIASx and PIASy. It has been proposed that PIAS1 and PIAS3 function by blocking the DNA-binding activity of STAT1 and STAT3, respectively (152, 153). In contrast, PIASx and PIASy repress the transcriptional activity of STAT1 and STAT4 by recruiting corepressor molecules such histone deacetylases (HDACs) (154, 155). Interestingly, functions of the PIAS proteins are not restricted only to STATs. They can also function as SUMO (small ubiquitin-related modifier) ligases targeting transcription factors to nuclear bodies where they become transcriptionally active or inactive (156).

1.5 CYTOKINE-INDEPENDENT ACTIVATION OF JANUS KINASES

Due to the fact that Jak-dependent pathways such involving e.g. STATs, Erks, and AKT regulate cell growth, it is very likely that any kind of deregulation may lead to disease. Indeed, a number of diseases including cancer are linked to a dysfunctional Jak/STAT pathway activation.

1.5.1 Jak fusion proteins

It is well established that recombination of genes encoding members of the Jak family can result in their intrinsic activation. The Jak2 gene has been shown to be recombined with parts of several other genes leading to the formation of a fused oncogenic allele and the expression of hyperactive Jak2 fusion proteins. These have been associated with lymphoid and myeloid leukemia or with unclassified myeloproliferative neoplasms (MPN-U). Several fusion genes that involve the kinase domain alone or together with the pseudokinase domain of Jak2 have been described: ETV6-Jak2 (157), TEL-Jak2 (158, 159), BCR-Jak2 (160, 161), SSBP2-Jak2 (162) and PCM1-Jak2 (163-166). In all cases it is believed that the kinase domain contributed by the *Jak2* gene drives the oncogenic transformation of these cells. So far Jak2 is the only member of the Jak family shown to be involved in such translocations with its oncogenic consequences. However, fusion genes constructed *in vitro* with other Jak family members are also constitutively active and transform BAF cells to factor-independent growth (167).

1.5.2 Jak2-V617F as an example of a gain-of-function mutant

Jak2 has been also described to be constitutively active in the myeloproliferative neoplasms polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). Within weeks in 2005, 4 different research groups from Cambridge (168), Boston (169), Paris (170) and Basel (171) published reports describing a novel Jak2 mutation affecting the pseudokinase domain. By sequencing DNA templates from PV patients, a guanosine (G) to thymidine (T) alteration at position 2343 could be identified. This results in a valine (V) to phenylalanine (F) substitution at the conserved residue V617 generating a constitutively active kinase that leads to cytokine-independent activation of various signaling proteins, like STATs, MAP kinases and

PI3K/AKT (170, 172). In bone marrow transplantation assays, introduction of the Jak2-V617F mutant into mouse was sufficient to induce a PV phenotype (173-176). Noteworthy, Jak2 harboring the V617F mutation was found in MPN patients with a high incidence: in 99% of PV patients, in 50% of ET patients and in 50% of PMF patients. However, another study by Scott and collegues has shown that Jak2-V617F expression is rather uncommon in cancers and myeloid malignancies other than classic BCR-ABL-negative myeloproliferative neoplasms (177). The transforming potency of Jak2-V617F mutant depends on binding to cytokine receptors (172) and, as very recently shown, on STAT5 activation (178). A study on the Jak2-V617F mutant-expressing erythroid leukemic cell line HEL has shown that specific inhibition of Jak2-V617F activity with a small molecule inhibitor leads to G_1 cell cycle arrest (179). This inhibition correlated with decreased expression of cyclin D2 and increased expression of p27; both proteins are involved in cell cycle regulation. Moreover, the Jak2-V617F mutant induces homologous recombination (HR) activity what results in genetic instability (180). It is hypothesized that the constitutively active Jak2 mutant deregulates the HR mechanism what initiates development of MPNs. The induction of genetic instability may also explain the evolution of MPN to acute myeloid leukemia. The Jak2-V617F mutation is not the only one identified in Jaks. A number of other mutations of Jak2 (Fig. 13), but also of Jak1 and Jak3 were found in patients suffering from different leukemias (acute lymphoblastic leukemia (ALL), acute megakaryoblastic leukemia (AMKL), and acute myeloid leukemia (AML)) (79). In addition to hematologic diseases, somatic Jak1 and Jak3 mutations were also found in solid tumors: Jak1 in lung and breast carcinoma, Jak3 in breast and gastric carcinoma (181). Interestingly these are tissues in which Jak3 is normally not expressed.

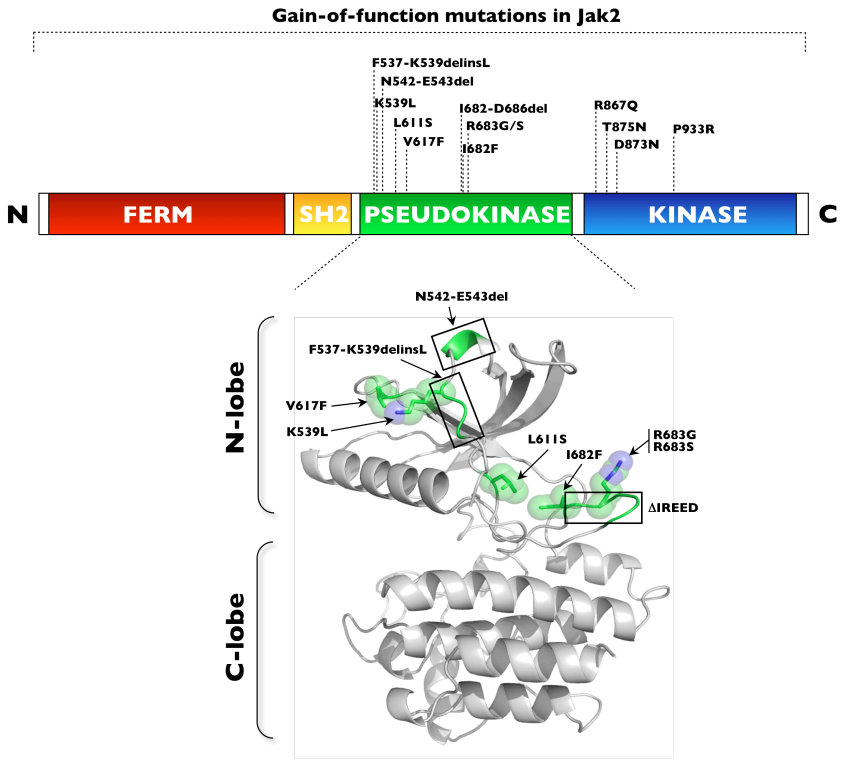

Figure 13

Domain structure of Jak2 and model structure of the Jak2 pseudokinase domain. Residues for which mutations were reported in patients resulting in constitutively active kinases are indicated. The point mutations are represented as green stick models with spheres indicating the Van-der-Waals radii of atoms. Regions carrying insertions (ins) and/or deletions are highlighted by a colored backbone. Adapted from (79).

1.5.3 Non-canonical Jak/STAT signaling caused by gain-of-function mutants

It is known that massive remodeling of the chromatin precedes Jak/STAT-mediated transcriptional activation (182, 183). It is also widely assumed that the effects of STAT activation are mediated by direct transcriptional induction of STAT target genes. Chromatin and higher order chromosome structures play a central role in a number of aspects of DNA biology in eukaryotes. Processes ranging from gene expression to chromosome dynamics during cell division are regulated by the folding of DNA into chromosomes. Heterochromatin and euchromatin are two types of chromatin found in chromosomes. Heterochromatic domains are in general inaccessible to DNA-binding factors and are transcriptionally silent. Euchromatin in contrast, defines more accessible and transcriptionally active portions of the genome. Very interestingly, using the *Drosophila hop*$^{Tum\text{-}l}$ hematopoietic tumor model, signaling via Jaks and STATs has been

reported to directly affect the stability of heterochromatin (184, 185). The *Drosophila* genome contains only one Jak, called Hopscotch (Hop) and a single STAT protein (STAT92E). These two proteins are most homologous to Jak2 and STAT5, respectively (186, 187). The *hop*$^{Tum\text{-}l}$ gene encodes an allele of Hop that is hyperactive due to a G341E mutation located presumably in a regulatory region of the protein (188-190). In this hematopoietic tumor model based on the hyperactive Jak mutant, Hop$^{Tum\text{-}l}$ globally disrupts heterochromatic gene silencing, which enables expression of genes that are not necessarily under direct STAT transcriptional regulation. It has been hypothesized, that Jak-mediated constitutive activation of STATs enables STAT-binding partners like p300/CBP, which possess HAT activity, to acetylate surrounding histones resulting in chromatin decondensation and expression of other than the canonical STAT-responsive genes (191).

Very interestingly, similarly to human leukemias, Hop$^{Tum\text{-}l}$ causes overproduction and clonal expansion of particular blood cell types what deregulates the *Drosophila* blood cell development (189) and results in a high incidence of hematopoietic tumors. The tumorigenicity of Hop$^{Tum\text{-}l}$ strongly depends on the disruption of heterochromatic gene silencing (184). Given the evolutionary conservation of the canonical Jak/STAT pathway among different species, the non-canonical pathway affecting heterochromatin might also operate in vertebrates. To date, a number of constitutively active gain-of-function Jak mutants and Jak fusion proteins were found in human leukemias (79), so it would be of great interest to investigate this issue in mammalian cells.

2 OBJECTIVES

My objectives were the following:

1) Elucidation of the relative contribution of Jak1 and Jak2 in the signal transduction process of IFNγ

a) Establishment of a cellular system suitable to study analogue-sensitive Janus kinases

i) Generation of the analogue-sensitive mutants of Jak1 and Jak2 and their stable expression in Jak1- or Jak2-deficient cells

ii) Biochemical characterization of the analogue-sensitive kinases

b) Studying the outcome of specific pharmacologic inhibition of Jak1 and Jak2 in the context of IFNγ-mediated signal transduction

2) Contribution to a better understanding of the mechanisms underlying signal transduction mediated by the Jak2-V617F mutant

a) Studying the effects of inhibition of the constitutively active Jak2 and Jak3 mutants in leukemic cells

b) Studying negative feedback mechanisms counteracting signaling through hyperactive Jak2 mutants

3) Characterization of protein kinase inhibitors designed to inhibit Jak kinase activity or reported to have suppressive properties against cells expressing Jak mutants

3 MATERIAL AND METHODS

Material and methods:

a) *In vitro* experiments were performed with the following cell lines or primary cells:

i) Leukemic cell lines

(1) HEL – human erythroleukemia (Jak2-V617F)

(2) UKE-1 – cell line derived from a patient with transformed myelodysplastic syndrome (Jak2-V617F)

(3) SET-2 – human essential thrombocythemia (Jak2-V617F)

(4) CMK – human acute megakaryocytic leukemia (Jak3-A572V)

(5) CMY – human acute megakaryoblastic leukemia (Jak3-A573V)

ii) Human fibrosarcoma cell lines

(1) 2C4 (parental cell line for U4C and γ2A)

(2) U4C (Jak1-deficient)

(a) U4C-FRT-Jak1-WT

(b) U4C-FRT-Jak1-M955A and M955G (analogue-sensitive)

(c) U4C-FRT-Jak1-D1002N (kinase-inactive)

(3) γ2A (Jak2-deficient)

(a) γ2A-FRT-Jak2-WT

(b) γ2A-FRT-Jak2-M929A and M929G (analogue-sensitive)

(c) γ2A-FRT-Jak2-D976N (kinase-inactive)

iii) Human embryonic kidney cell lines

(1) HEK-Flp-In-293-EpoR

(a) HEK-Flp-In-293-EpoR-Jak2-WT

(b) HEK-Flp-In-293-EpoR-Jak2-K539L, -V617F and -T875N

(2) HEK-Flp-In-YFP

(a) HEK-Flp-In-YFP-Jak2-WT, -V617Fand -T875N

iv) Other cancer cell lines

(1) A431 – human epidermoid carcinoma (EGFR overexpressing cell line)

(2) HepG2 – human hepatocellular carcinoma

v) Primary CD14+ monocytes

b) Expression and phosphorylation of proteins were assessed by:

i) Immunoprecipitation *(page 132)*

ii) Western blot *(page 132)*

c) Gene expression assessed by:

i) Whole-genome DNA microarray *(pages 46 and 63)*

ii) Immunology DNA microarray *(page 64)*

iii) Quantitative PCR *(pages 46 and 62)*

iv) Semi-quantitative PCR *(page 86)*

d) Cloning of Jak1 or Jak2 mutant expression constructs:

i) Mutations introduced by site-directed mutagenesis *(page 62)*

ii) Constructs cloned into the pcDNA5/FRT/TO expression vector *(page 62)*

e) Cellular growth was monitored by:

i) WST-1 assay *(page 86)*

f) Cellular apoptosis was monitored by:

i) FACS Annexin-V assay *(page 87)*

g) Protein localization was followed by:

i) Confocal cell live microscopy *(page 129)*

Detailed information about each method can be found in the results section on the indicated pages.

Growth condition for the above-mentioned cells can be found on the following pages:

- Human leukemic cell lines – *page 106*
- Human fibrosarcoma cell lines – *page 62*
- Human embryonic kidney cell lines – *page 132*
- Human epidermoid carcinoma A431 – *page 106*
- Human hepatocellular carcinoma HepG2 – *page 168*
- Primary CD14+ cells – *page* 87

4 RESULTS

4.1 "JAK1 AND JAK2 IN IFNγ SIGNALING: A CHEMICAL GENETIC APPROACH TO DISSECT THEIR RELATIVE CONTRIBUTIONS"

4.1.1 Preamble

Although it is well documented that Jak1 and Jak2 are the key components of IFNγ signal transduction, the respective contribution of Jak1 and Jak2 to initiate and sustain the IFNγ response remains to be fully understood.

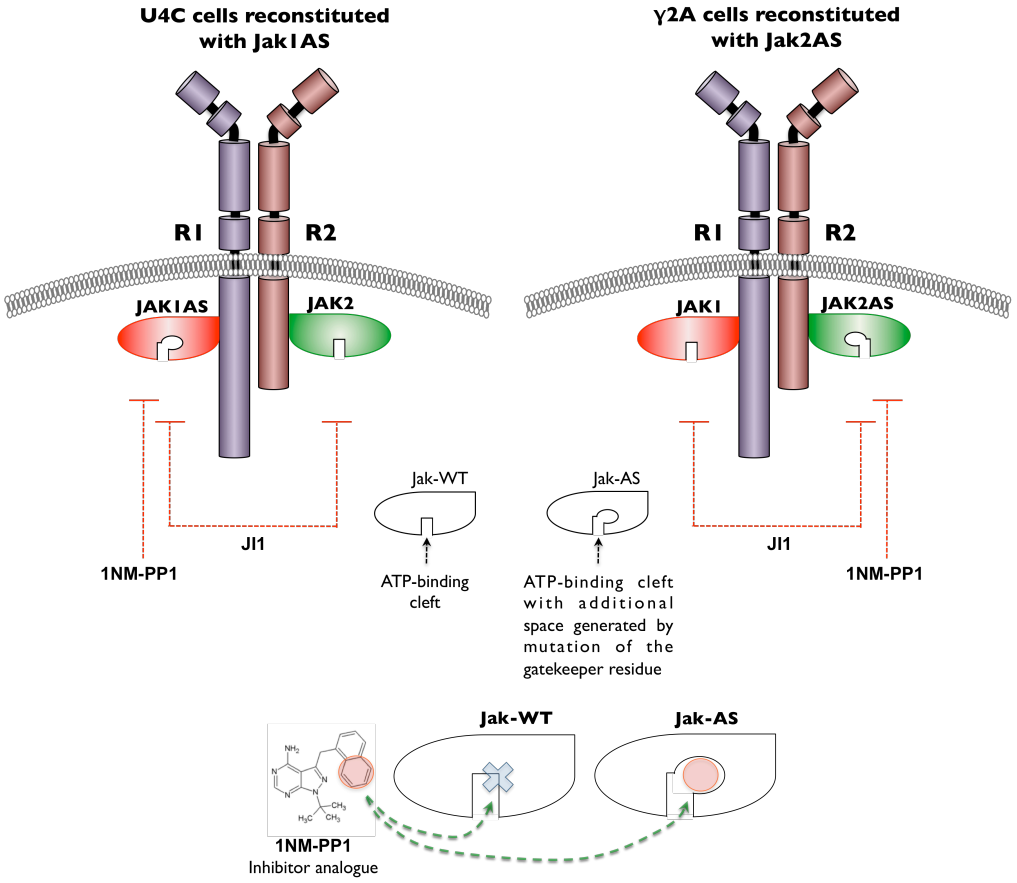

Figure 14

Diagram representing the principle of the chemical genetics approach used to dissect the relative roles of Jak1 and Jak2 in the IFNγ-mediated signaling response. The chemically modified inhibitor 1NM-PP1 contains a bulky substituent (highlighted by a red circle) that precludes binding of the inhibitor to the wild-type Jak. 1NM-PP1 can inhibit only kinases with the extra space inside the ATP-binding pocket generated by mutation of the gatekeeper residue, what means that in U4C cells only Jak1AS would be suppressed and only Jak2AS in γ2A cells. JI1 targets both analogue-sensitive and wild-type kinases. This approach enables the generation of signaling and gene expression profiles characteristic for the inhibition of either Jak1 (U4C) or Jak2 (γ2A).

In the following work we have addressed the question concerning the requirements of Jak1 and of Jak2 activity for an efficient IFNγ response using a chemical genetics approach. The analogue-sensitive mutants of Jak1 and Jak2 were stably expressed in U4C and γ2A cells that are deficient in Jak1 and Jak2, respectively (Fig. 14).

Both analogue-sensitive mutants of Jak1 and Jak2, in contrast to wild-type Jaks, were inhibitable by the inhibitor analogue 1NM-PP1, what gave us the possibility to tightly control the activity of either Jak1 (in U4C cells) or Jak2 (in γ2A cells) and monitor the response to IFNγ in early as well as late phases. Specific inhibition of Jak1 or Jak2 by 1NM-PP1 was compared with the effects of the pan-Jak inhibitor JI1.

We could demonstrate that inhibition of either Jak1AS or Jak2AS potently reduces STAT1 tyrosine phosphorylation what had a strong repercussion on IFNγ-mediated target gene expression. The suppression of STAT1 phosphorylation was consistently stronger upon inhibition of Jak2AS compared to the inhibition of Jak1AS. Interestingly, expression of STAT1 and IRF1 only seemed to depend on Jak2 activity; Jak1 activity was not necessary to induce STAT1 and IRF1 protein up-regulation. Importantly, U4C and γ2A cells expressing kinase-inactive mutants of Jak1 and Jak2, respectively, showed a similar regulation of STAT1 and IRF1, thereby supporting the results obtained by the chemical genetics approach.

4.1.2 Article

Jak1 and Jak2 in IFNγ signaling: a chemical genetic approach to dissect their relative contributions.

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Abstract

Interferon γ (IFNγ) is involved in many biological processes including antiviral responses and immune surveillance. IFNg signals though the receptor-associated Janus kinases Jak1 and Jak2. Using a chemical genetics approach we investigated the relative contribution of Jak1 and Jak2 in IFNγ signal transduction. We constructed analogue-sensitive mutants of Jak1 and Jak2. They have normal activity in IFNγ-mediated signaling and can be specifically inhibited by kinase inhibitor analogues. Single inhibition of either Jak1 or Jak2 activity by an inhibitor analogue showed differential effects on IFNγ-mediated STAT1 phosphorylation, Jak2 inhibition having a stronger suppressive effect. Analysis of the transcriptome of IFNγ-stimulated fibrosarcoma cells revealed that both active Jak1 and Jak2 are required for full expression of many genes, e. g. those involved in antiviral responses and antigen presentation. Furthermore, we identified STAT1 and IRF1 as genes, whose increased expression upon IFNγ stimulation strongly depends on active Jak2 but not Jak1. Exploiting the quick and reversible pharmacological inhibition of analogue-sensitive kinases we address the temporal requirement of Jak2 activity and the importance of different phases of the IFNγ response.

Introduction

IFNγ is involved in many biological processes including antiviral responses or immune surveillance. IFNγ signaling involves phosphorylation of the protein tyrosine kinases Jak1 and Jak2 associated with IFNγR1 and IFNγR2, respectively. Upon IFNγ stimulation, STAT1 is recruited to tyrosine-phosphorylated Y440 of (human) IFNγR1. STAT1 is then phosphorylated on Y701, and dimers of Y-phosphorylated STAT1 molecules translocate to the nucleus where they bind to GAS (gamma activated sequences) elements in promoter regions of IFNγ target genes (1-4). The relative role of the two Jaks for signal transduction is not entirely clear yet. Cellular and animal knockout models (5-11) have shown that lack of Jak1 or Jak2 expression prevents IFNγ signaling. However, the requirement of the expression of a Janus kinase in a certain cytokine signaling pathway does not automatically prove that its kinase activity is essential. Studies with kinase-inactive Jaks have yielded valuable data but in similar cellular systems differences have been reported (12, 13).

During the last years a number of inhibitors targeting the Jak family of kinases have entered clinical trials for different indications (14). A minority of these inhibitors has good specificity within the Jak family. This raises the question whether inhibitors affecting more than one Jak might have significant side effects due to inhibition of other cytokine signaling pathways: e. g., inhibition of IFNγ signal transduction is likely to enhance the risk of viral infections and to reduce cancer immune clearance.

To address the relative importance of Jak1 and Jak2 in IFNγ signal transduction we used a recently described chemical genetic approach (15, 16). This involves a genetic re-engineering of the kinase of interest, which is specifically rendered sensitive to a chemically modified ATP or inhibitor. To achieve this, the gatekeeper residue in the ATP binding pocket of the kinase is mutated to a smaller residue (e.g. glycine). Thereby, the space of the ATP-binding pocket is increased and is now accessible to a bulky derivative of a kinase inhibitor (or of ATP), which does not fit into the ATP-binding pocket of the wild-type kinase of interest, nor into the one of other kinases present in the cell. The possibility to specifically regulate the activity of a kinase by an ATP-competitive inhibitor has obvious advantages over e. g. the regulation of the expression of the whole protein or the use of kinase-inactive mutants (17, 18). The quick and reversible pharmacological inhibition of analogue-sensitive kinases by bulky inhibitors prevents the possible occurrence of compensatory processes often observed in “knock out studies” (19).

Using a chemical genetics approach we investigated the relative contribution of Jak1 and Jak2 in IFNγ signal transduction. We demonstrate that single inhibition of either Jak1 or Jak2 activity by a bulky inhibitor has a strong though not complete suppressive effect on IFNγ-mediated STAT1 phosphorylation. Analysis of the transcriptome of IFNγ-stimulated cells revealed that both active Jak1 and Jak2 are required for full expression of many genes, e. g. involved in antiviral responses

and antigen presentation. Furthermore, we have identified STAT1 and IRF1 as genes, whose expression upon IFNγ stimulation strongly depend on active Jak2 but not Jak1 and address the temporal requirement of Jak2 activity for their expression.

Material and Methods

Inhibitors

Jak Inhibitor 1, 1Na-PP1 and 1NM-PP1 were obtained from Calbiochem.

Cell lysis and Western blot analysis

Cell lysis was performed as described before (20). Proteins were separated by SDS-PAGE, followed by electroblotting onto a PVDF (Roth) or Nitrocellulose membrane (GE Healthcare). Anti-STAT1, anti-phospho-STAT1, anti-Jak1 and anti-Fin13 antibodies were from Transduction Laboratories. Anti-IRF1 and anti-α-Tubulin was from Santa Cruz and anti-actin from Millipore. Anti-Jak2 and the HRP-conjugated secondary antibodies were purchased from Cell Signaling. The fluorescently labelled secondary antibodies (IRDye®680 and IRDye®800CW) were from Li-COR® Biosciences. Signals were detected using an ECL solution containing 2.5 mM luminol, 2.6 mM hydrogen peroxide, 100 mM Tris/HCl pH8.8 and 0.2 mM para-coumaric acid (21) and a CCD camera system (Intas) or an Odyssey Infrared Imaging System (Li-COR® Biosciences). Quantitation was performed on single channels with the analysis software provided by the manufactor (Li-COR® Biosciences). Briefly, the signals to be quantitated were normalized with respect to the loading control for each lane and the signal intensity was then represented as fold induction or as % of the strongest signal.

Quantitative PCR

Total RNA was extracted using the NucleoSpin RNA II (Macherey-Nagel) and 1 µg of total RNA was reversely transcribed with the ThermoScript RT-PCR System (Invitrogen) in a volume of 20 µL, using 50 mM oligo$(dT)_{20}$ according to the manufacturer's instructions. The real-time PCR was carried out on a CFX96 Detection System (BioRad) using a total volume of 20 µL containing cDNA corresponding to 50 ng RNA template, 10 pmol of each forward and reverse primer and 10 µL of 2x Absolute QPCR SYBR Green Fluorescein Mix (ThermoScientific). For further information please consult the supplemental information section.

Whole genome microarray analysis

U4C-Flp-In-Jak1AS and γ2A-Flp-In-Jak2AS cells were left untreated or stimulated with IFNγ or IFNγ + inhibitors (1NM-PP1 or JI1) for 24h. Total RNA was extracted using the miRNeasy kit

(Qiagen) and subjected to whole human genome microarray analysis (Agilent Biotechnologies). Detailed methodologies and bioinformatic analyses are described in the supplemental material.

Results

Characterization of the gatekeeper mutants of Jak1 and Jak2.

The gatekeeper residue in the catalytic cleft of Jak1 and Jak2 was genetically modified by site-directed mutagenesis. Jak1M955 and Jak2M929 were mutated to either glycine or to alanine. Expression constructs encoding wild-type Jak1 and Jak2 as well as the potential analogue-sensitive mutants were stably inserted into the FRT (Flp-In system, Invitrogen) site of human fibrosarcoma cell lines deficient in Jak1 (U4C-Flp-In cells) or deficient in Jak2 (γ2A-Flp-In cells). The use of the FRT site allows the isogenic expression of WT and mutated Jaks from the same site in the genome. To test the functionality of the different gatekeeper mutants of Jak1 and Jak2, U4C-Flp-In cells expressing either Jak1WT or Jak1 mutants and γ2A-Flp-In cells expressing either Jak2WT or Jak2 mutants were monitored for IFNγ-mediated target gene expression upon IFNγ stimulation. Quantitation of STAT1 and IRF1 protein expression upon IFNγ treatment revealed no major differences between U4C-Jak1WT and U4C-Jak1M955G (Jak1AS) or γ2A-Jak2WT and γ2A-Jak2M929A (Jak2AS) (Figure 1A) expressing cells. Furthermore, quantitative analysis of LMP2 and TAP2 mRNA expression by real-time PCR confirmed the functionality of the mutated Jaks (Figure 1B). Moreover, the functionality of Jak1AS was further verified by a focused microarray containing 243 immunology-related genes showing a similar response of Jak1M955G (Jak1AS) and Jak1WT cells to IFNγ (Supplemental figure 2).

To verify if bulky inhibitors can indeed explore the extra space generated by mutation of the gatekeeper residue and inhibit these Jak1 and Jak2 mutants, the tyrosine phosphorylation of STAT1 upon IFNγ treatment with or without inhibitors was monitored in U4C and γ2A cells stably expressing WT or mutant Jaks. STAT1 phosphorylation in all cell lines was effectively abrogated by Jak Inhibitor 1 (JI1), a pan-Jak inhibitor (22) targeting the wild-type forms of Jak1 and Jak2 as well as the analogue-sensitive mutants. Of the two inhibitor analogues that were tested, 1NM-PP1 (23) was able to inhibit the Jak1M955G (Jak1AS) and Jak2M929A (Jak2AS)-dependent IFNγ-mediated signal transduction (Figure 2, supplemental figure 1). Pre-treatment with the bulky inhibitor 1NM-PP1 led to a dose-dependent down-regulation of IFNγ-mediated STAT1 phosphorylation only in cells expressing analogue-sensitive Jaks (data not shown). Moreover, 1NM-PP1 only suppressed the phosphorylation of the analogue-sensitive Jaks, in contrast to wild-type Jaks (data not shown). The characterization of the mutants Jak1M955A, Jak2M929G, which were not further used in this study and experiments using a second inhibitor analogue, 1Na-PP1, are shown in supplemental figure 1 and supplementary table I.

Thus, in response to IFNγ treatment, U4C-Jak1WT and -Jak1M955G (Jak1AS) or γ2A-Jak2WT and -Jak2M929A (Jak2AS) cells showed a similar pattern of target gene expression, indicating that the introduced gatekeeper residue mutation does not compromise protein function. Both analogue-sensitive Jak mutants were also inhibitable by the 1NM-PP1 compound while the corresponding WT Jaks were not inhibited. Therefore, these mutants hereafter called Jak1AS and Jak2AS were selected for further study.

Jak2 and Jak1 are essential for full phosphorylation of STAT1 but Jak2 plays the more important role.

In the chemical genetic approach the activity of an analogue-sensitive kinase can be inhibited quickly in a time-dependent manner. To address the issue of temporal requirements of both Jaks for STAT1 phosphorylation, cells expressing either Jak1AS or Jak2AS were stimulated with IFNγ for 20 min or left untreated. To inhibit only the analogue-sensitive kinase, 1NM-PP1 was used and administrated 30 min prior to, simultaneously with or 10 min after cytokine stimulation (Figure 3A). Regardless of the time of the 1NM-PP1 treatments, STAT1 phosphorylation was efficiently, though not completely down regulated. Very interestingly, only 10 min of inhibitor treatment (+10) was as effective to reduce the phospho-STAT1 signal as longer treatments (-30: pre-treatment; 0: co-stimulation), which is indicative of a quick dephosphorylation of phospho-STAT1 (24). Quantitation of the STAT1 phosphorylation revealed that inhibition of Jak2AS resulted in a stronger suppression of STAT1 activity compared to Jak1AS (down-regulation to below 20 % for selective Jak2 inhibition compared to 40 % for selective Jak1 inhibition)

The enzymatic activity of a kinase can be perturbed in two different ways: by small molecule inhibitors, which have to compete with cellular ATP for binding to the catalytic cleft or by the introduction of kinase-inactivating mutation. We compared the analogue-sensitive kinases that could be selectively inhibited by 1NM-PP1 with the kinase-inactive mutants Jak1DN (Jak1D1002N) and Jak2DN (Jak2D976N) in the context of STAT1 activation upon IFNγ treatment. As shown before (Figure 2), 1NM-PP1 inhibition of Jak1AS and Jak2AS resulted in significant, though not complete suppression of STAT1 phosphorylation upon IFNγ treatment (Figure 3A) and Jak2 inhibiton was again more efficient in mediating this down-regulation. Cells expressing the kinase-inactive Jak1 mutant (Jak1DN) showed also residual STAT1 phosphorylation upon IFNγ stimulation, in contrast to Jak2DN expressing cells where STAT1 activation was not observed (Figure 3B). Expectedly, pharmacological inhibition of the analogue-sensitive Jaks was slightly less effective than mutation-based inactivation of kinase activity. However, the qualitative comparison shows that the specifically inhibited state reflects the kinase-inactive state. Taken together the data indicate that Jak2 is more important in IFNγ-mediated STAT1 activation than Jak1.

Full regulation of the vast majority of IFNγ-regulated genes requires both active Jak1 and Jak2.

To investigate the general impact of Jak1 and Jak2 inhibition on IFNγ-mediated target gene expression, a whole genome microarray analysis was carried out. U4C-Jak1AS and γ2A-Jak2AS cells were stimulated with IFNγ and treated with either 1NM-PP1 (to inhibit the activity only of the analogue-sensitive mutant) or JI1 (to suppress both wild-type and analogue-sensitive Jaks) for 24h. IFNγ stimulation resulted in the differential expression of 345 genes that were common for both U4C-Jak1AS and γ2A-Jak2AS cell lines of which 305 were up-regulated and 40 were down-regulated compared to the untreated control. Selective inhibition of either Jak1 or Jak2 resulted in down-regulation of the expression of the majority of genes regulated by IFNγ (Supplemental figure 3 (blue dots highlight a few well known examples) and Supplemental table VI); among others those involved in the antiviral response (OAS, PML, IRF1) (25-28), antigen presentation (HLA-A, -B and -C), and negative regulation of IFNγ signaling (SOCS1) (29). We confirmed this partial down-regulation of IFN-responsive genes by qPCR (Figure 4). Suppression of both Jaks by Jak inhibitor 1 (JI1) completely abrogated the inducible expression of these genes (Figure 4) while specific inhibition of Jak1 or Jak2 resulted in significant but not complete down-regulation. Taken together, the microarray and qPCR data suggest that both active Jak1 and Jak2 are indispensable for full expression of the vast majority of IFNγ -regulated genes. Supplemental tables II-VII provide an overview of the statistically relevant differentially regulated genes in the setting of the microarray study.

IFNγ-mediated STAT1 and IRF1 gene expression depends on active JAK2 but not JAK1.

Microarray analysis revealed that some genes might be regulated by Jak1 or Jak2 only (Supplemental Figure 3 (red dot highlights STAT1) and Supplemental tables II-V). Interestingly, STAT1 was among the genes depending on Jak2 activity only. To investigate this, we treated cells expressing Jak1AS and Jak2AS with 1NM-PP1 30' prior to, simultaneously with or 1 h, 3 h and 6 h after cytokine stimulation. Phospho-STAT1, IRF1 and STAT1 were analyzed at 24 h post IFNγ stimulation. Jak1AS-expressing cells show persistent phospho-STAT1 signals after 24 h even if Jak1AS is inhibited by 1NM-PP1. This is in contrast to Jak2AS-expressing cells treated in the same way. Only inhibition of Jak2AS blocked the protein up-regulation of STAT1 and IRF1 (which is dependent on PY-STAT1) regardless of the time of the inhibitor treatment (Figure 5A), whilst inhibition of Jak1AS did not reduce STAT1 and influenced IRF1 expression only slightly. As a control, the same experiment was performed for Jak1WT and Jak2WT expressing cells showing no influence of 1NM-PP1 on the IFNγ-mediated STAT1 and IRF1 response (Supplemental Figure 4). Cells expressing the kinase-inactive Jak1DN or Jak2DN mutants show the same Jak2-dependence of STAT1 upregulation as the specifically inhibited analogue-sensitive Jaks (Figure 5B).

Reversible inhibition of Jak2 reveals differences in the regulation of IFNγ-mediated STAT1 and IRF1 protein expression.

In contrast to experiments involving dominant negative kinases, experiments with analogue sensitive kinases allow for a reversible inhibition of their activity. Inhibition of IFNγ-stimulated Jak2 activity over a defined time of 3 h in the initial stage of cytokine stimulation shows clear differences in the behaviour of IRF1, an immediate early gene induced by phospho-STAT1 (30), compared to the late phase response gene STAT1, which is regulated by IRF1 (31, 32). For this, cells were stimulated with IFNγ, 1NM-PP1 was added 5 min later and at 3 h the cells were washed and further cultivated in medium containing IFNγ but no inhibitor (Figure 6, supplemental figure 6). The initial phospho-STAT1 signal was rapidly suppressed by selective Jak2 inhibition, again showing the very short half-life of phosphorylated STAT1. Still, this level of STAT1 activity seems sufficient to lead to a modest but significant upregulation of IRF1 expression. Removal of the inhibitor after 3 h led to a strong peak in STAT1 phosphorylation, followed by a peak in IRF1 expression 2 h later. Cells not treated with inhibitor (but with medium replaced after 3 h, upper panel of Figure 6) displayed essentially a pattern as observed in IFNγ-stimulated 2C4 cells (the parental cells to U4C- and γ2A-cells) (Supplemental figure 5A, supplementary figure 5B for comparison of IFNγ kinetics in 2C4 and γ2A-Jak2AS cells). This shows that the expression of IRF-1 closely follows phospho-STAT1 levels. In contrast, STAT1 expression seems rather independent from fluctuations in Jak2 activity during the early phase of IFNγ stimulation in which the initial phospho-STAT1 peak is observed.

Discussion

In this study we generated analogue-sensitive mutant kinases for Jak1 and Jak2 to analyze their respective contributions to signal transduction of IFNγ. We could demonstrate that inhibition of either Jak1 or Jak2 strongly reduces STAT1 tyrosine phosphorylation. Moreover, inhibition of either Jak1 or Jak2 activity similarly reduces expression of most target genes, pointing at an equally important role for both kinases in IFNγ signaling. Expression of STAT1 and IRF1, however, only depends on Jak2 activity.

To generate analogue-sensitive kinases of Jak1 and Jak2, the gatekeeper residue in both kinases was mutated to a smaller residue to generate space for the accommodation of a “bulky” inhibitor, which cannot be used either by wild-type Jaks or ideally by any other kinase present in the cell (15, 33). The wild type or analogue-sensitive forms of Jak1 and Jak2 were introduced into U4C (Jak1-deficient) or γ2A (Jak2-deficient) cells, respectively, using the Flp-In system (13, 34). Importantly, both analogue-sensitive mutants of Jak1 and Jak2 performed similarly to the wild-type Jak1 and Jak2 kinases in the context of IFNγ-mediated target gene expression (Figure 1). In

contrast to wild-type Jaks, the analogue-sensitive forms of Jak1 as well as Jak2 were selectively inhibited by 1NM-PP1 (Figure 2) what gave us the unique opportunity to study the outcome of specific pharmacologic inhibition of Jak1 and Jak2 in the context of the IFNγ response in Jak reconstituted U4C or γ2A cells.

Inhibition of either Jak1AS or Jak2AS drastically reduced IFNγ-mediated STAT1 tyrosine phosphorylation (see Figures 2 and 3A/B). We consistently observed that the reduction in STAT1 phosphorylation was somewhat stronger upon inhibition of Jak2AS compared to the inhibition of Jak1AS. Although both AS-kinases were fully dephosphorylated upon immunoprecipitation after 1NM-PP1 treatment, we could initially not exclude that this "residual" STAT1 phosphorylation might be the result of a difference in the effectiveness of kinase inhibition within the cell where an inhibitor has to compete with ATP for binding to the catalytic cleft of the kinase in its active state. For reasons of comparison we therefore included kinase-inactive versions of Jak1 or Jak2 into the study, which carry an amino acid exchange precluding phosphotransferase activity. Jak1DN significantly affected the IFNγ-induced STAT1 phosphorylation although a partial STAT1 phosphorylation was still observed (Figure 3B). On the other hand, STAT1 phosphorylation was completely abolished in cells expressing Jak2DN. Taken together our results indicate that STAT1 tyrosine phosphorylation in response to IFNγ relies more on Jak2 than Jak1, but both kinases have to be active to observe a full activation. Importantly, the comparison of the kinase-inactive mutants and the pharmacologically inhibited analogue-sensitive mutants shows that the inhibition with 1NM-PP1 is efficient and reflects the kinase-inactive state.

To assess the impact of specific Jak1AS or Jak2AS inhibition on the transcriptome, a microarray analysis was performed. The expression of the majority of genes induced by IFNγ was suppressed to some extent upon selective inhibition of either Jak1AS or Jak2AS (Figure 4, Supplemental figure 3, Supplemental table VI). These included genes encoding SOCS1 and genes associated with the antiviral response (Fig. 4). Former studies (12) using different kinase-inactive Jak mutants and higher expression levels of reconstituted Jaks also find an important role for both kinases but attribute a much higher importance to Jak2 than we deduced from our system and the microarray data. Although in general STAT1 phosphorylation is reduced more efficiently by selectively inhibiting Jak2, many genes are influenced similarly by inhibition of Jak1 and Jak2. Only a small subset of genes shows a regulation dependent on only one of the kinases (Supplemental tables II-V). Very interestingly, the IFNγ-induced expression of the genes encoding the transcription factors STAT1 and IRF1 seems to strongly depend on Jak2 activity; Jak1 activity was not necessary to induce STAT1 and IRF1 protein up-regulation (Figures 5A and B). These data are supported by the fact that STAT1 and IRF1 protein up-regulation was also seen in cells expressing the kinase-inactive mutant Jak1-DN (Figure 5B) and another variant of kinase-inactive mutant, Jak1-KE (12, 13). In contrast, Jak2 inhibition by a small molecule inhibitor (Jak2AS+1NM-PP1) or by an inactivating mutation (Jak2DN) has a suppressive effect on STAT1 and IRF1 gene

expression (Figures 5A and B), as on the bulk of other genes (Supplemental figure 3). Thus, Jak1 seems to play a less important role in IFNγ-mediated STAT1 and IRF1 gene expression.

The availability of the specific and reversible inhibitors targeting only one Jak in our system gave us a possibility to specifically inhibit one Jak in a defined way over time and thus we addressed the temporal requirement of activated Jak2 for IFNγ-induced IRF1 and STAT1 expression in γ2A cells. The expression of IRF-1 closely followed phospho-STAT1 levels. It is well known that the gene encoding IRF1 contains GAS element(s) in its promoter and is upregulated shortly after IFNγ treatment. In contrast, STAT1 expression proved rather independent from fluctuations in Jak2 activity during the early phase of IFNγ stimulation. STAT1 gene regulation crucially depends on IRF1 (31, 32). Possibly the low levels of IRF1 induced upon IFNγ stimulation for only the first 5 min may have been sufficient to allow for a similar time course of STAT1 expression as in cells not treated with the bulky inhibitor between 5 min and 3 h. Suppression of the early STAT1 activation phase for different time lapses (30 min pre-stimulation to 1 to 3 h post-stimulation (Supplemental figure 7)) and experiments of inhibition of the late phase of signal transduction (Figure 5A) show that a sustained activation of phospho-STAT1 and a sustained IRF1 expression are essential for STAT1 protein expression through Jak2.

Taken together, the analogue-sensitive Jak1 and Jak2 mutants are specifically inhibitable by 1NM-PP1 and their inhibited state closely reflects the kinase-inactive state. In contrast to kinase-inactive mutants, the analogue-sensitive mutants can be inhibited reversibly in a temporally defined way. It will be interesting to further investigate the requirements of the different phases of Jak activity and their repercussion on specific gene read-outs.

Footnotes

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Abbreviations used in this paper: IRF, IFN regulatory factor; AS, analogue-sensitive; WT, wild-type

Figures

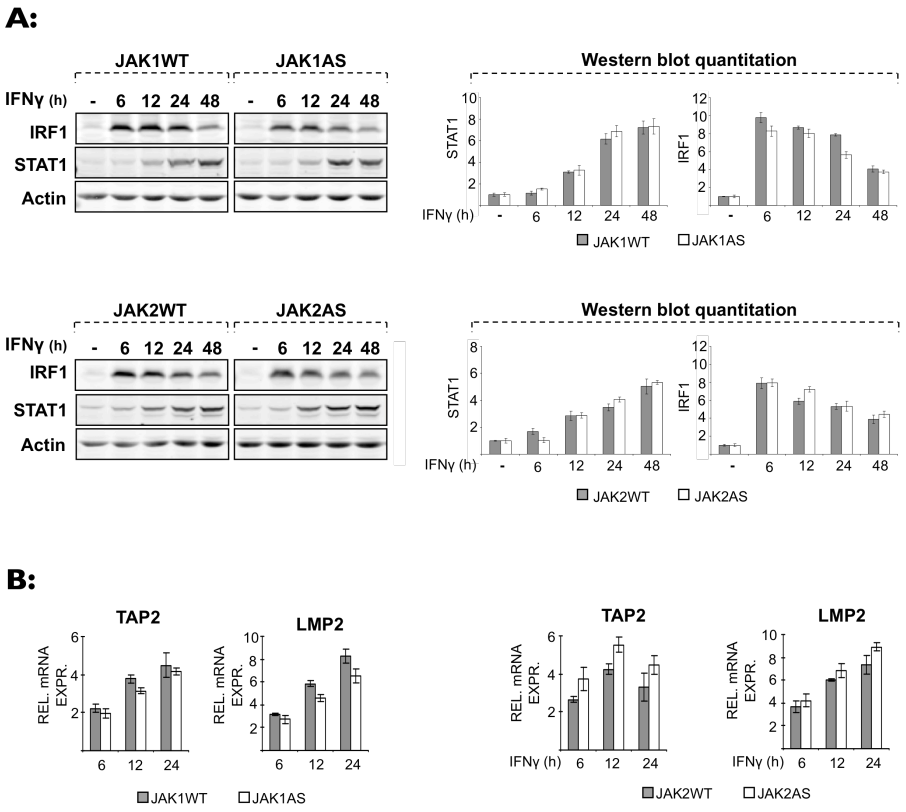

Figure 1: Jak1AS (Jak1M955G) and Jak2AS (Jak2M929A) are functional and promote IFNγ-induced gene regulation

A: U4C-Jak1WT and U4C-Jak1AS (-Jak1M955G) or γ2A-Jak2WT and γ2A-Jak2AS (-Jak2M929A) were stimulated with IFNγ (10 ng/mL) for the indicated periods of time. Lysates were resolved by SDS-PAGE and subjected to quantitative Western blot analysis LI-COR® biosciences). IFNγ-mediated up-regulation of IRF1 and STAT1 was quantified using actin as reference. Bar diagrams represents the mean and SD values of three independent experiments. **B:** U4C-Jak1WT and U4C-Jak1AS or γ2A-Jak2WT and γ2A-Jak2AS were stimulated with IFNγ. Total RNA was prepared and TAP2 and LMP2 expression was analyzed by qPCR. The experiment was performed in triplicate and standard deviations are given.

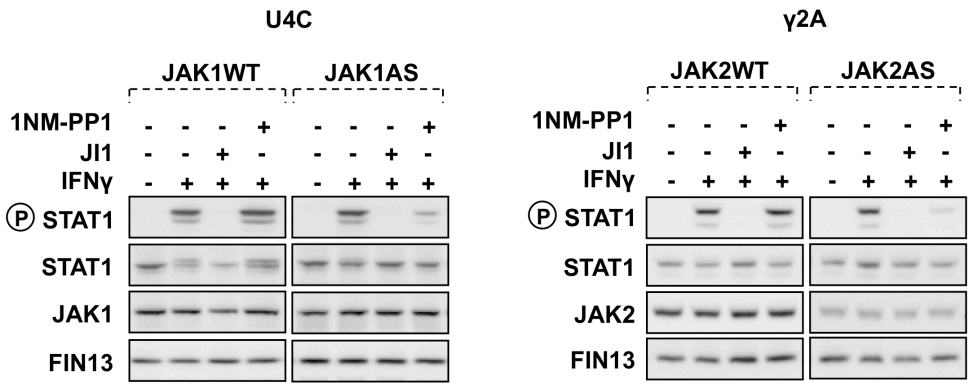

Figure 2: The activity of Jak1AS and Jak2AS can be specifically suppressed by the inhibitor analogue 1NM-PP1

U4C-Jak1WT and U4C-Jak1AS or γ2A-Jak2WT and γ2A-Jak2AS cells were pretreated or left untreated for 30 min with JI1 (1 μM) or 1NM-PP1 (40 μM) before IFNγ was added (10 ng/mL for 30 min). After lysis, proteins were resolved by SDS-PAGE and analyzed by Western blot. Phosphorylation of STAT1 was detected and equal loading was verified by redetection of the blot with antibodies against STAT1 and FIN13.

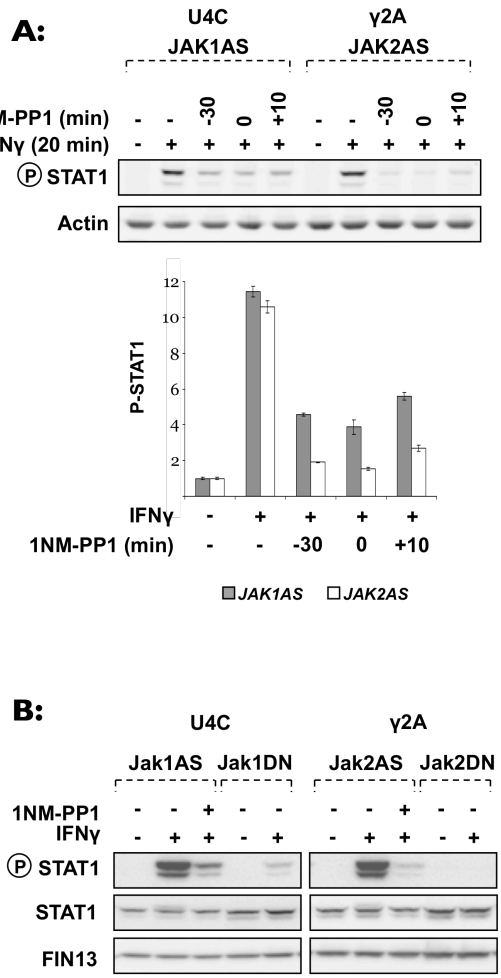

Figure 3: Full IFNγ-induced STAT1 phosphorylation requires both active Jak1 and Jak2

A: U4C-Jak1AS and γ2A-Jak2AS cells were left untreated or stimulated with IFNγ for 20 min and treated with 1NM-PP1 that was administrated 30 min prior to, simultaneously with or 10 min after cytokine stimulation. Western blots were prepared and phospho-STAT1 signals were quantified using actin as reference. The bar diagram represents the mean and SD values of three independent experiments. **B:** U4C-Jak1AS and γ2A-Jak2AS cells were left untreated or stimulated with IFNγ for 20 min and treated with 1NM-PP1 administrated 30 min prior to IFNγ. U4C-Jak1DN and γ2A-Jak2DN cells were stimulated with IFNγ for 20 min or left untreated. Phosphorylation of STAT1 was detected and equal loading was verified by redetection of the blot with antibodies against STAT1 and FIN13.

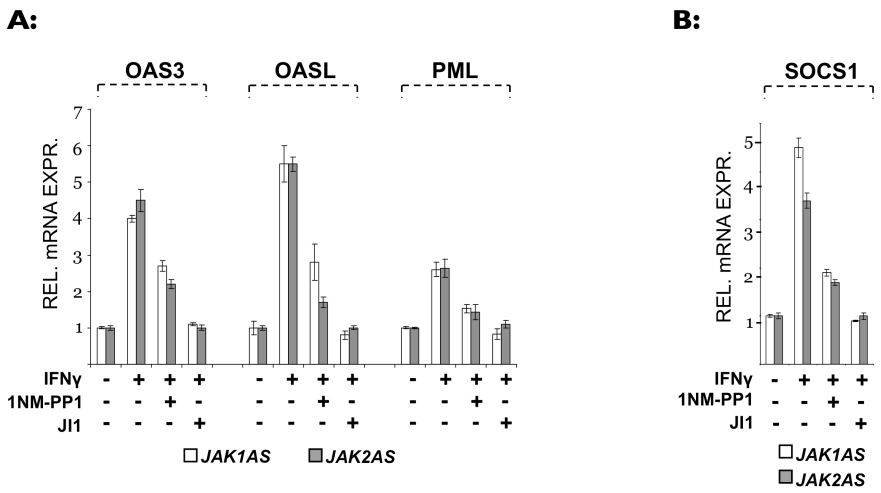

Figure 4: Both active Jak1 and Jak2 are required for full IFNγ-mediated gene expression

U4C-Jak1AS and γ2A-Jak2AS cells were pretreated or left untreated for 30 min with JI1 (1 μM) or 1NM-PP1 (40 μM) before IFNγ was added (10 ng/mL for 24 h). Total RNA was prepared and OAS3, OASL, PML and SOCS1 mRNA levels were analyzed by qPCR. The experiment was performed in triplicate and standard deviations are given.

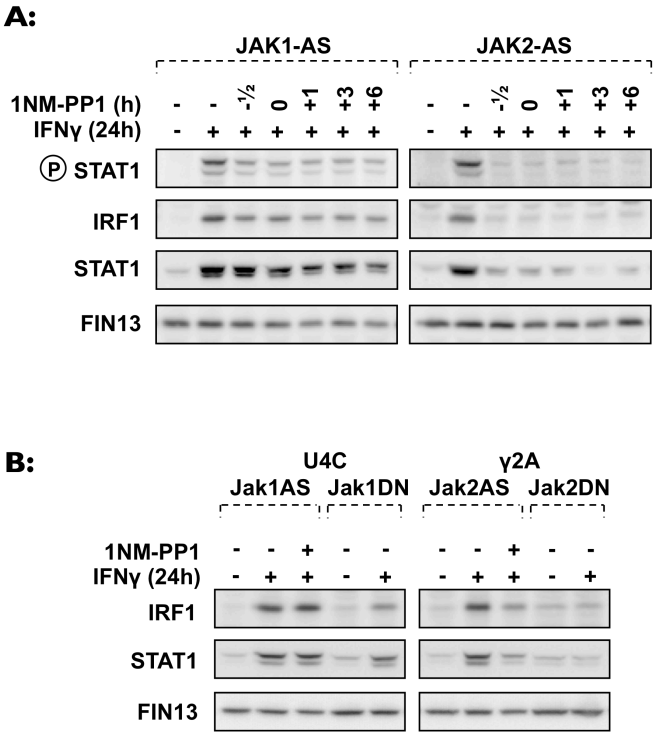

Figure 5: IFNγ-mediated STAT1 and IRF1 protein expression strongly depends on active Jak2

A: U4C-Jak1AS and γ2A-Jak2AS cells were left untreated or stimulated with IFNγ for 24 h and treated with 1NM-PP1 that was administrated 30 min prior to, simultaneously with or 1, 3 or 6 h after cytokine stimulation. Lysates were resolved by SDS-PAGE and subjected to Western blot analysis. Phospho-STAT1 and the expression of STAT1 and IRF1 were monitored and FIN13 was used as loading control. **B:** U4C-Jak1AS, U4C-Jak1DN, γ2A-Jak2AS and γ2A-Jak2DN cells were left untreated or stimulated with IFNγ for 24 h and treated with 1NM-PP1 where indicated. Western blots were detected for the expression of IRF1 and STAT1. Equal loading was verified by detection with a FIN13 antibody.

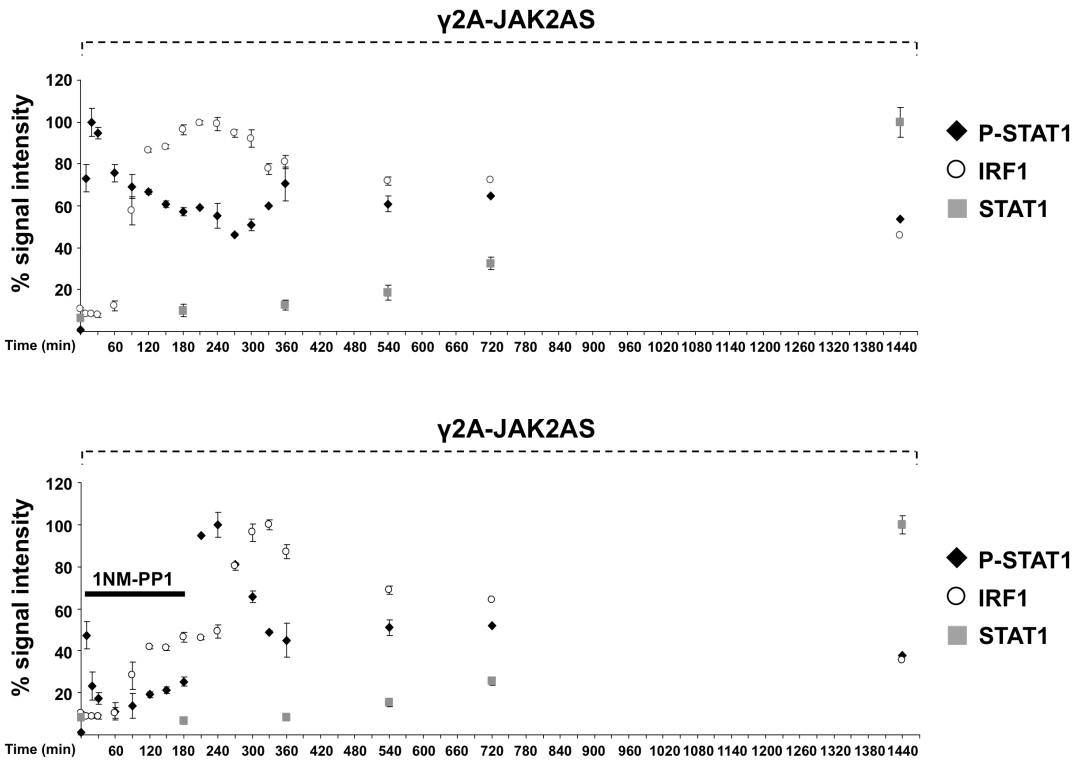

Figure 6: In contrast to IRF1, IFNγ-induced STAT1 protein expression is insensitive to early fluctuations in Jak2 activity

γ2A-Jak2AS cells were left untreated or stimulated with IFNγ (10 ng/mL). One part of IFNγ-stimulated cells (lower panel) was additionally treated with the inhibitor analogue 1NM-PP1 (40 µM) administrated 5 min after the cytokine stimulation. The time lapse of inhibitor treatment (5 min to 3 h) is indicated in the lower panel by a bar subscribed 1NM-PP1. 3 h later cells were washed with PBS and new medium containing fresh IFNγ (10 ng/mL), but not supplemented with 1NM-PP1 any more, was added. Lysates were resolved by SDS-PAGE and subjected to Western blot analysis. P-STAT1, IRF1, and STAT1 signals were quantified using α-Tubulin as reference (see Suppl. Fig. 6 for examples of original data). The scatter plot represents the mean of signal intensities obtained for each collected time point. The highest signal for P-STAT1, IRF1 and STAT1 was set to 100%. The experiment was performed three times and SD values are given.

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Supplements:

Supplementary Methods

Cell culture and materials

2C4, U4C and γ2A cells (human fibrosarcoma cells provided by Dr. I. M. Kerr, Cancer Research U.K., London, U.K.) were maintained in DMEM (Lonza). All media were supplemented with 10% FCS, 100 mg/L streptomycin, and 60 mg/L penicillin. U4C-Flp-In and γ2A-Flp-In cells (13, 34) stably expressing Jak1, Jak1-M955G (Jak1-AS), Jak1-D1002N (Jak1-DN) and Jak2, Jak2-M929A (Jak2-AS), Jak2-D976N (Jak2-DN), respectively, were generated using the Flp-In system from Invitrogen Life Technologies according to the manufacturer`s recommendations.

U4C-Flp-In-Jak1, U4C-Flp-In-Jak1AS, U4C-Flp-In-Jak1DN, γ2A-Flp-In-Jak2, γ2A-Flp-In-Jak2AS, γ2A-Flp-In-Jak2DN cells were cultured with 250 µg/mL hygromycin. Cells were grown at 37°C in a water-saturated atmosphere at 5% CO_2. IFNγ was obtained from Peprotech.

Generation of Jak1 and Jak2 mutant constructs

Standard cloning procedures were performed throughout this study. The mutations of Jak1 (M955G, M955A, D1002N) and Jak2 (M929G, M929A, D976N) were introduced by PCR technique using a site-directed mutagenesis kit (Stratagene). All procedures were performed according to the manufacturer`s instructions. The mutations were introduced into pcDNA5/FRT/TO-Jak1 and pcDNA5/FRT/TO-Jak2 vectors containing the cDNA sequence of mJak1 or mJak2, respectively. The integrity of all constructs was verified by DNA sequencing at GATC Biotech, Constance, Germany.

Quantitative PCR

Total RNA was extracted using the NucleoSpin RNA II (Macherey-Nagel)) following the manufacturer`s instructions. The concentration of isolated RNA was measured using a NanoDrop spectrophotometer. Constant amounts of 1 µg of total RNA were reversely transcribed with the ThermoScript RT-PCR System (Invitrogen) in a volume of 20 µL, using 50 mM oligo$(dT)_{20}$ according to the manufacturer's instructions. DNA contaminations were removed using the RNAse-free DNAse Set (Qiagen). The real-time PCR was carried out on a CFX96 Detection System (BioRad) using a total volume of 20 µL containing cDNA corresponding to 50 ng RNA template, 10 pmol of each forward and reverse primer and 10 µL of 2x Absolute QPCR SYBR Green Fluorescein Mix (ThermoScientific). Thermal cycling conditions for all assays were: 95ºC for 3 min, 40 cycles at 95ºC for 10 s and 60ºC for 30 s. Quantitative PCR results were normalized to TBP (TATA-binding protein). Relative fold changes were calculated by the comparative threshold cycles (C_T) method, $2^{-\Delta\Delta CT}$. The specificity of qPCR products was documented by the

melting curve analysis with the following cycling conditions: 95ºC for 1 min, 60ºC for 1 min and 60ºC for 30 sec followed by steps of increases of 0.5ºC for 30 sec until 95ºC is reached. Gene-specific primers used in qPCR analysis were purchased from Eurogentec (Belgium), as follows:

TBP	For: 5`- ACCCAGCAGCATCACTGTT -3` Rev: 5`- CGCTGGAACTCGTCTCACTA -3`
SOCS1	For: 5`- AGCTCCTTCCCCTTCCAG -3` Rev: 5`- CAAAATAACACGGCATCCC -3`
LMP2	For: 5`- CGTTGTGATGGGTTCTGATT -3` Rev: 5`- GCTTGTCAAACACTCGGTTC -3`
TAP2	For: 5`- GTAAGGAGGGTGCTGCACTT -3` Rev: 5`- CACGCTCTCCTGGTAGATCA -3`
OASL	For: 5`- GAGTGGAACGAAGAGGTGCT -3` Rev: 5`- ATGCTCCTGCCTCAGAAACT -3`
OAS3	For: 5`- GGAGAGGTTGTCATCTGGGT -3` Rev: 5`- CCTGGAATGAAATACACCCC -3`
PML	For: 5`- ACAGAGCACAGAGAGCCATC -3` Rev: 5`- GGGAGACCAAGTCCGAATAG -3`

Microarray analysis

Gene expression profiling experiments were performed using the Agilent 44K whole human genome microarrays (Agilent Biotechnologies, Diegem, Belgium) according to the two-color gene expression analysis (Quick Amp labeling) protocol (version 5.7) from the manufacturer. Hybridizations were performed by comparing cDNA derived from 300 ng of total RNA extracted from U4C-Jak1-M955G or γ2A-Jak2-M929A cells treated for 24h with IFNγ (10 ng/mL) and 40 μM of 1NM-PP1 or 1 μM of JI1 with that obtained from untreated cells. For each treatment, three biological replicates were analyzed in duplicate including one dye swap. Only high quality RNAs with a ribosomal RNA ratio greater than 1.9 and no evidence of degradation, as evaluated using the Agilent Bioanalyzer 2100 RNA 6000 nano assay, were used for analysis. Microarray images were quantified using the GenePix Pro 6.1 software (Molecular Devices, Sunnyvale, CA). The background level was detected using the morphological method “closing followed by openings”. GenePix flagging, able to filter out low-quality spots, was optimized to reduce the number of false negative and false positive spots in the analysis. The following GenePix script was used to determine the good quality spots.

[Dia.] >= 45 and

([F532 CV] + [F635 CV])/2 < 80 and

([SNR 532] + [SNR 635])/2 > 4 and
[F635 % Sat.] < 25 and
[F532 % Sat.] < 25 and
([% > B635+2SD] > 60 or [% > B532+2SD] > 60) and
[Flags] <> [Bad]

Information was imported from GPR files, processed and further analyzed by R/Bioconductor tools. First, various normalization methods were applied to remove experimental bias in the dataset. Spatial effects were removed using two-dimensional approximation of zero log-ratio level by radial basis functions. Dye-effects were removed by a Lowess normalization with the smoothing parameter *f*=0.67 (35). Finally, between-array normalization was performed by median-based centering and scaling of log-ratio distributions. The quality of microarrays was controlled by distributions of log-ratio values of the good-flagged spots, the total number of good-flagged spots per array, the average correlation with other arrays and spatial homogeneity. Replicate spots were summarized and additionally filtered before the statistical analysis: only spots with intensities significantly higher than the background level were considered. To find genes with statistically significant regulation in each class (IFNγ, IFNγ+1NM-PP1, IFNγ+JI1) and between the three classes, the empirical Bayes method (36), implemented in Bioconductor's limma package, was used. Genes with an adjusted p-value less then 0.05 are considered as significantly regulated. Microarray raw data were deposited in the ArrayExpress public repository with the reference number E-MEXP-2621 (Username: Reviewer_E-MEXP-2621; Password: 1269281492172).

PIQOR™ Immunology microarray

U4C-Flp-In-Jak1WT and U4C-Flp-In-Jak1AS were left untreated or stimulated with IFNγ for 24h. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and analyzed by the PIQOR™ Immunology Microarray provided by Miltenyi Biotec. Bioinformatic analysis was performed by Miletnyi's Biotec service.

Supplemental results

Characterization of the gatekeeper mutations in Jak1 and Jak2 and the sensitivity of the mutants to the inhibitor analogues 1Na-PP1 and 1NM-PP1.

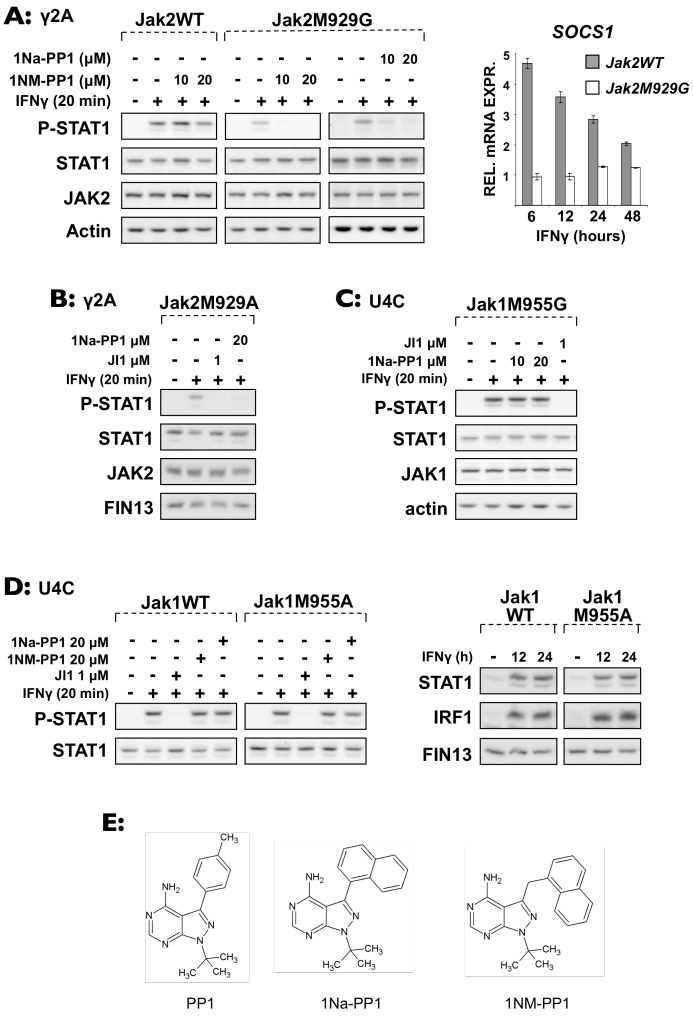
To further test the functionality of the gatekeeper mutants of Jak1 and Jak2, U4C-Flp-In cells expressing either Jak1WT or Jak1 mutants and γ2A-Flp-In cells expressing either Jak2WT or Jak2 mutants were monitored for IFNγ-mediated STAT1 phosphorylation or target gene expression upon IFNγ stimulation. To verify if inhibitor analogues 1Na-PP1 and 1NM-PP1 inhibit these Jak1- and Jak2-gatekeeper-mutants, the tyrosine phosphorylation of STAT1 upon IFNγ and inhibitor treatment was analyzed.

The naphtyl (1Na-PP1) and methylnaphtyl (1NM-PP1) groups (23) (Supplemental figure 1E) are the ones exploiting the extra space generated by the mutation of the gatekeeper residue, thereby ensuring that the wild type Jaks are not inhibited by these compounds. Jak-specific inhibitors with modifications exploiting this extra space would be optimal compounds to use in this kind of study. Such compounds are, however, not available commercially. WT Jaks are not inhibited by the PP1 and PP2 inhibitors of the src family kinases since they harbor a bulkier gatekeeper residue, which clashes with the phenylmethyl group of PP1. The naphtyl- and methylnaphtyl-derivatives of PP1 are still bulkier and the chance that these compounds would inhibit wild type Jaks was even lower.

Supplemental Figure 1A shows that the Jak2M929G mutant does not sustain a robust STAT1 phosphorylation which equals the one of Jak2WT. Concomitantly, SOCS1 gene transcription is not observed. Thus, this mutant is functionally impaired and cannot be further used to study IFNγ signal transduction events. Nevertheless, Jak2M929G was inhibitable by both 1Na-PP1 and 1NM-PP1 (Supplemental Figure 1A) but this is of no further interest since functional integrity is a prerequisite. Jak2M929A, which is shown to sustain IFNγ signal transduction and target gene transcription (Figure 1) is also inhibited by 1Na-PP1 and is thus inhibitable by both bulky inhibitor analogues (Figure 1 and supplemental figure 1B). Both the Jak1M955G and Jak1M955A mutants showed a similar signal transduction compared to Jak1WT upon IFNγ (Figure 1; Supplemental figure 1C and D) and target gene upregulation comparable to Jak1WT (Figure 1; Supplemental figure 1D right panel). However, only Jak1M955G was inhibitable by 1NM-PP1 while Jak1M955A was not. Both, Jak1M955G and -M955A were not inhibitable by 1Na-PP1 (Supplemental figure 1C and D).

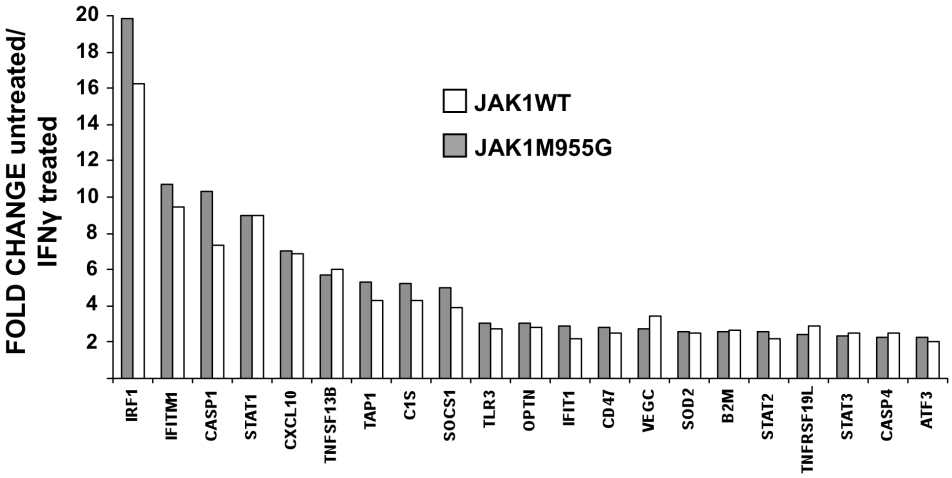
As a conclusion, the Jak1M955G (Jak1AS) and Jak2M929A (Jak2AS) mutants are functional and are both inhibited by the same inhibitor analog (1NM-PP1) so that they were chosen to conduct this study. The two mutations were therefore designated as Jak1AS and Jak2AS (AS = analog sensitive).

Supplemental figures



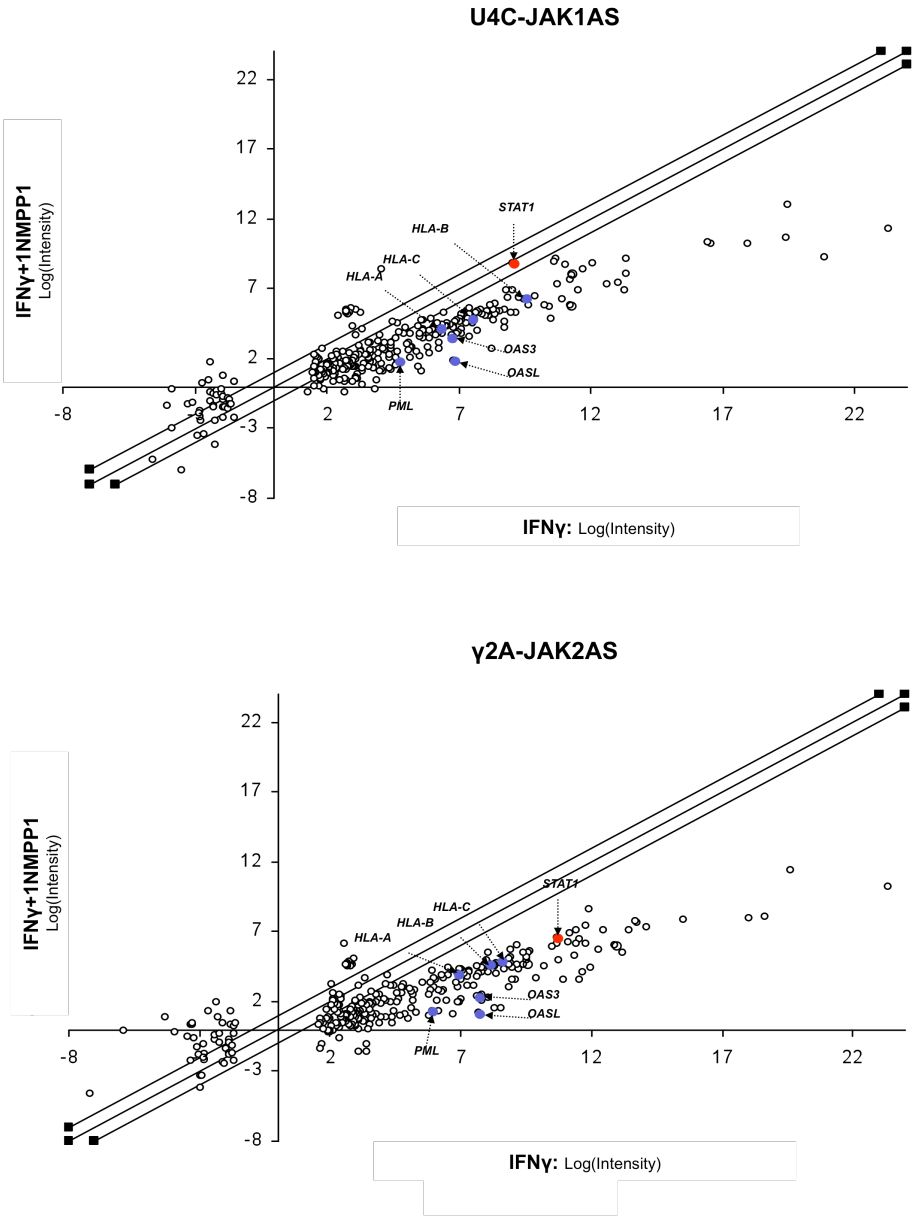
Supplemental figure 1: Further characterization of the Jak1 and Jak2 analogue-sensitive mutants and their sensitivity to the inhibitor analogues 1Na-PP1 and 1NM-PP1.

A: γ2A-Jak2WT and γ2A-Jak2M929G were treated with IFNγ and 1NM-PP1 or 1Na-PP1 as indicated. Western blots were detected for P-STAT1, STAT1, Jak2 and actin (left panel). SOCS1 mRNA expression was investigated by qPCR upon IFNγ–treatment for the indicated times (right panel). **B:** γ2A-Jak2M929A cells were treated with IFNγ, JI1 and 1Na-PP1 as indicated. Western blots were detected for P-STAT1, STAT1, Jak2 and FIN13. **C:** U4C-Jak1M955G cells were stimulated with IFNγ, JI1 and 1Na-PP1 as indicated. Western blots were detected for P-STAT1, STAT1, Jak1 and actin. **D:** U4C-Jak1WT and U4C-Jak1M955A were treated with IFNγ, JI1, 1Na-PP1 and 1NM-PP1 as indicated. Western blots were detected for P-STAT1, STAT1 (left panel) or for STAT1, IRF1 and FIN13 (right panel). **E:** The structures of PP1 and its derivatives 1Na-PP1 and 1NM-PP1.



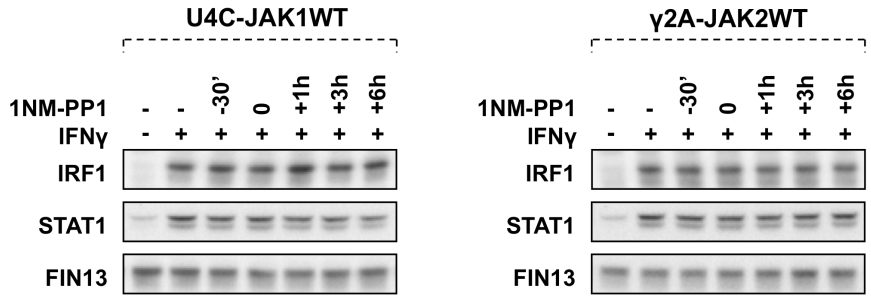
Supplemental figure 2: Jak1M955G induces a similar cellular response to IFNγ compared to Jak1WT when expressed in U4C cells.

Microarray analysis (PIQOR™ Immunology Microarray, Miltenyi Biotec) of IFNγ (24 h)-stimulated U4C-Jak1WT and U4C-Jak1M955G cells. The 21 most regulated genes are shown as fold upregulated versus unstimulated cells.



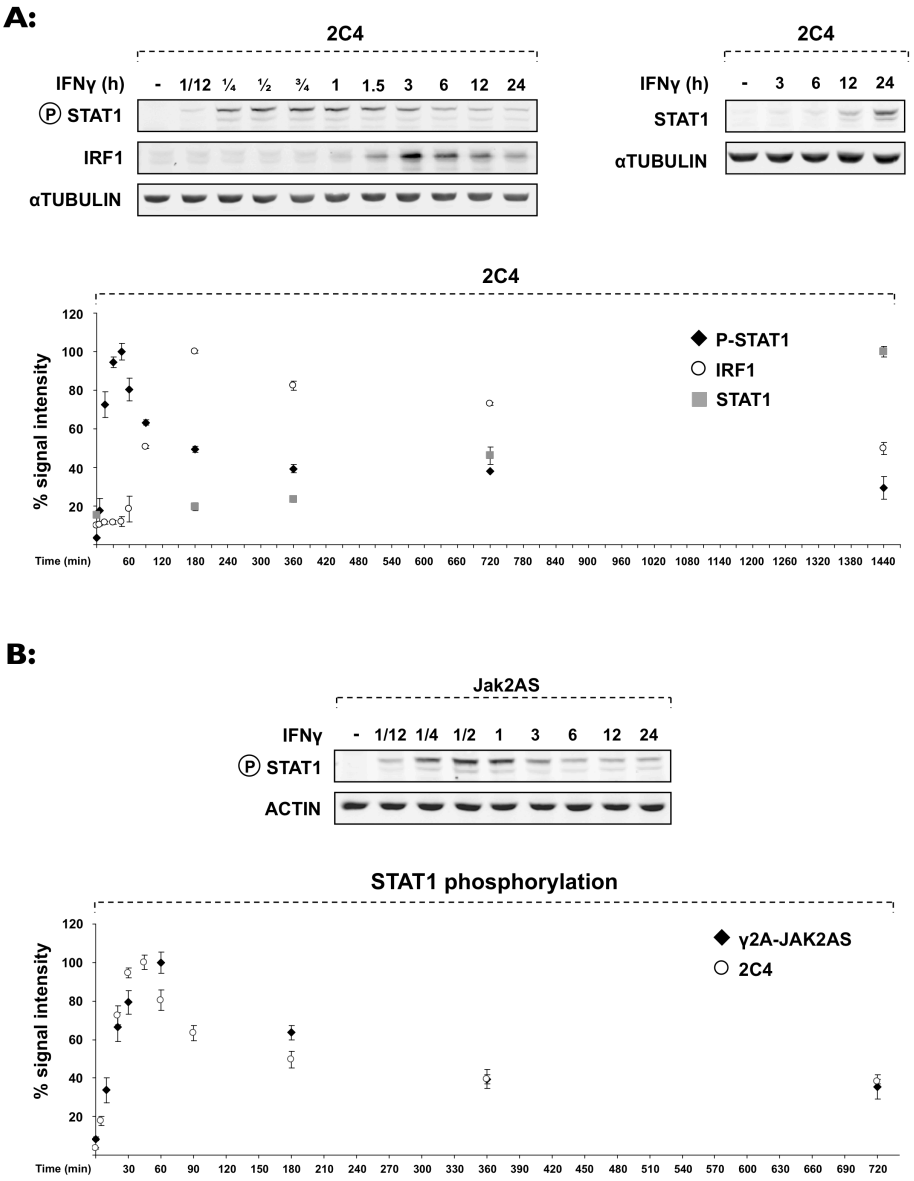
Supplemental figure 3: Selective inhibition of Jak1 or Jak2 results in down-regulation of the expression of the majority of genes regulated by IFNγ.

The scatter plots were generated by plotting normalized Log expression data obtained with U4C-Jak1AS or γ2A-Jak2AS cells treated with IFNγ for 24 h against those found with cells pretreated with 1NM-PP1 for 30 min and then stimulated with IFNγ for 24 h. Each dot in the graph corresponds to the expression value of a particular gene found to be significantly differentially expressed upon IFNγ-treatment in both U4C-Jak1AS cells and γ2A-Jak2AS cells (FDR<0.05). The scatter plots were generated using BRB-ArrayTools developed by Dr. Richard Simon and BRB-ArrayTools Development Team (*http://linus.nci.nih.gov/BRB-ArrayTools.html*).



Supplemental figure 4: Jak1WT and Jak2WT are insensitive to the inhibitor analogue 1NM-PP1.

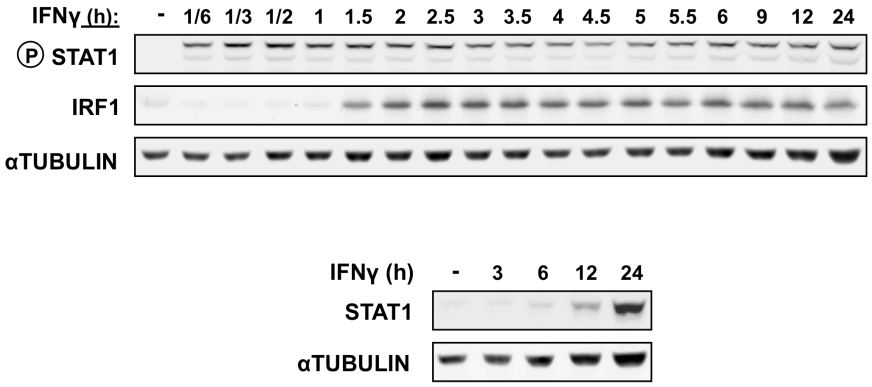
U4C-Jak1WT and γ2A-Jak2WT cells were left untreated or stimulated with IFNγ for 24 h and treated with 1NM-PP1 that was administrated 30 min prior to, simultaneously with or 1 h, 3 h, or 6 h after cytokine stimulation. Lysates were resolved by SDS-PAGE and subjected to Western blot analysis. Expression of IRF1 and STAT1 was detected using specific antibodies.



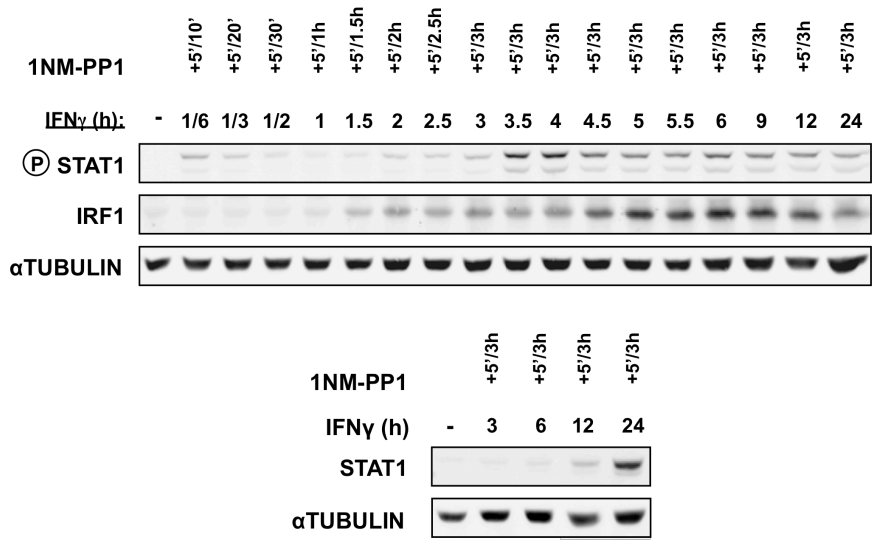
Supplemental figure 5: IFNγ-mediated response in 2C4 and γ2A-Jak2AS cells.

A: 2C4 cells were left untreated or stimulated with IFNγ (10 ng/mL) for the indicated periods of time. Western blots were detected with antibodies against P-STAT1, IRF1 and STAT1. Signals were quantified using α-Tubulin as reference. The scatter plot represents the mean of signal intensities obtained for each collected time point. The highest obtained signals for P-STAT1, IRF1 and STAT1 were set to 100%. The experiment was performed three times and SD values are given. **B:** γ2A-Jak2AS were stimulated or not with IFNγ (10 ng/mL) for the time indicated. Western blots were detected with antibodies against P-STAT1, whose signal was quantified using actin as reference. The scatter plot represent the mean of signal intensities obtained for time points up to 12 h collected in γ2A-Jak2AS cells and 2C4 cells (5A). The highest signal obtained for P-STAT1 was set to 100%.

γ2A-JAK2AS kinetics without inhibitor treatment

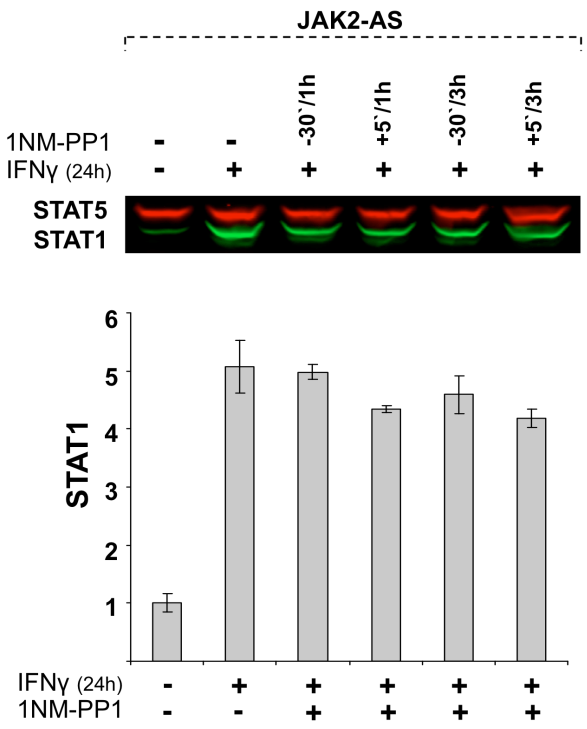


γ2A-JAK2AS kinetics with inhibitor treatment (5 min to 3 hours)



Supplemental figure 6: IFNγ-mediated upregulation of IRF1 and STAT1 is differentially affected by fluctuations in Jak2 kinase activity during early time points of stimulation.

Representative Western blots used for the quantitation in Figure 6 are represented (see also Figure 6 for treatment details).



Supplemental figure 7: The upregulation of STAT1 protein expression is not affected by the lack of Jak2 activity in the early phase of IFNγ response.

γ2A-Jak2AS cells were left untreated or stimulated with IFNγ (10 ng/mL) as indicated. Additionally, cells were treated with the inhibitor analogue 1NM-PP1 administrated for limited intervals beginning 30 minutes prior interferon stimulation to 1or 3 hours post interferon stimulation. Alternatively 1NM-PP1 was administered for the intervals beginning 5 minutes after cytokine stimulation to 1 or 3 hours post interferon stimulation. At the end of these intervals cells were washed with PBS and new medium containing fresh IFNγ (10 ng/mL) was added. Lysates were resolved by SDS-PAGE and subjected to quantitative Western blot analysis. STAT1 signals were quantified using STAT5 as reference. The bar diagram shows the mean of signal intensities where SD values of three independent experiments are given.

Supplemental references

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Supplemental table I

Characteristics of the different gatekeeper mutations in Jak1 and Jak2.

	PY-STAT1 upon IFNγ	Gene induction upon IFNγ	1Na-PP1 sensitive	1NM-PP1 sensitive	Conclusion
Jak1WT	√	√	No	No	
Jak1M955G	√	√	No	√	Functional and analogue-sensitive
Jak1M955A	√	√	No	No	Not suited
Jak2WT	√	√	No	No	
Jak2M929G	reduced	reduced	n.d.	√	Not suited
Jak2M929A	√	√	√	√	Functional and analogue-sensitive

Supplemental table II

The inhibitor analogue 1NM-PP1 reduces the IFNγ-mediated response only in Jak1AS cells.

		JAK1-AS (LogFC)			JAK2-AS (LogFC)		
Systematic Name	Gene	IFNγ	IFNγ + 1NM-PP1	IFNγ + JI1	IFNγ	IFNγ + 1NM-PP1	IFNγ + JI1
A_23_P133133	ALPK1	6.22	4.49	#N/A	3.38	3.64	#N/A
A_23_P32404	ISG20	5.62	4.68	0.81	4.22	3.81	1.46
A_23_P381714	CA13	2.90	1.66	-0.38	2.44	2.08	0.96
A_23_P27556	EMR1	2.12	0.94	#N/A	1.93	1.39	-0.34
A_24_P44462	TPM1	-1.89	-1.06	1.34	-1.61	-2.36	-0.06
A_24_P413126	TMEPAI	-1.87	-0.76	-0.19	-2.90	-3.35	-0.76

Supplemental table III

The inhibitor analogue 1NM-PP1 enhances the IFNγ-mediated response only in Jak1AS cells.

		JAK1-AS (LogFC)			JAK2-AS (LogFC)		
Systematic Name	Gene	IFNγ	IFNγ + 1NM-PP1	IFNγ + JI1	IFNγ	IFNγ + 1NM-PP1	IFNγ + JI1
A_23_P4821	JUNB	3.00	4.27	1.97	2.08	1.65	1.28
A_23_P86653	PRG1	1.88	2.58	0.39	2.14	2.01	0.70
A_23_P93641	AKR1B10	-2.88	-3.61	-0.80	-2.80	-2.12	-1.28
A_24_P940006	EFNB3	-1.47	-2.30	-1.48	-1.69	-1.78	-0.50

Supplemental table IV

The inhibitor analogue 1NM-PP1 reduces the IFNγ-mediated response only in Jak2AS cells.

		JAK2-AS (LogFC)			JAK1-AS (LogFC)		
Systematic Name	Gene	IFNγ	IFNγ + 1NM-PP1	IFNγ + JI1	IFNγ	IFNγ + 1NM-PP1	IFNγ + JI1
A_23_P56630	STAT1	10.55	5.86	2.26	9.07	8.75	2.69
A_23_P207999	PMAIP1	3.95	2.43	1.94	4.57	4.54	2.44
A_23_P34915	ATF3	5.13	2.66	1.49	4.06	3.63	2.62
A_24_P167642	GCH1	3.61	2.60	1.07	3.28	2.90	0.53
A_24_P319635	MCL1	2.93	1.35	1.19	3.16	3.60	1.36
A_23_P121898	PARP8	3.95	0.39	0.69	2.89	2.35	0.84
A_24_P394865	GOLPH2	3.18	2.16	1.04	2.66	2.40	1.18
A_32_P38003	PKR	3.29	1.31	#N/A	2.29	1.93	1.10
A_23_P63668	IFIT5	2.94	1.31	0.56	2.10	1.53	0.09
A_23_P212475	SCOTIN	2.19	1.35	0.10	1.65	1.45	0.62
A_24_P408772	PBEF1	2.64	1.05	1.29	1.64	1.70	1.50
A_23_P321261	ME2	2.29	0.14	0.62	1.56	1.36	1.11
A_23_P170058	PSMB2	2.34	0.31	0.09	1.53	0.95	-0.07
A_23_P57658	HRASLS	-2.19	-1.06	-0.73	-1.63	-1.39	-0.03

Supplemental table V

The inhibitor analogue 1NM-PP1 enhances the IFNγ-mediated response only in Jak2AS cells.

		JAK2-AS (LogFC)			JAK1-AS (LogFC)		
Systematic Name	Gene	IFNγ	IFNγ + 1NM-PP1	IFNγ + JI1	IFNγ	IFNγ + 1NM-PP1	IFNγ + JI1
A_24_P44462	TPM1	-1.61	-2.36	-0.06	-1.89	-1.06	1.34

Supplemental table VI

The inhibitor analogue 1NM-PP1 reduces the IFNγ-mediated response in Jak1AS and Jak2AS cells.

		JAK1-AS (LogFC)			JAK2-AS (LogFC)		
Systematic Name	Gene	IFNγ	IFNγ + 1NM-PP1	IFNγ + JI1	IFNγ	IFNγ + 1NM-PP1	IFNγ + JI1
A_23_P74290	GBP5	23.31	11.18	#N/A	23.39	10.13	#N/A
A_23_P166797	RTP4	19.44	10.56	#N/A	15.54	7.80	0.78
A_32_P107372	GBP1	16.61	10.15	2.77	18.09	7.86	1.30
A_23_P29237	APOL3	13.39	8.02	2.18	12.49	7.01	2.21
A_23_P48513	IFI27	13.36	6.81	#N/A	13.73	7.70	1.24
A_32_P44394	AIM2	13.08	7.32	#N/A	13.46	7.05	1.25
A_23_P75786	SLC15A3	12.68	7.23	#N/A	12.32	5.63	#N/A
A_32_P209960	CIITA	12.15	8.73	#N/A	11.94	8.58	#N/A
A_23_P59005	TAP1	11.73	8.28	1.25	11.58	6.35	1.30
A_23_P41765	IRF1	11.34	7.90	2.27	9.56	4.84	1.10
A_23_P370682	BATF2	11.30	7.61	1.38	11.14	6.22	1.35
A_23_P85693	GBP2	11.26	5.73	-2.18	12.90	5.94	-0.90
A_23_P64173	COP1	11.11	8.59	#N/A	11.46	7.04	#N/A
A_23_P30913	HLA-DPA1	10.96	6.16	#N/A	11.48	6.09	0.80
A_23_P51487	GBP3	10.77	7.26	1.63	8.38	3.92	0.23
A_23_P65651	WARS	10.73	9.04	4.01	9.67	5.49	2.29
A_23_P153745	IFI30	10.52	4.79	-1.78	11.71	4.05	-0.28
A_23_P14174	TNFSF13B	10.49	5.64	#N/A	11.41	5.20	#N/A
A_23_P35412	IFIT3	10.30	5.72	#N/A	12.01	4.32	0.48
A_23_P125107	HLA-B	9.50	6.21	0.91	9.42	5.45	0.60
A_23_P202978	CASP1	9.34	6.31	1.77	9.21	5.92	1.91
A_23_P111000	PSMB9	9.10	6.83	1.59	9.55	5.15	0.60
A_23_P1962	RARRES3	9.04	5.16	#N/A	8.94	4.65	#N/A
A_24_P161018	PARP14	8.79	4.12	-0.18	8.89	2.93	0.45
A_23_P23074	IFI44	8.77	4.61	0.11	11.61	3.45	-0.40
A_23_P75741	UBE2L6	8.51	5.35	1.52	8.32	4.61	0.95
A_23_P145264	HLA-F	8.24	5.71	0.53	8.63	4.83	0.56
A_23_P69383	PARP9	8.06	4.96	1.08	10.75	4.86	0.77
A_24_P941167	APOL6	7.92	5.15	3.85	7.65	4.41	2.01
A_24_P326082	HLA-E	7.89	5.43	0.36	8.86	4.53	0.34
A_23_P314024	HLA-F	7.81	5.26	0.71	7.90	4.49	0.54
A_24_P113674	HLA-B	7.75	5.27	0.64	8.12	4.66	0.52
A_32_P351968	HLA-DMB	7.74	3.95	#N/A	11.85	6.03	#N/A
A_23_P2492	C1S	7.65	4.83	2.10	11.19	6.77	1.68
A_23_P65442	ISGF3G	7.58	4.50	3.66	10.74	6.05	3.29
A_23_P140807	PSMB10	7.46	4.59	-0.72	8.07	4.03	-0.27
A_23_P24004	IFIT2	7.44	5.56	1.04	8.25	4.50	1.46
A_24_P298409	HLA-C	7.40	4.61	0.68	8.35	4.44	0.71
A_32_P234459	HLA-H	7.39	4.73	0.39	7.04	4.22	0.49
A_23_P138680	IL15RA	7.30	4.42	1.55	6.96	4.22	0.95

Supplemental table VI (continued)

		JAK1-AS (LogFC)			JAK2-AS (LogFC)		
Systematic Name	Gene	IFNγ	IFNγ + 1NM-PP1	IFNγ + JI1	IFNγ	IFNγ + 1NM-PP1	IFNγ + JI1
A_23_P126908	TNFRSF14	7.17	4.01	0.27	4.58	1.83	-0.17
A_23_P819	ISG15	7.14	4.96	-0.97	9.21	4.57	0.62
A_23_P18604	LAP3	7.13	3.74	0.11	6.76	0.98	0.00
A_32_P460973	HLA-E	7.12	5.15	0.38	8.31	4.51	0.32
A_23_P203498	TRIM22	7.01	3.77	0.68	7.27	3.07	1.25
A_23_P152782	IFI35	6.98	4.71	0.04	7.73	3.97	0.55
A_23_P47955	OAS3	6.87	3.45	0.92	7.76	2.28	0.13
A_24_P87931	APOL1	6.84	4.22	0.17	6.18	2.69	-0.45
A_23_P72737	IFITM1	6.83	3.25	-0.61	9.00	3.44	-0.42
A_23_P139786	OASL	6.82	1.82	#N/A	7.72	1.15	-1.46
A_23_P211488	APOL2	6.77	3.56	0.64	6.97	1.87	-0.21
A_24_P376483	HLA-A	6.71	4.38	0.37	6.90	3.92	0.59
A_24_P311926	HLA-G	6.59	4.26	0.53	7.35	4.04	0.54
A_32_P132206	USP18	6.43	3.68	0.03	13.21	5.45	1.99
A_23_P350295	HLA-H	6.41	4.37	0.42	6.37	3.59	0.59
A_23_P68155	IFIH1	6.32	3.82	1.12	8.73	4.21	0.70
A_23_P47691	TRIM21	6.29	3.02	0.30	3.84	1.05	-0.58
A_23_P250629	PSMB8	6.17	3.59	0.53	6.32	2.62	-0.27
A_23_P154235	NMI	6.06	4.02	1.87	5.88	2.77	0.75
A_23_P368681	GIMAP2	5.93	3.91	#N/A	5.11	2.87	0.97
A_23_P143713	APOBEC3G	5.91	3.35	0.67	5.16	1.47	0.00
A_23_P201459	IFI6	5.86	2.71	-0.90	11.00	3.51	#N/A
A_23_P36700	TAPBPL	5.72	3.61	#N/A	3.84	2.43	0.14
A_23_P142849	RND3	5.68	3.43	0.75	7.83	4.33	2.09
A_23_P29953	IL15RA	5.57	3.72	-0.19	5.26	2.73	-0.76
A_23_P120002	SP110	5.55	2.04	-0.30	5.84	0.89	-0.62
A_23_P167096	VEGFC	5.43	2.47	-1.19	4.09	2.07	0.61
A_24_P311917	BTN3A3	5.29	2.76	#N/A	5.82	2.42	-0.47
A_23_P39465	BST2	5.24	2.77	0.10	14.14	7.25	#N/A
A_23_P61688	SLC12A7	5.20	2.70	1.59	5.24	2.45	0.74
A_23_P156687	CFB	5.19	1.98	-0.23	7.85	2.37	-0.22
A_24_P48898	APOL2	5.18	2.55	0.24	5.19	1.94	-0.30
A_23_P1461	OPTN	5.07	2.92	1.11	4.77	2.48	0.99
A_23_P37441	B2M	5.04	4.11	0.63	6.58	4.37	1.10
A_23_P111804	PARP12	4.95	3.65	1.34	4.87	3.00	1.05
A_24_P329065	BTN3A1	4.78	3.82	-0.01	5.60	3.29	0.68
A_23_P217866	IFI16	4.76	2.47	1.61	4.64	2.07	1.15
A_24_P207139	PML	4.71	1.62	-1.32	5.92	1.24	-0.78
A_24_P16124	IFITM4P	4.68	1.43	-0.74	5.37	1.10	-0.52
A_23_P21207	UBE1L	4.64	3.37	-0.36	6.03	2.95	0.34

Supplemental table VI (continued)

		JAK1-AS (LogFC)			JAK2-AS (LogFC)		
Systematic Name	Gene	IFNγ	IFNγ + 1NM-PP1	IFNγ + JI1	IFNγ	IFNγ + 1NM-PP1	IFNγ + JI1
A_23_P134176	SOD2	4.51	1.14	-0.92	3.52	-0.10	-0.33
A_23_P47304	CASP5	4.47	2.80	1.05	4.33	3.04	1.71
A_23_P30243	LRAP	4.46	2.88	-0.41	3.85	2.14	0.26
A_23_P35912	CASP4	4.45	3.04	1.26	4.16	3.20	1.57
A_23_P42306	HLA-DMA	4.45	1.97	-1.49	6.86	3.23	-0.18
A_23_P69109	PLSCR1	4.43	2.21	0.43	4.51	1.21	0.12
A_24_P385611	SP100	4.17	2.68	1.39	2.47	0.98	0.53
A_24_P254933	IFITM3	4.12	1.12	-0.85	4.44	0.76	-0.70
A_23_P319792	XRN1	4.00	1.92	0.34	2.93	0.96	-0.26
A_24_P252078	BTN3A2	3.98	3.20	-0.83	4.83	3.19	0.01
A_23_P6935	CD47	3.82	2.53	-0.60	3.83	1.90	0.47
A_23_P136478	IRF2	3.81	1.62	#N/A	2.83	1.38	-0.68
A_24_P49260	SPTLC3	3.79	1.19	#N/A	1.81	-0.05	-0.68
A_23_P69310	CCRL2	3.72	2.29	0.14	3.70	1.79	1.13
A_23_P151610	PSME1	3.71	3.05	0.78	4.63	2.49	0.77
A_23_P142424	TMEM149	3.69	2.51	-1.69	4.51	3.04	-0.08
A_23_P26815	RILP	3.66	1.91	-0.97	1.98	0.80	-0.54
A_24_P116805	STAT3	3.66	2.00	#N/A	3.59	0.30	-0.31
A_23_P350107	TRIM56	3.55	2.25	1.05	2.99	1.15	-0.12
A_23_P142676	DNPEP	3.32	2.41	0.44	3.30	1.88	0.22
A_24_P349117	GPR158	3.30	2.23	1.51	3.58	1.91	0.70
A_23_P76145	CLIF	3.21	0.83	#N/A	2.95	0.51	-0.70
A_23_P355244	SAMD9	3.18	1.89	0.29	3.60	1.63	-0.19
A_23_P31810	CEBPD	3.14	0.57	-0.79	3.53	1.73	0.36
A_23_P75220	SLC25A28	3.14	1.33	-0.07	3.11	0.67	0.18
A_32_P167592	1-8U	3.13	0.49	-1.07	4.20	0.84	-0.50
A_23_P52266	IFIT1	3.05	0.30	-3.28	8.35	1.43	-2.08
A_23_P92073	PARP3	3.03	1.36	0.92	2.70	1.07	0.75
A_23_P12572	CASP7	3.02	1.77	0.24	2.31	1.15	-0.07
A_23_P201097	GUK1	2.91	1.34	-0.75	3.06	1.01	-0.18
A_24_P23034	ZNFX1	2.88	1.66	1.34	3.13	1.10	0.64
A_24_P287043	1-8D	2.81	0.40	-0.91	3.60	0.73	-0.47
A_23_P97064	FBXO6	2.77	1.13	0.16	2.88	1.12	-0.82
A_23_P365719	TAPBP	2.76	1.76	0.35	3.03	1.88	0.36
A_23_P66017	PRRT2	2.75	0.22	-2.65	2.40	-0.65	-2.02
A_23_P62642	CCDC19	2.75	-0.43	-1.06	3.14	-1.69	-1.79
A_23_P129486	SEPX1	2.72	1.19	-1.52	2.00	0.61	-0.81
A_24_P343233	HLA-DRB1	2.70	0.44	#N/A	5.90	3.27	-0.30
A_23_P216655	TRIM14	2.70	1.11	0.33	2.67	0.75	0.45
A_24_P362317	ADAR	2.53	1.74	0.66	3.15	0.93	0.08

Supplemental table VI (continued)

		JAK1-AS (LogFC)			JAK2-AS (LogFC)		
Systematic Name	Gene	IFNγ	IFNγ + 1NM-PP1	IFNγ + JI1	IFNγ	IFNγ + 1NM-PP1	IFNγ + JI1
A_24_P263623	PTGES3	2.45	1.12	0.86	3.68	0.88	0.78
A_23_P212089	NFKBIZ	2.39	1.77	#N/A	3.15	-0.09	0.27
A_23_P88819	MVP	2.39	1.74	-0.40	2.33	1.62	0.13
A_24_P30194	IFIT5	2.37	1.57	0.10	2.97	1.22	0.13
A_23_P135548	DPYD	2.34	0.84	0.13	3.40	1.00	1.20
A_23_P142750	EIF2AK2	2.32	1.21	-0.32	3.19	1.25	-0.16
A_23_P60479	DNAJA1	2.17	1.40	0.09	2.50	0.39	-0.44
A_23_P123343	NUDCD1	2.13	1.19	0.87	1.75	0.58	0.82
A_23_P123672	TDRD7	2.08	0.97	-0.21	2.47	0.84	-0.15
A_23_P140301	PSMA3	2.08	0.77	-0.70	1.96	-0.14	-0.75
A_32_P184916	GNB4	1.92	0.84	0.18	3.01	0.30	0.09
A_23_P12044	TMEM51	1.90	0.98	-0.25	1.95	0.63	0.03
A_23_P212397	LARS2	1.83	1.09	-0.02	1.60	0.30	0.19
A_23_P359245	MET	1.81	0.46	0.84	1.81	0.37	0.27
A_23_P251480	NBN	1.77	0.68	0.41	2.10	0.20	-0.10
A_23_P104046	BPNT1	1.76	-0.10	-2.25	3.03	0.15	-0.73
A_23_P86216	PSMA5	1.63	0.93	0.22	2.04	-0.22	0.30
A_23_P53345	ARNTL2	1.60	0.94	0.36	1.92	0.71	0.63
A_23_P31143	TPD52L1	-3.98	-1.42	0.91	-5.88	-0.12	0.19
A_23_P422212	SLC35F3	-3.80	-0.27	0.53	-4.24	0.82	1.51
A_23_P14083	AMIGO2	-3.00	-1.25	4.01	-2.99	-1.93	1.07
A_23_P30275	PCYOX1L	-2.64	0.14	-0.12	-2.25	0.79	0.87
A_23_P35456	SH3PXD2A	-2.44	0.39	0.17	-1.65	0.20	-0.17
A_23_P388168	RAB3B	-2.23	-1.55	-1.54	-2.17	-0.40	-0.04
A_23_P111995	LOXL2	-2.13	-0.42	-0.13	-1.63	-0.43	0.36
A_23_P97990	HTRA1	-1.96	-0.60	-1.01	-1.74	0.24	-0.03
A_23_P251841	NRXN3	-1.95	-0.30	-1.40	-3.26	0.31	-0.35
A_23_P422911	HS6ST3	-1.87	0.70	-0.79	-2.33	1.88	0.73
A_23_P165840	ODC1	-1.85	0.70	0.52	-3.43	-0.62	0.96
A_23_P120883	HMOX1	-1.45	0.24	3.17	-3.26	0.26	1.55
A_23_P24433	CTSF	-1.45	0.30	0.49	-1.74	1.23	0.87

Supplemental table VII

The inhibitor analogue 1NM-PP1 enhances the IFNγ-mediated response in both Jak1AS and Jak2AS cells.

		JAK1-AS (LogFC)			JAK2-AS (LogFC)		
Systematic Name	Gene	IFNγ	IFNγ + 1NM-PP1	IFNγ + JI1	IFNγ	IFNγ + 1NM-PP1	IFNγ + JI1
A_23_P214080	EGR1	4.14	8.35	2.17	2.60	6.01	2.84
A_23_P60933	MT1G	3.13	5.40	-0.19	2.96	4.95	2.10
A_23_P303242	MT1X	2.96	5.51	0.29	2.70	4.61	2.28
A_23_P414343	MT1H	2.83	5.14	0.19	2.83	4.41	2.28
A_23_P54840	MT1A	2.78	5.36	0.40	2.66	4.52	2.15
A_23_P37983	MT1B	2.76	5.33	-0.15	2.84	4.68	1.91
A_23_P206724	MT1E	2.50	4.94	0.01	2.73	4.64	2.07
A_24_P937405	PRSS23	-3.43	-6.05	-1.68	-2.91	-4.23	-1.55

4.2 *"ERLOTINIB SUPPRESSES GROWTH OF LEUKEMIC CELLS EXPRESSING JAK2-V617F OR JAK3-A572V BY A MECHANISM INVOLVING EIF2α"*

4.2.1 Preamble

Since several gain-of-function mutants of Jaks have been discovered in various hematological malignancies, the interest in developing specific Jak kinase inhibitors has increased. Since almost all kinase inhibitors are ATP-competitive compounds targeting the catalytic cleft of the kinase domain, many inhibitors available on the market show a certain unspecificity. Erlotinib, which was designed to inhibit epidermal growth factor receptor (EGFR) activity, was reported to possibly suppress many other kinases, including the Janus kinases (Fig. 15).

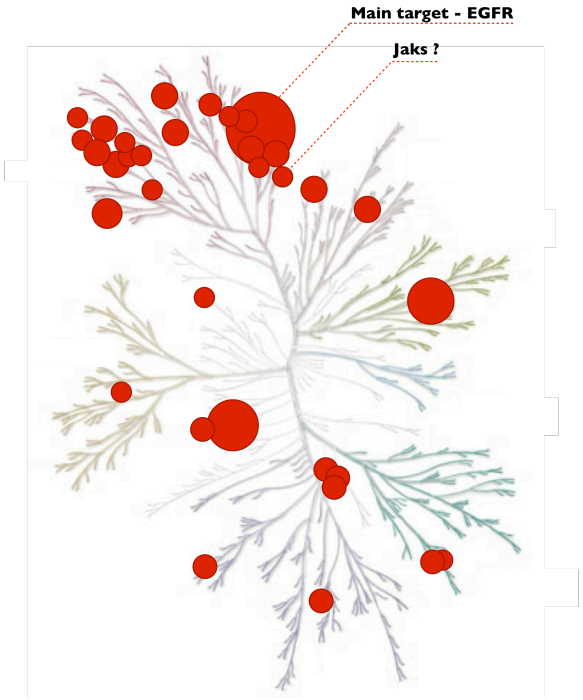

Figure 15

The kinase dendrogram represents possible targets of Erlotinib. Kinases found to bind Erlotinib are marked with red circles, where larger circles represent higher affinity. The interaction protein kinases of Erlotinib were determined by performing an in-vitro competition binding assay with a panel of 317 protein kinases. Adapted from (26).

Hovewer, a relatively high concentration of Erlotinib had to be used in an *in-vitro* kinase assay to obtain an inhibitory effect on Jak2 (192). In contrast to that, Erlotinib effectively suppressed growth of leukemic HEL cells expressing the Jak2-V617F mutant at much lower concentrations. This discrepancy was suggestive of other targets of Erlotinib than Jak2-V617F. Molecular modeling approaches also suggested that Erlotinib binding to Jak2 is rather unlikely, since Jaks have a more spacious gatekeeper residue than the EGFR kinase domain (see Supplementary figure 4 of Kaczor *et al.*, submitted; page 114).

In the present study the activity of Erlotinib was investigated on a panel of leukemic cell lines expressing constitutively active Jak2-V617F or Jak3 mutants (A572V or A573V). Erlotinib-mediated effects on cellular signaling, gene expression, proliferation and apoptosis were compared with those mediated by known Jak inhibitors (TG101348, CP-690550 and JI1). We could show that the Jak inhibitors but not Erlotinib were able to suppress constitutive activity of STAT5 and Erk1/2 phosphorylation. Regardless of that, growth of leukemic cells driven by the constitutively active Jak2 or Jak3 mutants was effectively inhibited by Erlotinib. Since the EGF receptor is not expressed on leukemic cells we tested if Erlotinib treatment had an effect on related kinase targets but failed to identify a possible target. By microarray analysis we determined gene expression profiles, which were quite different for cells treated either with Erlotinib or Jak Inhibitor 1. Interestingly, Erlotinib induced a number of genes involved in the ER stress response. Moreover, all leukemic cell lines and also polycythemia vera patient cells treated with Erlotinib displayed enhanced phosphorylation of eIF2α. Transformed cells are known to react to ER stress and the activation of the eIF2α-ATF4 pathway with growth arrest or apoptosis (193, 194). Since Erlotinib targets a pathway independent of Jak2 we investigated whether the combination of Jak inhibitors with Erlotinib has beneficial effects on growth suppression of leukemic cells. Interestingly, we found that combined treatment of Jak-specific inhibitors together with Erlotinib was much more efficient in suppressing the growth of leukemic cells than either agent alone.

4.2.2 Article

Erlotinib suppresses growth of leukemic cells expressing Jak2-V617F or Jak3-A572V by a mechanism involving eIF2α.

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Running title: Erlotinib suppresses leukemic growth by inactivating eIF2α

Key words: Jak2-V617F, signal transduction, small molecule inhibitors, myeloid leukemia, Erlotinib

SUBMITTED

Abstract

Several hyperactive Janus kinase mutants were recently discovered in myeloproliferative neoplasms and leukemia, which lead to an aberrant signal transduction and growth. These constitutively active kinases are interesting targets for ATP-competitive kinase inhibitors, some of which we set out to further characterize. We analyzed the suppressive effects of Erlotinib and three Jak inhibitors on constitutive activity of Jak2 and Jak3 mutants in leukemic cell lines and cells from patients with polycythemia vera. The properties of these kinase inhibitors were analyzed by monitoring cell growth, apoptosis, protein phosphorylation, and gene expression profiling experiments. Growth of Jak2-V617F-(HEL, UKE-1, SET-2) and Jak3-A572V-positive (CMK) leukemic cells was efficiently inhibited by Janus kinase inhibitors (Jak Inhibitor 1, TG101348 and CP690550) as well as by Erlotinib. However, only Jak inhibitors but not Erlotinib dose-dependently abrogated constitutive STAT5 and Erk1/2 phosphorylation and inhibited STAT- and AP-1-dependent target gene expression. Microarray analysis revealed that Jak Inhibitor 1 and Erlotinib affected the expression of strikingly different sets of genes in HEL cells with only a marginal overlap. Erlotinib was found to affect the eIF2α/ATF4 pathway. By targeting different growth inhibitory mechanisms the combination of a Jak inhibitor and Erlotinib showed an enhanced effect on cell growth suppression and apoptosis induction of leukemic cells.

Introduction

Janus kinases are involved in a variety of biological processes including hematopoiesis and the regulation of the immune system. Upon cytokine-induced receptor aggregation, the cytokine receptor-associated Janus kinases (Jaks) auto-activate and phosphorylate the receptor and various signaling proteins leading to the activation of STATs (signal transducers and activators of transcription), MAPK (mitogen activated protein kinase) and other signaling pathways (1). Since cytokines and Jaks regulate processes like cell growth and survival, it is not surprising that a deregulation of cytokine signaling pathways is often found in cancer.

Mutations in the Janus kinase 2 gene were found with high incidence in patients with myeloproliferative neoplasms (MPNs) (Jak2-V617F and a number of point mutations and deletions in exon 12) (2), in myeloid leukemia (Jak2-T875N) (3), in acute lymphoblastic leukemia (ALL) (Jak2-L611S) (4), and in acute megakaryoblastic leukemia (AMKL) (Jak2-V617F and Jak2-M535I) (5). These constitutively active Jak2 mutants have been described to activate STAT5 and STAT3, MAP kinases and PI3K/AKT. Gain of function mutations of Jak3 (A572V, A573V) were found in ALL and AMKL patients (6, 7) while activating mutations in Jak1 have also been reported for ALL (8, 9).

More and more genetic alterations within Jak genes are discovered in MPN and leukemia. Moreover, Jaks are interesting targets as they are involved in graft rejection and autoimmune disease. Thus, the interest in developing small molecule inhibitors has risen and some compounds are presently tested in clinical trials (10). Compounds like Jak Inhibitor 1, TG101348 and CP690550 were identified to suppress Janus kinase activity (11). Very recently, an epidermal growth factor receptor inhibitor, Erlotinib (Tarceva) approved for the treatment of advanced non–small cell lung cancer (NSCLC) (12) and advanced or metastatic pancreatic cancer (13), was described to potently inhibit growth of Jak2-V617F harbouring cells (14). By *in-vitro* kinase and binding competition assays, Erlotinib was shown to interact with Jak2 and Jak3, respectively (14, 15). The number of compounds that are reported to inhibit growth of cells harbouring Jak mutants is increasing but some of these compounds, including Erlotinib, were not biochemically characterized in depth. Thus we set out to investigate the effects of this inhibitor in greater detail.

In the present study, we explored the inhibitory effects of Erlotinib and several Jak inhibitors, Jak Inhibitor 1, TG101348 and CP690550, on constitutive activity of Jak2 mutants (V617F, K539L, T875N) and Jak3 mutants (A572V, A573V) in leukemic cell lines as well as in cells from polycythemia vera patients. We present evidence that Erlotinib does not directly inhibit Jak-dependent signal transduction although it potently inhibits growth of leukemic cell lines. In contrast to Jak inhibitors, Erlotinib induced phosphorylation of eukaryotic initiation factor 2-alpha (eIF2α). Interestingly, combined treatment of Jak-specific inhibitors together with Erlotinib was

much more efficient in suppressing growth of Jak mutant-positive leukemic cells than either agent alone.

Materials and methods

Information on materials, cell culture, constructs, cell lysis and Western blot as well as microarray analysis is provided as Supplementary Information.

Quantitative PCR

Total RNA from HEL cells was extracted using the NucleoSpin RNA II (Macherey-Nagel) following the manufacturer's instructions. DNA contaminations were removed from RNA preparations using the RNAse-free DNAse Set (Qiagen, Valencia, CA). The concentration of isolated RNA was measured using a NanoDrop ND1000 spectrophotometer. Constant amounts of 1 µg of total RNA were reversely transcribed with the ThermoScript RT-PCR System (Invitrogen) in a volume of 20 µL, using random hexamer primers according to the manufacturer's instructions. The real-time PCR was carried out on a CFX96 Detection System (Bio-Rad) using a total volume of 20 µL containing cDNA corresponding 50 ng RNA template, 10 pmol of each forward and reverse primer and 10 µL of 2x Absolute qPCR SYBR Fluorescein Mix (ThermoScientific). For further information please see the Supplemental information section.

XBP1 mRNA RT-PCR

Total RNA from HEL cells was extracted using the NucleoSpin RNA II (Macherey-Nagel, Düren, Germany) following the manufacturer`s instructions. The concentration of isolated RNA was measured using a NanoDrop ND1000 spectrophotometer. Constant amounts of 1 µg of total RNA were reversely transcribed with the ThermoScript RT-PCR System (Invitrogen) following the manufacturer`s instructions. The PCR was performed using Platinum Taq polymerase under the following conditions: 95°C for 5 min, followed by 35 cycles of 50 sec 95°C, 50 sec 50°C and 30 sec at 72°C. The last step was incubation at 72°C for 10 min. The XBP1 primers (forward: CCTTGTAGTTGAGAACCAGG; reverse: GGGGCTTGGTATATATGTGG) amplify the unspliced XBP1 mRNA resulting in a 442 bp band and the spliced form with a band at 416 bp.

Cell proliferation assay

All experiments were done in triplicate and the results normalized to growth of untreated cells. For the WST-1 assay, $5x10^3$ cells were plated into microtiterplate wells in 100 µL growth medium with the indicated concentrations of inhibitor. The relative growth of cells was quantified after 72 h using the WST-1 Cell Proliferation Kit (Invitrogen) according to the manufacturer's instructions.

Apoptosis assay

For the apoptosis assay, $5x10^5$ cells were grown in 6-well plates with addition of the indicated concentration of inhibitors for 48 h. Cells were harvested and processed as described in the Annexin V-FITC Apoptosis kit (Miltenyi Biotec). FITC and propidiumiodide fluorescences were measured using a BD FACS Canto II (Becton Dickinson). As positive control for apoptosis, Etoposide (50 μM) was used. All experiments were done in triplicate and standard deviations are given.

Isolation of primary cells

For the isolation of primary CD14+ monocytes, primary PBMC were isolated by a Ficoll-Paque PLUS (GE Healthcare) gradient centrifugation according to the provider's protocol. CD14+ cells were purified using CD14 MicroBeads on LS columns and a QuadroMACS Separator (all from Miltenyi Biotec) according to the manufacturer's instructions.
CD14+ cells were then cultured for further experiments in IMDM (Lonza) supplemented with 10% human serum, 100 mg/mL streptomycin, 60 mg/mL penicillin, and 0.05 % L-Gln.

Functional classification of microarray regulated genes

Lists of significantly differentially expressed genes containing gene identifiers and associated Log expression values were submitted to Ingenuity Pathway Analysis (Ingenuity Systems®, www.ingenuity.com) for functional analysis. Each identifier was mapped to its corresponding gene object in the Ingenuity knowledge base. A Fisher's exact test was used to calculate a p-value determining the probability that each biological function is assigned just by chance. Functions with a p-value lower than 0.05 were considered as significantly enriched.

Results

Erlotinib exerts anti-proliferative and slight pro-apoptotic effects on Jak2-V617F- or Jak3-A572V-expressing leukemic cells.

A panel of leukemic cell lines carrying either the Jak2-V617F mutation (UKE-1, SET-2, HEL) or the Jak3-A572V mutation (CMK) were used to evaluate the effect of Erlotinib and known Jak inhibitors such as Jak Inhibitor 1, TG101348 and CP690550 on cell growth and apoptosis. Cells were grown in the presence of increasing concentrations of inhibitors and cell proliferation as well as apoptosis was assessed by WST-1 or Annexin V assays, respectively. The Jak2-V617F-driven growth of the leukemic cell lines (HEL, UKE-1, SET-2) was effectively suppressed by Jak Inhibitor 1 and TG101348, while the Jak3-A572V-positive cell line CMK was potently inhibited by CP690550 (Figure 1A, Supplemental figure 1). The EGFR inhibitor Erlotinib was very effective in inhibiting proliferation of all tested cell lines, however, at slightly higher concentrations than the

Jak inhibitors. Of note, the primary target of Erlotinib, i. e. the EGFR, is not expressed in leukemic cells lines (16). Specific inhibition of Jak2-V617F in HEL cells or Jak3-A572V in CMK by TG101348 or CP690550, respectively, induced modest up-regulation of apoptosis (Figure 1B). In contrast, Erlotinib treatment had almost no effect on cell death induction.

Taken together, the three Jak-specific inhibitors and Erlotinib potently suppress growth of Jak2-V617F- or Jak3-A572V-positive leukemic cells although the concentrations of Erlotinib need to be slightly higher than those of the Jak inhibitors to have similar potency. In comparison, Erlotinib induces less apoptosis than the three Jak inhibitors.

The constitutive signaling mediated by Jak mutants is effectively inhibited by Jak inhibitors but not by Erlotinib.

The constitutively active Jak mutants lead to cytokine-independent activation of various signaling proteins including STATs and Erk1/2 kinases (6, 7, 18). We used leukemic cell lines carrying the Jak2-V617F mutant or one of the Jak3 mutants (A572V or A573V) as well as Jak2-V617F-positive cells from polycythemia vera (PV) patients to monitor the activation of Jak downstream targets. Treatment of HEL cells with Jak Inhibitor 1 led to a dose-dependent reduction of STAT5 and Erk1/2 phosphorylation (Figure 2A). Interestingly, even the highest concentration of Erlotinib (15 μM) had no effect on constitutive phosphorylation of STAT5 and Erk1/2 (Figure 2A), although it suppresses growth of HEL, SET-2 and UKE-1 cells at even lower concentrations. Also in SET-2 and UKE-1 cells, phosphorylation of STAT5 and Erk1/2 was not affected by Erlotinib treatment (Supplemental figure 2A), but was readily abrogated by Jak Inhibitor 1 treatment. It is known that the highest achievable concentration of Erlotinib in blood of treated patients is around 5 μM (17). Thus for most of the following experiments 5 μM of Erlotinib was used. To validate these results on the level of readout genes, we performed quantitative real-time PCR to monitor the expression of known STAT5 target genes (PIM1 or CIS) (19, 20) or the Erk/AP1 target gene MCP1 (21). PIM1, CIS and MCP1 are expressed in all Jak2-V617F-expressing leukemic cell lines (HEL, UKE-1, SET-2) due to the constitutive signaling. Treatment with Jak Inhibitor 1 suppressed the expression of PIM1, CIS and MCP1 mRNA (Figure 2B) while Erlotinib treatment had little effect. Moreover, Erlotinib had no effect on STAT5 activation in polycythemia vera patient-derived cells (Supplemental figure 2B) or in CMK (Jak3-A572V) and CMY (Jak3-A573V) leukemic cells (Figure 2C) while Jak Inhibitor 1 or CP690550 were effectively inhibiting STAT5 phosphorylation.

In line with the results obtained for leukemic cells, Erlotinib was also ineffective in suppressing STAT5 phosphorylation mediated by Jak2WT, Jak2-V617F, Jak2-T875N and Jak2-K539L (Supplemental figure 2C) in the previously described inducible Hek-EPOR cell system (22). Treatment with Jak Inhibitor 1 again led to an inhibition of STAT5 phosphorylation in HEK cells expressing Jak2-WT or the different constitutively active mutants found in myeloid leukemia or MPN. The same was observed for Gö6976, an additional inhibitor with Jak inhibitory activity (29).

Since Erlotinib was described to have inhibitory activity against Jak2WT and Jak2-V617F (14, 16) we also tested it against its primary target, EGFR, to assure that our compound was functional. In A431 human epidermoid carcinoma cells the EGF-dependent activation of EGFR was totally abrogated by 1 µM of Erlotinib (Supplemental figure 2D) showing that our compound had good activity. To furthermore assess the potency of Erlotinib in Jak inhibition we tested it in *in-vitro* kinase assays in comparison to Jak Inhibitor 1. Erlotinib had an IC50 concentration of 3600 nM compared to 1.6 nM for Jak Inhibitor 1 in *in vitro* kinase assays at 13 µM ATP (data not shown). This explains why Erlotinib does not inhibit Jak2 at millimolar ATP concentrations in cells.
Taken together, Erlotinib fails to inhibit Jak2- and Jak3-dependent STAT5 and Erk1/2 phosphorylation while known Jak inhibitors readily do.

Erlotinib affects cellular phosphorylation events in a way distinct from specific Jak inhibitors.

Erlotinib was not able to interfere with Jak-mediated signaling but still inhibited growth of cells driven by the constitutively active Jak2 or Jak3 mutants. Therefore, we further investigated the underlying mechanism. We first compared the phosphorylation pattern of lysates from Jak Inhibitor 1- and Erlotinib-treated HEL cells. Western blots of these lysates were detected using a general phosphotyrosine antibody or a specific phosphotyrosine antibody for the activation loop tyrosines of Jak2. The band patterns of these detections showed that Jak Inhibitor 1 and Erlotinib affected different phosphorylation targets in HEL cells (Figure 3A). Even detection with the Jak2-phosphotyrosine-specific antibody leads to an “unspecific” detection of further tyrosine phosphorylated bands, which might represent targets of Jaks since their epitope may well be sequentially related to the autophosphorylation epitope in the Jak2 activation loop. We then tested Western blots of these lysates with a panel of well characterized phosphospecific antibodies against proteins known to be involved in Jak/STAT signaling and against other targets known to be involved in leukemia. The phosphorylation of the receptor tyrosine kinases PDGFR, cKIT, FLT3 as well as EGFR (which was not expressed) was not regulated by either of the inhibitors (data not shown). Src also was not involved (data not shown). However, we found that Jak Inhibitor 1 also suppresses the phosphorylation of p38 and SHP2 (in addition to the one of STAT5 and Erk1/2) while Erlotinib did not affect the phosphorylation of these proteins (Figure 3B). SHP2 is a phosphatase that can downregulate Jak/STAT signaling and is also known to mediate MAPK activation by adopting an adapter function in Jak/STAT signaling. P38 is also known to be activated cytokine-dependently. This again argues against an involvement of Erlotinib in the inhibition of the oncogenic Jak2-V617F.
Interestingly, a hyperphosphorylation of Jak2 could be observed upon treatment with Jak Inhibitor 1 in Western blots detected with the phospho-Jak2-specific antibody (directed against the activation loop tyrosines) although Jak-dependent signaling was abrogated (Figure 3A, right panel). This was not seen in blots detected with a general phosphotyrosine antibody. The

observation was reproducible in SET-2 cells and macrophages derived from PV patients (Figure 3C). We corroborated the results seen from the lysates by performing immunoprecipitations (IP) and detecting Western blots of those IPs with the Jak-specific phosphotyrosine antibody. Consistent with the previous observations, phosphorylation increased upon Jak inhibitor treatment (Figure 3C, lower panel).

Although we identified proteins involved in mediating Jak2-V617F-dependent signal transduction and further provide evidence for a hyperphosphorylation "priming" of Jak2-V617F by Jak-specific inhibitors, we failed to identify a possible target of Erlotinib with the approaches described above.

Erlotinib induces phosphorylation of eIF2α in cells expressing constitutively active Jak mutants.

To further characterize the differences of Jak Inhibitor 1 (JI1) and Erlotinib treatment, we subjected HEL cells to a whole human genome DNA microarray analysis. In total 128 genes were found to be significantly differentially expressed upon treatment with JI1, expression of 77 genes being downregulated while 51 genes were upregulated (Figure 4A). Erlotinib treatment resulted in the differential expression of 57 genes of which 15 were down-regulated and 42 were up-regulated compared to the untreated control (Figure 4A, Supplemental table 1). Expression of only three genes was affected by both inhibitors. Hierarchical clustering of induced or repressed genes involved in cell growth showed that there was no overlap in the gene expression signatures associated with Jak Inhibitor 1 and Erlotinib (Figure 4B). Altogether, and in line with all previous results, the data suggest that growth inhibition triggered by Jak Inhibitor 1 and Erlotinib occurs through distinct pathways.

Further analysis of the microarray data revealed that a number of the Erlotinib-regulated genes were related to the ER-stress response (23, 24), more precisely to genes associated with the phospho-eIF2α/ATF4 pathway (Table 1). The ER stress response has been described to activate several signaling pathways, i.e. the PERK/eIF2α/ATF4, the IRE1/XBP1, and ATF6 pathways (25). Phosphorylation of eIF2α leads to a general attenuation of protein translation and to enhanced transcription and translation specifically of ATF4-dependent targets. These involve genes regulating cell growth and apoptosis. Since ATF4-dependent genes seemed to be regulated in the array, we investigated whether Erlotinib induced eIF2α phosphorylation in the HEL cells, an initial event leading to activation of this pathway. Erlotinib induced eIF2α phosphorylation dose-dependently, while Jak Inhibitor 1 and the src kinase inhibitor PP2 (used as another unrelated control compound) did not do so (Figure 5A). Again, Jak Inhibitor 1 was the only compound able to suppress STAT5 phosphorylation. ATF4, the crucial transcription factor, regulated transcriptionally (26) and translationally (27) upon eIF2α phosphorylation, was also up-regulated upon Erlotinib treatment as investigated by qPCR (Figure 5B). As positive controls, HEL cells were treated with tunicamycin and thapsigargin, known inducers of ER stress and ATF4. A target gene downstream of eIF2α phosphorylation and ATF4 is CHOP/DDIT3, which has

been shown to contribute to cell growth inhibition upon ER stress induction (28). CHOP mRNA was up-regulated in HEL cells upon Erlotinib treatment but not upon Jak Inhibitor 1 treatment, and up-regulation was evident from 3 hours to 24 hours of treatment (Figure 5C).

We also investigated whether the IRE1/XBP1, another ER stress-regulated pathway, was activated upon Erlotinib treatment. XBP1 mRNA is spliced (XBP1s) upon ER stress induction, to yield the active transcription factor involved in the ER stress response. Only the ER stress inducers tunicamycin and thapsigargin could induce the splicing of XBP1 mRNA, whereas Erlotinib did not (Figure 5D). Thus, Erlotinib does not seem to induce a classical ER stress response, but rather selectively induces the eIF2α/ATF4 pathway by a yet unknown mechanism.

The other Jak2-V617F positive leukemic cells UKE-1 and SET-2 (Supplemental figure 3A), cells derived from PV patients (Supplemental figure 3B) and leukemic CMK cells carrying the Jak3-A572V mutation (Supplemental figure 3C) also responded to Erlotinib treatment by increased eIF2α phosphorylation. In fact, the induction of eIF2α phosphorylation upon Erlotinib could be a general phenomenon in myeloid leukemia cells as we could observe it also in leukemia cells not harbouring constitutively active Jak mutations (data not shown).

Taken together, Erlotinib induces phosphorylation of eIF2α by a mechanism independent of Jak2 activity since the gene induction profile and the phosphoprotein regulation pattern of Erlotinib and Jak Inhibitor 1 are drastically different.

<u>The combination of a Jak inhibitor and Erlotinib leads to greater antiproliferative effects and stronger apoptosis induction than either agent alone.</u>

Since the specific Jak inhibitors (Jak Inhibitor 1, TG101348, and CP690550) and Erlotinib suppress leukemic cell growth while having different targets we hypothesized that the combination of a Jak inhibitor and Erlotinib could have an enhanced effect on leukemic cell growth by targeting different growth inhibitory mechanisms. Jak2-V617F- or Jak3-A572V-positive leukemic cells were treated with Jak inhibitors (JI1, TG101348, and CP690550) or Erlotinib alone or a combination of them (Figure 6). Indeed, when the Jak inhibitor and Erlotinib treatments were combined, greater antiproliferative effects were achieved in HEL, UKE-1, SET-2 and CMK cells (Figure 6A). Importantly, very low doses of each inhibitor could be used to achieve good antiproliferative effects. Interestingly, Erlotinib, which alone does not have a strong potential to stimulate apoptosis in HEL or CMK cells (Figure 1B), enhanced the Jak inhibitor-mediated cell death induction. Combination of Erlotinib and TG101348 or CP690550 in HEL and CMK cells, respectively, was more effective in inducing apoptosis (Figure 6B) than each compound alone.

Discussion

We used the patient-derived Jak2-V617F or Jak3-A572V expressing leukemic cell lines HEL, SET-2, UKE-1 and CMK, respectively, to evaluate the capacity of Jak inhibitors (Jak Inhibitor 1, TG101348, and CP690550) and Erlotinib to influence Jak-dependent signaling and cell growth. The three known Jak inhibitors and Erlotinib effectively suppressed cell growth at low micromolar concentrations. We set out to investigate the effects of Erlotinib, compared to the Jak-specific inhibitors, in detail in the context of constitutively active Janus kinase signaling.

Cells expressing Jak2-V617F or the Jak3 mutants (Jak3-A572V or Jak3-A573V) show constitutive activation of signaling pathways, e.g. constitutive STAT5 and Erk1/2 phosphorylation, which was effectively abrogated by the Jak inhibitor treatment. In contrast, however, Erlotinib did not reduce STAT5 or Erk1/2 phosphorylation up to concentrations of 5 μM (maximal concentration achievable in patients: 6 μM (17)) in any of the Jak2-V617F- or Jak3-mutant expressing cells (Figure 2, Supplemental figure 2A). Erlotinib also failed to inhibit Epo-dependent Jak2-WT signaling or cytokine-independent signaling through other constitutively active Jak2 mutants (Jak2-T875N and Jak2-K539L, Supplemental figure 2C). Consistently, STAT5 signaling was also not affected by Erlotinib in PV patient cells (Supplemental figure 2B). This compound was previously reported to target Jak2-V617F and to inhibit growth of V617F-positive cells (see also Figure 1A and Supplemental figure 1) and PV hematopoietic progenitor cells at low micromolar concentrations (14). However, Erlotinib effects were not characterized in depth in the cellular context. Indeed, the reported IC_{50} concentrations of Erlotinib determined in *in vitro*-kinase assays (4000 nM) and in cell growth assays (2000 nM) rather suggest that the primary target of Erlotinib is not Jak2-V617F (14), as the IC_{50} concentration of an ATP-competitive kinase inhibitor determined in *in vitro*-kinase assays is lower than the concentration determined in assays with intact cells, mainly due to higher intracellular ATP levels (29-31). These observations are also supported by a molecular modeling approach. Using structural data on the Jak2 kinase domain we tried to fit Erlotinib into the binding pocket of Jak2 (32, 33). As shown in Supplemental figure 4, Erlotinib cannot be accommodated into the Jak2 ATP binding pocket due to the bulkier "gatekeeper" residue in Jak2 (Methionine in Jak2; threonine in EGFR). Interestingly, it has been reported that mutation of the EGFR threonine to methionine renders the EGFR insensitive to Erlotinib inhibition (34). In the same line of evidence some formerly described micromolar inhibitors of Jaks which are structurally related to Erlotinib (WHI-P131 (also called Janex1 or Jak3-inhibitor-I) and WHI-P154 (also called Jak3-inhibitor-II) have been recognized to be in fact low nanomolar inhibitors of EGFR (35).

Erlotinib also did not inhibit Jak2-V617F-dependent target gene expression. CIS and PIM1, two genes controlled at the transcriptional level downstream of Jak2-V617F/STAT5, and MCP1,

which is transcriptionally regulated by Jak2-V617F/Erk/AP1, were strongly down-regulated by Jak Inhibitor 1, but not by Erlotinib (Figure 2B).

Surprisingly, and regardless of its inability to inhibit Jak2-dependent signaling, Erlotinib potently inhibited growth of Jak2-V617F-harbouring HEL, SET-2, and UKE-1 cells as well as of Jak3-A572V-carrying CMK cells (Figure 1A, Supplemental figure 1). So far, several reports showed that Erlotinib and another EGFR inhibitor, Gefitinib, induce differentiation and inhibit proliferation of AML and MDS cells (16). It is noteworthy that those cells do not express EGFR, thereby indicating that Erlotinib and Gefitinib function through a previously unrecognized, EGFR-independent mechanism.

Moreover, overall tyrosine phosphorylation of HEL cells was differentially affected by Jak Inhibitor 1 and Erlotinib (Figure 3A) what strongly suggested that the two inhibitors do not have the same target. We tested a number of potential target kinases using phosphospecific antibodies for kinases involved in hematologic disease and for signaling molecules often regulated by these kinases. We failed to find an evidence for the Erlotinib target by this approach but could show that SHP2 and p38 are activated by Jak2-V617F in HEL cells. We also further characterized the hyperphosphorylation observed upon inhibition of Jak2 and showed that it appears in several cell lines. This phenomenon of inhibitor priming of kinases has been described before (36-38) and is thought to occur when the inhibitor binds the kinase in the active conformation. This is the case for the Jaks since solved structures with the inhibitor compounds Jak Inhibitor 1 and CP690550 have shown that Jaks bind these inhibitors in their active conformation (33, 39).

cDNA microarray analysis of HEL cells again suggested that Erlotinib seems to be very different from Jak Inhibitor 1 with regard to its molecular targets. Of the total genes significantly and differentially expressed by JI1 (128 genes) and Erlotinib (57 genes), only 3 genes were common (Figure 4A), and various genes associated with the ER-stress response were regulated by Erlotinib. Erlotinib, in contrast to Jak Inhibitor 1 and CP690550, can induce activation of the eIF2α/ATF4 pathway by induction of eIF2α phosphorylation in Jak2-V617F- or Jak3-A572V-positive leukemic cells as well as in cells from PV patients (Figure 5A, Supplemental figure 3). ATF4 and the ATF4-dependent gene CHOP were up-regulated as shown by qPCR (Figure 5B, 5C). Moreover 7 of the 10 ER stress-related genes found to be up-regulated in the microarray analysis are known ATF4 target genes (Table 1). eIF2α phosphorylation leads to a general transcriptional block and ATF4 induction followed by transcription of ATF4-regulated genes (40-42). ATF4-dependent genes, e. g. CHOP/DDIT3, have been shown to contribute to cell growth inhibition upon ER stress induction (28). To further investigate if ER stress was induced by Erlotinib we checked whether XBP1 mRNA was spliced as described under ER stress conditions in particular as it has been reported that the RNAse activity of IRE-1, the enzyme catalyzing the splicing event of XBP1 mRNA, can be activated by conformational changes by kinase inhibitor binding to the kinase domain directly adjacent to the RNAse catalytic domain (43). However, no

splicing of XBP1 mRNA could be detected (Figure 5D), so that we conclude that Erlotinib does not induce a classical ER stress response, but rather selectively the eIF2α/ATF4 pathway.

We tested whether Erlotinib, a known kinase inhibitor, activates kinases which target eIF2a, notably PERK and PKR. It cannot be excluded that a kinase inhibitor can have an allosteric activating effect on another kinase, different from its primary target. However, investigation of the phosphorylation status of the PERK and PRK kinases upon Erlotinib does not suggest an involvement of these kinases in the phosphorylation of eIF2α (data not shown).

The PERK/eIF2α/ATF4 pathway of ER stress was recently shown to lead to impaired cell proliferation or apoptosis (44). Thus, it is likely that Erlotinib induces growth inhibition of the leukemic cell lines with involvement of the eIF2α phosphorylation-dependent pathway through yet unknown upstream regulators. Since Erlotinib and Jak-specific inhibitors affect cell growth by distinct pathways we set out to investigate whether a combined treatment of these compounds was more efficiently inhibiting growth than either compound alone. Salubrinal, a compound selectively activating the eIF2α/ATF4 pathway (45) was shown to enhance the action of proteasome inhibitors in leukemia cells and multiple myeloma (46, 47). Interestingly, simultaneous administration of a Jak inhibitor (JI1, TG101348, CP690550) and Erlotinib achieved greater anti-proliferative effects in HEL, UKE-1, SET-2 and CMK cells than one agent alone (Figure 6A). It has been shown that the reduced cell growth of HEL cells treated with Jak Inhibitor 1 is mainly due to G_1 cell cycle arrest and only marginal to apoptosis induction (48). Two other Jak inhibitors TG101348 and CP690550 also only moderately induced apoptosis of HEL and CMK cells, respectively, which was further enhanced by co-treatment with Erlotinib (Figure 6B). Erlotinib, used at this low concentration on its own, does not exert strong growth-suppressive or pro-apoptotic effects but it remarkably enhances those mediated by JI1, TG101348 or CP690550. It should be noted that both inhibitors TG101348 and CP690550 are currently in the clinical trials (*clinicaltrials.gov*). These Erlotinib concentrations are in the range of the achievable steady state plasma concentration (between 1 and 6 μM) in patients (17). Taken together, our study suggests that the combination of a Jak-targeting inhibitor together with Erlotinib, both inhibitors used at relatively low concentrations, might be an interesting approach for further clinical studies in the development of more efficient treatment strategies for MPNs or leukemias involving constitutively active Jak mutants.

Figures

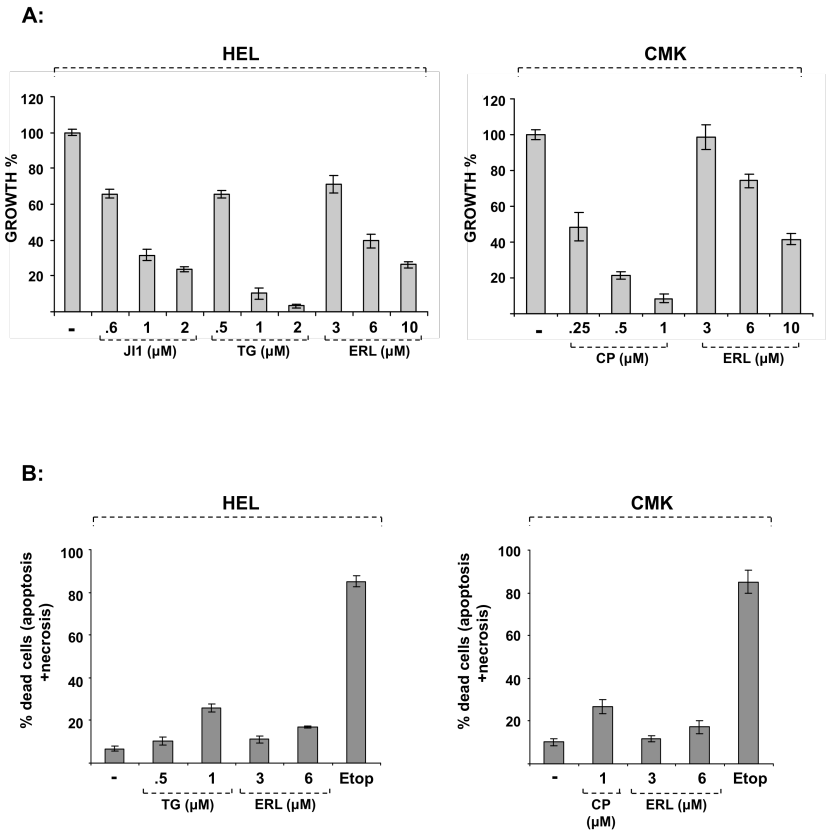

Figure 1: Erlotinib suppresses proliferation of Jak2-V617F-expressing leukemic cells but it does not exert strong pro-apoptotic effects.

(A) WST-1 proliferation assay of HEL and CMK cells incubated for 72 h with the indicated concentrations of Jak Inhibitor 1 (JI1), TG101348 (TG), CP690550 (CP), or Erlotinib (ERL). Experiments were done in triplicate and the results were normalized to the growth of untreated cells, which was set 100%. Standard deviations are indicated. (B) Annexin-V apoptosis assay of HEL and CMK cells incubated for 48 h with the indicated concentrations of TG101348, CP690550 or Erlotinib. Etoposide (50 μM, 48 h) treatment served as positive control for apoptosis induction. The bar diagrams represent the percentages of apoptotic and necrotic cells measured for each condition. The standard deviations of three experiments are given.

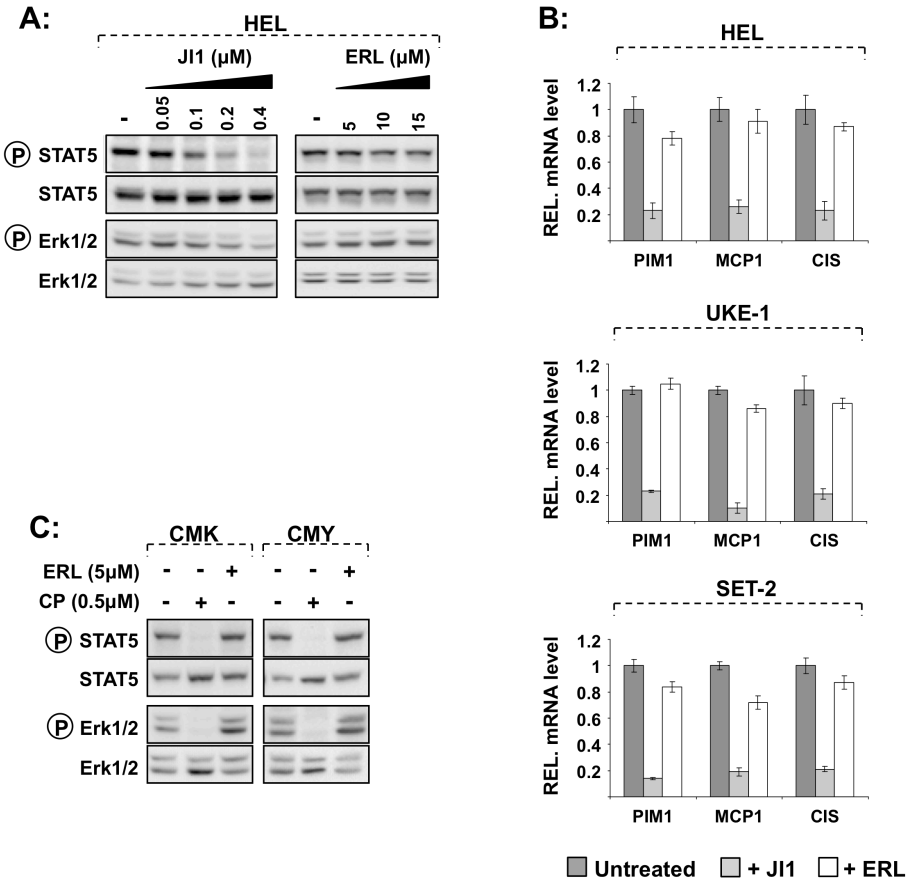

Figure 2: Signaling through the Jak2-V617F and Jak3-A572V mutants is effectively inhibited by Jak inhibitors but not by Erlotinib.

A: Leukemic HEL cells were treated for 3 h with the indicated concentrations of Jak Inhibitor 1 (JI1), or Erlotinib (ERL) or were left untreated. After lysis of the cells, the proteins were resolved by SDS-PAGE and subjected to Western blot analysis. Phosphorylation of STAT5 and Erk1/2 was detected using phospho-specific antibodies. Equal loading was verified by stripping the blot and reprobing it with antibodies directed against the STAT5 and Erk1/2. **B**: HEL, UKE-1 and SET2 cells were treated for 3 h with Jak Inhibitor 1 (1 μM) or Erlotinib (5 μM) or were left untreated. Total RNA was prepared and CIS, PIM1 and MCP1 expression was analyzed by qPCR. The experiment was performed in triplicate and standard deviations are given. **C:** CMK and CMY cells left untreated or treated with CP690550 or Erlotinib for 3h were lysed and analyzed as described in A.

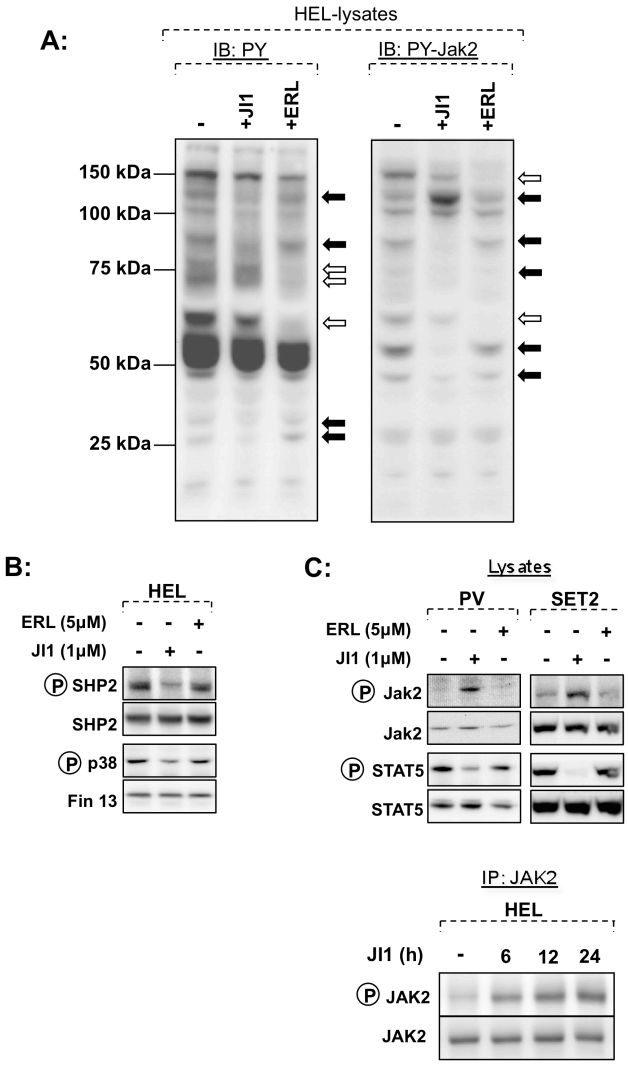

Figure 3: Erlotinib affects overall protein phosphorylation differentially from Jak Inhibitor 1.

A: HEL cells were left untreated or were treated with Jak Inhibitor 1 (1 μM) or Erlotinib (5 μM) for 3 h. After lysis and SDS-PAGE, Western blot was performed and protein phosphorylation was detected using the general phospho-tyrosine specific antibody (PY99) or an antibody recognizing the double tyrosine motif (Y1007/8) in the activation loop of Jak2. The black and white arrows indicate proteins whose phosphorylation was affected by Jak Inhibitor 1 or Erlotinib, respectively. **B:** HEL cells untreated or treated with JI1 (1 μM) or Erlotinib (5 μM) for 3 h were lysed and subjected to Western blot analysis with the indicated antibodies. **C:** *(Upper panel)* Macrophages isolated from PV patient blood and SET-2 cells were treated with JI1 or Erlotinib, lysed and Western blots were detected using phospho-specific antibodies against STAT5 (Y694) or Jak2 (Y1007/8). *(Lower panel)* HEL cells treated with Jak Inhibitor 1 for the indicated periods of time were lysed and Jak2 was immunoprecipitated using a Jak2-specific antibody. Immunoprecipitates were resolved on SDS-PAGE and the Western blot was detected with a P-Jak2 antibody. Equal loading was verified by stripping the blot and reprobing it with an antibody directed against Jak2.

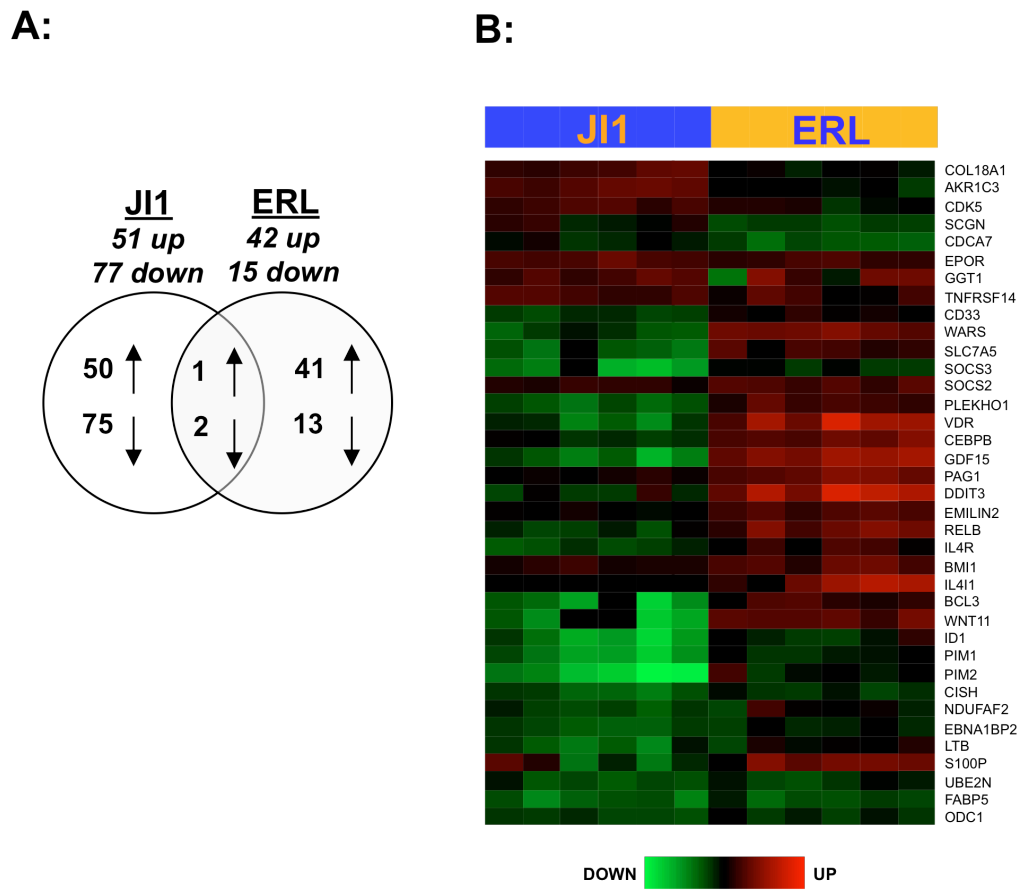

Figure 4: Microarray analysis reveals a minimal overlap of genes regulated after treatment of HEL cells with Jak Inhibitor 1 or Erlotinib.

A: Venn diagram showing the number of genes found significantly differentially expressed upon Jak Inhibitor 1 (JI1) or Erlotinib treatment. HEL cells were treated for 9 hours with JI1 or Erlotinib and the samples were processed as described in materials and methods. **B:** Hierarchical clustering of induced and repressed genes involved in cell growth. Genes, whose expression was enhanced or suppressed by the inhibitor, are indicated by the red or green color, respectively.

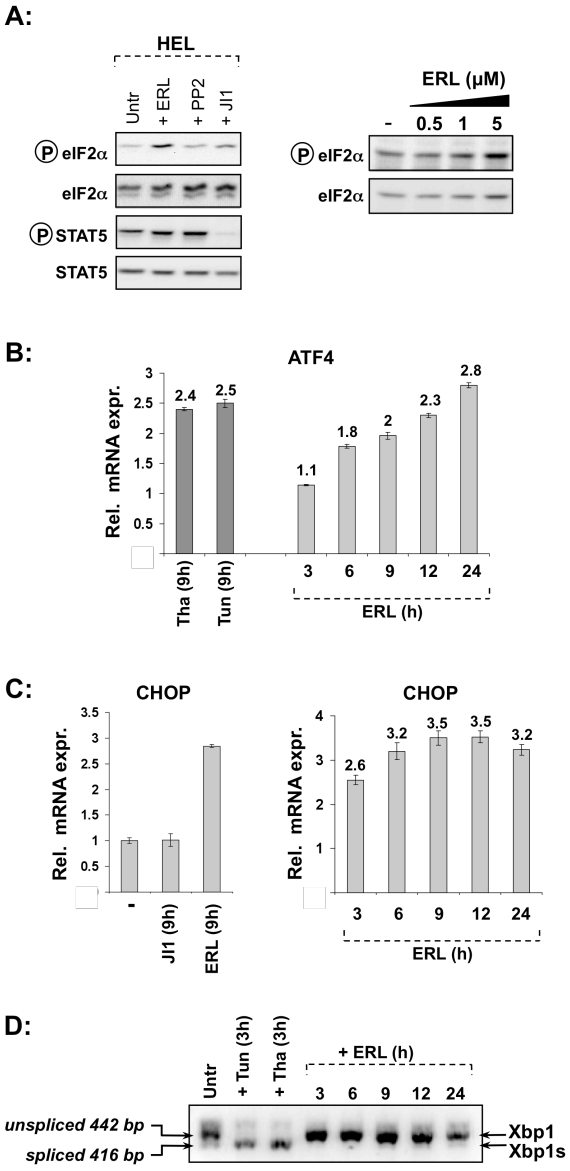

Figure 5: Erlotinib selectively induces activation of the eIF2α/ATF4 pathway

A: HEL cells were incubated for 3 h with the indicated inhibitors: Erlotinib (10 μM left panel, 0.5-5 μM right panel), JI1 (1 μM), PP2 (10 μM). Cells were lysed, resolved by SDS-PAGE and analysed by Western blot. Phosphorylation of eIF2α and STAT5 was detected using phospho-specific antibodies. Equal loading was verified by stripping the blot and reprobing it with antibodies directed against eIF2α and STAT5. **B:** HEL cells were treated with tunicamycin (Tun, 10 μM), thapsigargin (Tha, 1 μM) or Erlotinib (ERL, 5 μM) for the indicated times, RNA was extracted and processed as described in materials and methods. ATF4 and CHOP mRNA levels were investigated by qPCR. **C:** HEL cells were treated with Jak Inhibitor 1 (JI1, 1 μM) or Erlotinib (ERL, 5 μM) for the indicated times, RNA was extracted and CHOP expression was determined by qPCR. **D**: HEL cells were left untreated or treated with tunicamycin (Tun, 10 μM), thapsigargin (Tha, 1 μM), or Erlotinib (ERL, 10 μM) for the indicated periods of time before RNA was isolated. After RT-PCR, the samples were separated on an agarose gel to identify the spliced XBP1 mRNA.

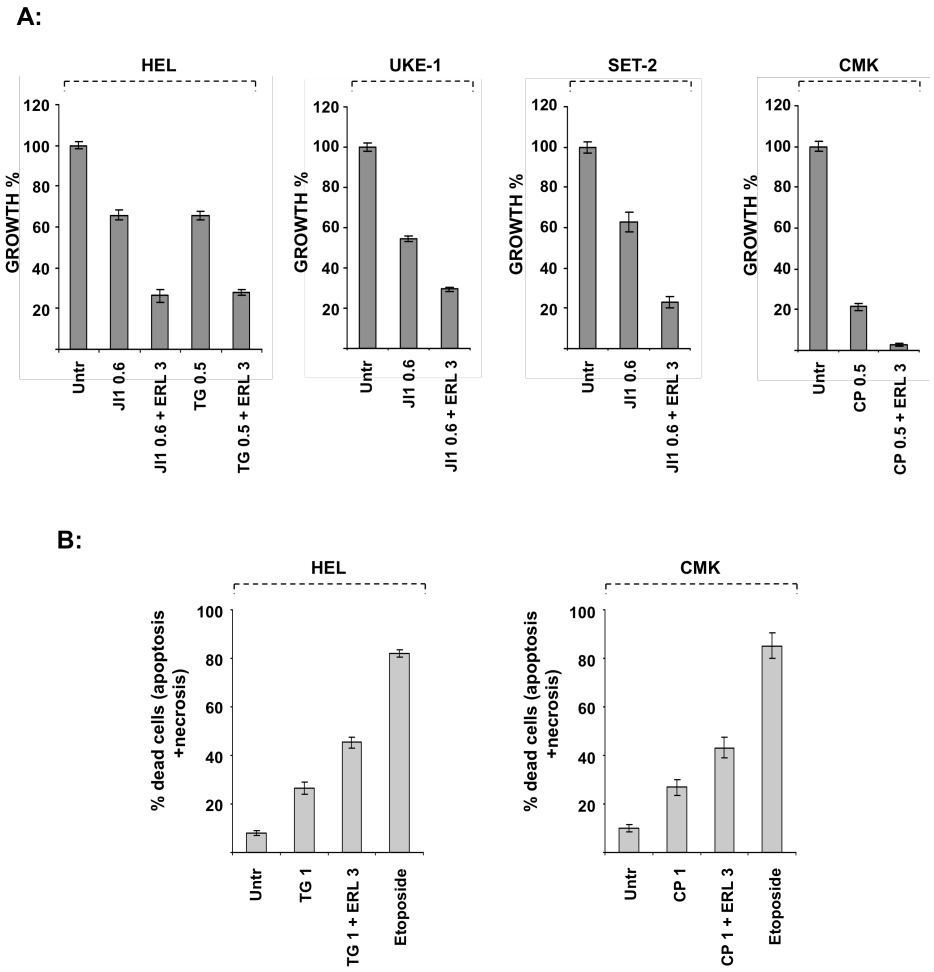

Figure 6: Erlotinib enhances growth-suppressive and pro-apoptotic effects mediated by Jak inhibitors.

A: WST-1 proliferation assay of HEL, UKE-1, SET-2 and CMK cells treated for 72 h with the indicated inhibitors, alone or in combinations: Jak Inhibitor 1 (JI1, 0.6 μM), TG101348 (TG, 0.5 μM), CP690550 (CP, 0.5 μM), Erlotinib (ERL, 3 μM). **B**: Flow cytometric Annexin-V apoptosis assay of HEL and CMK cells treated with TG101348, CP690550 or Erlotinib as indicated. Experiments were performed in triplicates and standard deviations are given.

Table 1: Analysis of the microarray data revealed that a number of the Erlotinib-regulated genes were related to the ER-stress response.

GENE	logFC	INFORMATION	LIT.
CHOP/DDIT3	4.141	ATF4-dependent, growth inhibition or apoptosis induction upon ER stress.	(49) (26)
RGS16	3.969	Induced upon UPR.	(50)
TRIB3	3.041	ATF4-dependent, negative feedback regulation of ATF4 pathway.	(49) (26)
DDIT4	2.986	ATF4-dependent, modulator of the mTOR pathway.	(49) (51)
WARS	2.293	Tryptophanyl-tRNA synthetase, regulated by ER-stress.	(49)
CEBPγ	2.183	ATF4-dependent, transcriptional regulation upon ER stress response	(49) (26)
S100P	2.032	ATF4-dependent.	(52)
SEC31B	2.01	Component of the COPII compex, essential for ER-Golgi transport.	(53) (54)
CEBPβ	1.954	ATF4-dependent, transcriptional regulation upon ER stress response	(49) (26) (51)
PPP1R15A	1.733	ATF4-dependent, regulation of PP1.	(49) (26)

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Supplements:

Supplemental material and methods

Cell culture and materials

HEL cells, SET-2, CMK, A431 cells (all from DSMZ, Braunschweig, Germany), and CMY cells (provided by Dr. Miura and Dr. Sato, Chiba University, Japan) were grown in RPMI 1640 medium. HEK293-Flp-In cells (Invitrogen, Carlsbad, CA) expressing EpoR were grown in Dulbecco´s modified Eagle´s medium supplemented with 1 µg/mL puromycin. UKE-1 cells (a kind gift of Dr. Fiedler, UKE Hamburg, Germany) were grown in IMDM medium. All media were supplemented with 10% or 20% fetal calf serum, 100 mg/L streptomycin, and 60 mg/L penicillin. Additionally, the medium for SET-2 and UKE-1 was supplemented with cytokine cocktails containing IL-3/IL-6/GM-CSF/SCF or IL-3/GM-CSF, respectively. Cells were grown at 37°C in a water-saturated atmosphere at 5% CO_2. The HEK293-Flp-In-EpoR stable transfectants expressing Jak2 and mutants thereof were described before (1). Epo (erythropoietin) and EGF (epidermal growth factor) were obtained from Peprotech (Rocky Hill, NJ). Jak Inhibitor 1, Tunicamycin and Thapsigargin were obtained from Calbiochem (Gibbstown, NJ), CP690550 was from Selleck Chemicals LLC (Houston, TX), Erlotinib and TG101348 were from Symansis (Washdyke, New Zealand) and Gö6976 was from Sigma (St. Louis, MO). Doxycycline (Sigma) was used at a concentration of 1 ng/mL.

Constructs

The EpoR expression construct was a kind gift of Dr. Stefan N. Constantinescu (Brussels, Belgium). Standard cloning procedures were performed throughout this study. The mutations of Jak2 resulting in amino acid substitutions K539L, V617F and T875N were described before (1).

Cell lysis, immunoprecipitation and Western blot analysis

Cell lysis, immunoprecipitation and Western blotting were performed as described previously (2). The anti-STAT5 and anti-SH-PTP2 (SHP-2) antibodies were from Santa Cruz Biotech (Santa Cruz, CA). Antibodies directed against phospho-Jak2, Jak2, phospho-SHP2, phospho-p38, p38, phospho-Erk1/2, Erk1/2, phospho-eIF2α, eIF2α and phospho-EGFR were purchased from Cell Signalling (Danvers, MA). Anti-FIN13 and anti-phospho-STAT5 antibodies were from Transduction Laboratories (Franklin Lakes, NJ). The horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signalling. Signals were detected using a self-made ECL solution described previously (3).

Quantitative PCR

The following experimental protocol was used: denaturation program (95ºC for 3 min), amplification and quantification program (95ºC for 10 sec, 60ºC for 30 sec), repeated 40 times. Quantitative PCR results were normalized to TBP (TATA-binding protein). Relative -fold changes were calculated by the comparative threshold cycles (C_T) method, $2^{-\Delta\Delta CT}$. The specificity of qPCR products was documented by melting curve analysis which resulted in single product-specific melting temperatures. The melting curve analysis was carried out as follows: 95ºC for 1 min, 60ºC for 1 min and 60ºC for 30 sec followed by steps of increases of 0.5ºC for 30 sec until 95ºC was reached. DNA contaminations were removed from RNA preparations using the RNAse-free DNAse Set (Qiagen). All investigated samples were transcribed in parallel. The plate was covered by microseal film (BioRad), centrifuged and placed into the real-time PCR detection system.

Microarray analysis

Gene expression profiling experiments were performed using Agilent 44K whole human genome microarrays (Agilent Biotechnologies, Diegem, Belgium) according to the two-color gene expression analysis (Quick Amp labeling) protocol (version 5.7) from the manufacturer. Hybridizations were performed by comparing 300 ng of total RNA extracted from HEL cells treated with either 5 μM of JI1 or 10 μM of Erlotinib with that obtained from untreated cells. For each treatment, three biological replicates were analyzed in duplicate including a dye swap. Only high quality RNAs with a ribosomal RNA ratio greater than 1.9 and no evidence of degradation, as evaluated using the Agilent Bioanalyzer 2100 RNA 6000 nano assay, were used in this study. Microarray images were quantified using the GenePix Pro 6.1 software (Molecular Devices, Sunnyvale, CA). Background level was detected using the morphological method “closing followed by openings”. GenePix flagging, able to filter out low-quality spots, was optimized to reduce the number of false negative and false positive spots in the analysis. The following GenePix script was used to determine the good quality spots.

[Dia.] >= 45 and
([F532 CV] + [F635 CV])/2 < 80 and
([SNR 532] + [SNR 635])/2 > 4 and
[F635 % Sat.] < 25 and
[F532 % Sat.] < 25 and
([% > B635+2SD] > 60 or [% > B532+2SD] > 60) and
[Flags] <> [Bad]

Information was imported from GPR files, processed and further analyzed by R/Bioconductor tools. First, various normalization methods were applied to remove experimental bias in the dataset. Spatial effects were removed using two-dimensional approximation of zero log-ratio level

by radial basis functions. The dye-effect was removed by a Lowess normalization with the smoothing parameter *f*=0.67 (4). Finally, between-array normalization was performed by median-based centering and scaling of log-ratio distributions. The quality of microarray was controlled by distributions of log-ratio values of the good-flagged spots, total number of good-flagged spots per array, average correlation with other arrays and spatial homogeneity. Replicate spots were summarized and additionally filtered before the statistical analysis: only spots with an intensity significantly higher than the background level were considered. To find genes with statistically significant regulation in each class (ERL, JI1) and between the two classes, the empirical Bayes method (5), implemented in Bioconductor's limma package was used. Genes with adjusted p-values less than 0.05 are considered as significantly regulated. Microarray raw data were deposited in the ArrayExpress public repository with the reference number E-MEXP-2244 (Username: Reviewer_E-MEXP-2244; Password: 1247176897450).

Supplemental results

Erlotinib does not inhibit signaling elicited by Jak2-WT or by the constitutively active mutants Jak2-V617F, -T875N, and -K539L.

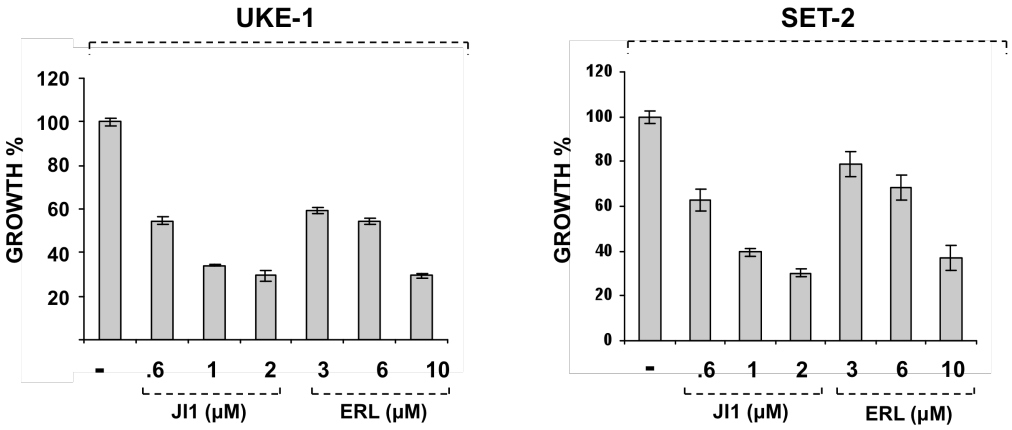
To investigate the effect of Erlotinib on other constitutively active Jak2 mutants (-T875N and -K539L) and on Jak2-WT, we generated stable transfectants inducibly expressing Jak2-WT, -V617F, -T875N and -K539L using the HEK Flp-In-EpoR cell line (1). In these cells the gene of interest can be inserted into a Flp recombinase target (FRT) site so that the different mutants are isogenically expressed from the same site in the genome. Induction by doxycycline (Dox) enables the expression of controlled levels of Jaks. Expression of Jak2-WT, -V617F, -T875N, or -K539L was induced with doxycycline for 12 hours (Supplemental figure 2C). The expression of the Jak2 mutants elicited constitutive signaling as demonstrated by the phosphorylation of STAT5. In contrast, expression of Jak2-WT did not lead to constitutive signaling events but the cells responded to Epo stimulation with STAT5 phosphorylation. In line with the results obtained for leukemic cells and PV patient cells (Figure 2, Supplemental figures 2A and 2B), treatment with Jak Inhibitor 1 and Gö6976 (6) led to an inhibition of STAT5 phosphorylation in HEK cells expressing Jak2-WT or the three constitutively active mutants while Erlotinib was ineffective.

The gatekeeper residue M929 may preclude binding of Erlotinib to the catalytic cleft of Jak2.

Analysis of the EGFR residues involved in Erlotinib binding reveals a very high conservation of these residues in Jak2 (Supplemental table 2). This strongly suggests that the binding mode of the quinazoline group of the inhibitor would be identical in Jak2 (Supplemental figure 4). However, the presence of a methionine residue in Jak2 (instead of the smaller threonine T790 in the EGFR) at the "gatekeeper" position of the kinase leads to a sterical clash with the

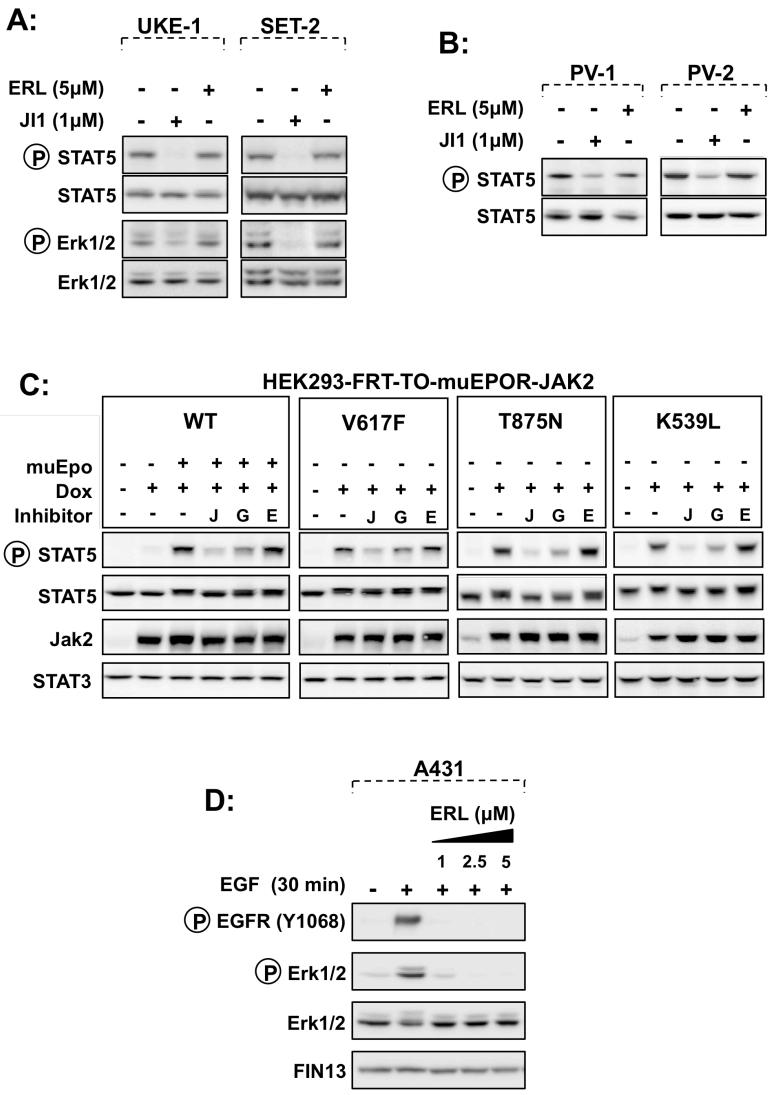
ethynylphenyl group of the inhibitor. Most interestingly, a cancer-associated mutation in the EGFR, T790 to methionine, was reported to cause resistance to both Erlotinib and Gefitinib (7). A solved crystal structure of this EGFR mutant T790M with a quinazoline-based inhibitor shows that the methionine can adopt an alternative conformation and still allows the binding of the inhibitor (8). It was proposed that resistance of the EGFR-T790M mutant to Erlotinib and Gefitinib is rather due to increased affinity for ATP than a sterical clash with the inhibitor. In the crystal structure of Jak2, however, the methionine residue M929 cannot adopt a conformation similar to M790 in the mutated EGFR and thereby evade a clash with the inhibitor. The comparison of the solved crystal structures thus suggests that the larger methionine residue M929 may not allow the accommodation of Erlotinib in the catalytic cleft and supports our biochemical data.

Supplemental figures



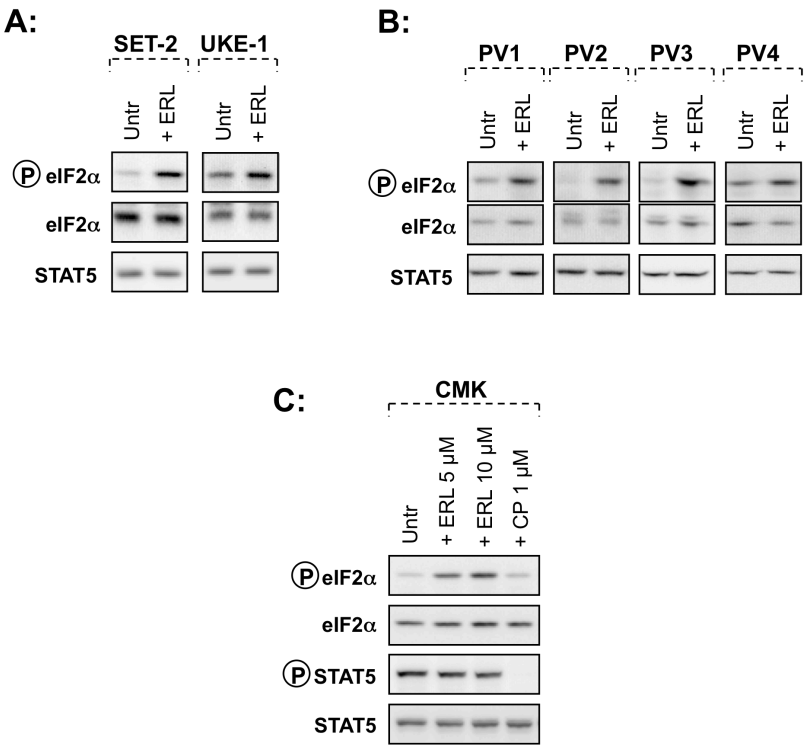
Supplemental figure 1: Erlotinib suppresses growth of Jak2-V617F-driven leukemic cells UKE-1 and SET-2.

WST-1 proliferation assay of UKE-1 and SET-2 treated for 72 h with the indicated concentrations of Jak Inhibitor 1 and Erlotinib. The results were normalized to the growth of untreated cells and standard deviations of three experiments are indicated.



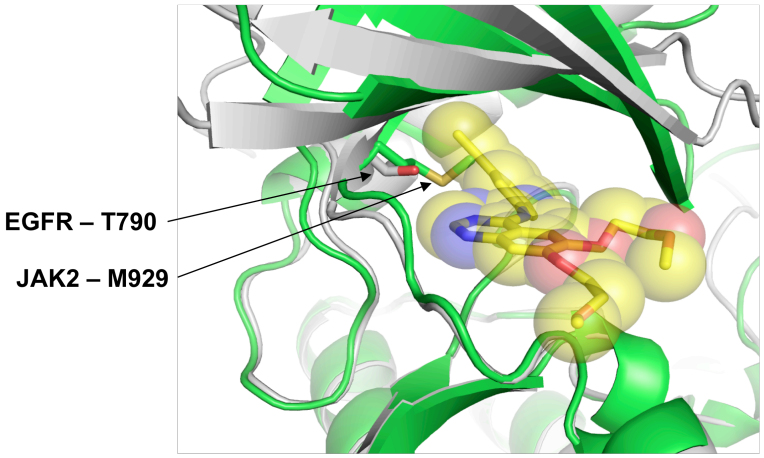
Supplemental figure 2: In contrast to Jak2 signaling, Erlotinib potently inhibits EGF-mediated activation of EGFR and its downstream target Erk1/2.

A: UKE-1 and SET-2 cells were left untreated or were incubated with the indicated concentrations of Jak Inhibitor 1 (JI1) or Erlotinib (ERL). Cells were lysed and subjected to Western blot analysis. Phosphorylation of STAT5 and Erk1/2 was detected using phospho-specific antibodies. Equal loading was verified by stripping the blot and reprobing it with antibodies directed against STAT5 and Erk1/2. **B:** Macrophages isolated from PV patient blood were left untreated or treated with 5 μM of Erlotinib or 1 μM of Jak Inhibitor 1 for 3 h. Cells were lysed and analyzed as in A. **C:** Stable, inducible HEK-Jak2-WT, -V617F, -T875N, and K539L cells were treated with 1 ng/mL doxycycline for 12 hours or were left untreated (-). The cells were treated with 10 ng/mL Epo, with Jak Inhibitor 1 (J, 1 μM), Gö6976 (G, 5 μM) or Erlotinib (E, 15 μM) as indicated. The cells were lysed, lysates were resolved by SDS-PAGE and subjected to Western blot analysis with the indicated antibodies. **D:** A431 epidermoid cancer cells were pretreated for 30 min with the indicated concentrations of Erlotinib before EGF was added (50 ng/mL). Western blots of lysates were detected with antibodies directed against P-EGFR, P-Erk1/2, Erk1/2, and FIN13.



Supplemental figure 3: Erlotinib induces eIF2α phosphorylation.

A: SET-2 and UKE-1 cells were left untreated or were incubated for 3 h with 5μM of Erlotinib. Cells were lysed, resolved by SDS-PAGE and analyzed by Western blot. Phosphorylation of eIF2α was detected using phospho-specific antibodies. Equal loading was verified by stripping the blot and reprobing it with antibodies directed against eIF2α and STAT5 **B:** Macrophages isolated from PV patient blood were left untreated or treated with 5μM of Erlotinib for 3 h. Cells were lysed and analyzed as in A. **C:** CMK cells were incubated with the indicated concentrations of Erlotinib (ERL) or CP690550 (CP) for 3 h. Cells were lysed and analyzed by Western blot analysis. Phosphorylation of STAT5 and eIF2α was detected using phospho-specific antibodies.



Supplemental figure 4: Structural aspects of Erlotinib binding to the EGFR and to Jak2.

The figure shows an overlay of the solved crystal structures of the EGFR/Erlotinib complex (highlighted in grey) ((9), PDB entry code 2ITY) and the solved structure of the Jak2 kinase domain (highlighted in green) ((10), PDB entry code 2B7A). The gatekeeper residues T790 of the EGFR and M929 of Jak2 are represented as rod models. The inhibitor Erlotinib is represented as rod model and van der Waals radii are shown.

Supplemental tables

Supplemental table 1: Erlotinib treatment resulted in the differential expression of 57 genes of which 42 were upregulated and 15 were downregulated compared to the untreated control.

SystematicName	Gene	logFC
NM_002928	RGS16	4.686
NM_004083	DDIT3	4.141
NM_002928	RGS16	3.969
NM_001017535	VDR	3.595
NM_172374	IL4I1	3.195
NM_004864	GDF15	3.166
NM_021158	TRIB3	3.041
NM_019058	DDIT4	2.986
AF011794	CPR8	2.389
NM_004184	WARS	2.293
NM_018420	SLC22A15	2.266
NM_001806	CEBPG	2.183
NM_018440	PAG1	2.154
NM_052966	FAM129A	2.139
NM_006509	RELB	2.043
NM_005980	S100P	2.032
NM_003204	NFE2L1	2.008
NM_015490	SEC31B	2.004
NM_173528	C15orf26	1.992
NM_005194	CEBPB	1.954
NM_178120	DLX1	1.949
NM_002079	GOT1	1.941
NM_145032	FBXL13	1.919
NM_145056	DACT3	1.861
NM_021642	FCGR2A	1.816
NM_080593	HIST1H2BK	1.776
NM_024523	GCC1	1.774
NM_017622	C17orf59	1.766
AL833530	AL833530	1.745
NM_014330	PPP1R15A	1.733
NM_004626	WNT11	1.722
NM_058172	ANTXR2	1.625
CR625561	CR625561	1.607
L13689	BMI1	1.590
ENST00000360896	CR614522	1.480
NM_004793	LONP1	1.439
NM_003877	SOCS2	1.401
NM_032048	EMILIN2	1.392
NM_018370	DRAM	1.367
NM_032947	MST150	1.360
CR609843	CR609843	1.343
NM_007240	DUSP12	1.320

SystematicName	Gene	logFC
XM_931256	CR1L	-2.197
NM_001034	RRM2	-2.091
NM_006047	RBM12; CPNE1	-2.081
NM_006597	HSPA8	-1.931
NM_006597	HSPA8	-1.839
NM_031942	CDCA7	-1.837
NM_006597	HSPA8	-1.786
ENST00000284676	C2orf77	-1.777
CR595668	CR595668	-1.761
NM_024094	DSCC1	-1.691
NM_031844	HNRNPU	-1.657
A_23_P21907	PGDS	-1.509
ENST00000338146	SPRYD4	-1.414
NM_018048	MAGOHB	-1.308
NM_006998	SCGN	-1.307

Supplemental table 2: EGFR residues that contact the 4-anilinoquinazoline-based inhibitors Erlotinib and Gefitinib and corresponding residues in Jak2.

Inhibitor region	EGFR residue*	Contact	Jak2 residue
ethynylphenyl-group	L788	hydrophobic	L927
	T790	hydrophobic	**M929**
	K745	hydrophobic	K882
	V726	hydrophobic	V863
	L844	hydrophobic	L983
	M766	hydrophobic	L902
quinazoline group	A743	hydrophobic	A880
	M793	hydrogen bond with mainchain amide group	L932
	L844	hydrophobic	L983
	L718	hydrophobic	L855
	V726	hydrophobic	V863
	L792	hydrophobic	Y931
	G796	hydrophobic	G935

*based on the crystal structures by Yun et al. (9), Stamos et al. (11), and Lucet et al. (10) (Pdb entry codes 1m17, 2ITY and 2B7A).

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4.3 "SOCS-MEDIATED DOWN-REGULATION OF MUTANT JAK2 (V617F, T875N AND K539L) COUNTERACTS CYTOKINE-INDEPENDENT SIGNALING"

4.3.1 Preamble

In 2005, an aquired gain-of-function mutation, V617F, has been found in the gene encoding Jak2. This activating mutation is believed to be a major event contributing to the pathogenesis of myeloproliferative neoplasms such as polycythemia vera, essential thrombocythemia and chronic idiopathic myelofibrosis. Shortly after the discovery of the Jak2-V617F mutants in MPNs, other gain-of-function mutations in Jak2 were found in patients suffering from different kind of leukemias. Due to that, the interest in understanding the molecular mechanism of action of the constitutively active Jak mutants and their inhibition has risen.

SOCS proteins are known to be strong negative regulators of Jak-mediated signaling. To date, no clear picture exists concerning the negative regulation of Jak mutants by the SOCS family proteins. It has been reported that Jak2-V617F cannot be regulated by SOCS1, SOCS2, or SOCS3. Moreover, SOCS3 was found to potentiate and modulate the activity of the Jak2-V617F mutant (195). On the other hand, elevated levels of SOCS1 and SOCS3 mRNA have been reported in patient cells expressing Jak2-V617F (196, 197) and these proteins are well known to down-regulate Jak activity and mediate their degradation (135, 198, 199). Interestingly, epigenetic silencing of SOCS1 and SOCS3 was found in around 40% of patients with Ph-negative chronic myeloid disorders (200).

To study different constitutively active Jak mutants in the same system; we generated the Hek Flp-In-293 cell line stably expressing either Jak2-WT, Jak2-V617F, Jak2-T875N or Jak2-K539L. Each Jak protein was inserted into a FRT (FLP recombinase target) site and its expression was not constitutive but stayed under the control of the Tet repressor (tetR) protein (Fig. 16). The tetR protein, upon binding to doxycycline (Dox), dissociates from the DNA what enables the expression of the inserted Jak gene. Importantly, levels of Jaks can be controlled depending on the Dox concentration used. We reveal that due to their constitutive activity the Jak2 mutant proteins (K539L, V617F, T875N) have a

decreased stability and their levels gradually decrease over time. Furthermore, they show increased ubiquitination and are efficiently targeted for degradation in the proteasome. We could show that SOCS1 and SOCS3 proteins are strongly induced in cells expressing the Jak2 mutants. Moreover, the SOCS3 mRNA level shows kinetics that parallels Jak2-V617F protein expression and the SOCS3 protein was found to bind Jak2-V617F. Importantly, treatment of cells expressing Jak2-V617F with SOCS3 or SOCS1 siRNA increased the Jak2 mutant protein level. We show that there is a certain threshold of the expression level of Jak2-V617F at which full cytokine-independent activation can occur despite a functioning SOCS-dependent negative feedback. Furthermore, increasing levels of the constitutively active Jak2 mutant lead to enhancement of STAT5 activation. This is in line with the observation, that the level of the Jak2-V617F protein seems to correlate with the disease severity (201).

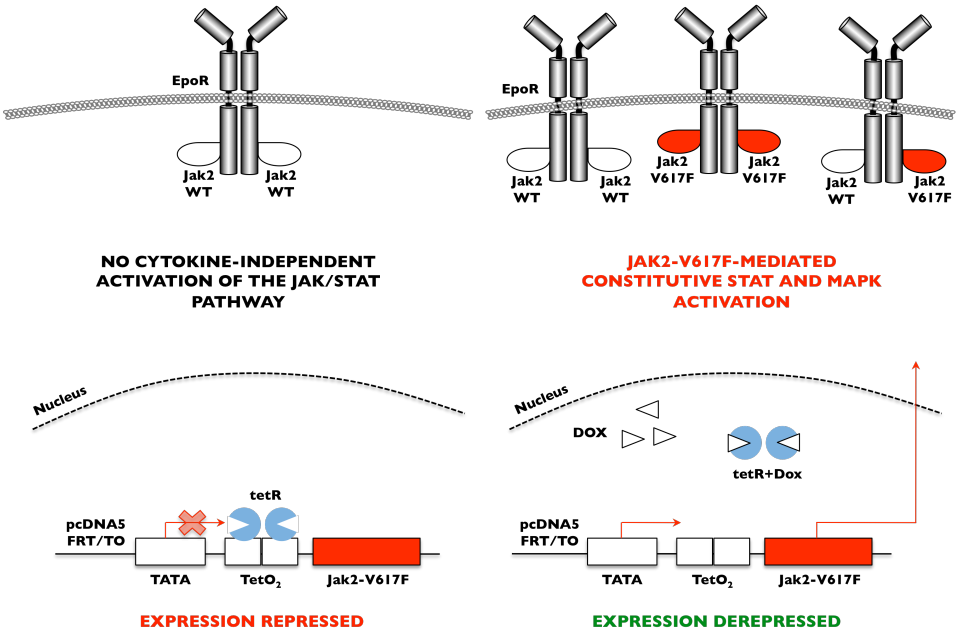

Figure 16

The figure illustrates the mechanism of doxycycline-regulated repression and derepression of the Jak2-V617F gene stably inserted into the Hek-Flp-In-293 cell line previously transfected with an expression vector encoding the erythropoietin receptor. The constitutively expressed tetR protein (blue) binds to Tet operator 2 ($TetO_2$) sequences in the pcDNA5/FRT/TO vector thereby repressing transcription of Jak2-V617F (red). Upon addition, doxycycline (Dox) binds to the tetR protein and that causes a conformational change in tetR and its release from $TetO_2$ sequences. The expression level of the Jak2-V617F mutant can be controlled by the concentration of Dox used. A low concentration of Dox ensures subendogenous levels of Jak2-V617F or other Jak proteins expressed from the pcDNA5 vector.

My contributions to this work published in the *Oncogene* journal were the following:

- establishment of cell lines employing the HEK-FRT/TO system
- assessment of the mRNA levels of SOCS1 and SOCS3 by quantitative PCR (Fig. 2A, 2B)
- monitoring the Jak2-V617F protein expression by Western blot (Fig. 2B)

4.3.2 Article

Oncogene (2009) 28, 3069–3080

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ORIGINAL ARTICLE

SOCS-mediated downregulation of mutant Jak2 (V617F, T875N and K539L) counteracts cytokine-independent signaling

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Recently, mutations in the gene of Janus kinase 2 (Jak2) were discovered in patients suffering from chronic myeloproliferative disorders (MPD) and leukemia. As suppressors of cytokine signaling (SOCS) proteins are potent feedback inhibitors of Jak-mediated signaling, we investigated their role in signal transduction through constitutively active Jak2 mutants. We selected two mutants, Jak2-V617F and Jak2-K539L, found in patients with MPDs and Jak2-T875N identified in acute megakaryoblastic leukemia. We found SOCS family members to be induced through Jak2-V617F in human leukemia cell lines expressing the mutant allele and in stable HEK transfectants inducibly expressing constitutively active Jak2 mutants. SOCS proteins were recruited to the membrane and bound to the constitutively active Jaks. In contrast to wild-type Jak2, the mutant proteins were constitutively ubiquitinated and degraded through the proteasome. Taken together, we show a SOCS-mediated downregulation of the constitutively active, disease-associated mutant Jak2 proteins. Furthermore, a threshold level of mutant Jak expression has to be overcome to allow full cytokine-independent constitutive activation of signaling proteins, which may explain progression to homozygocity in MPDs as well as gene amplification in severe phenotypes and leukemia.
Oncogene (2009) **28,** 3069 3080; doi:10.1038/onc.2009.155; published online 22 June 2009

Keywords: SOCS; Janus kinases; proteasomal degradation; myeloproliferative disorders

Introduction

A somatic mutation in Janus kinase 2, Jak2-V617F, was recently described to be present in the majority of patients suffering from polycythemia vera (PV). In addition, the mutation was also found in other myeloproliferative disorders (MPDs) such as essential thrombocythemia (ET) and idiopathic myelofibrosis (IMF) (Baxter *et al.*, 2005; James *et al.*, 2005; Levine *et al.*, 2005; Zhao *et al.*, 2005; Kralovics *et al.*, 2005a). The genetic mutation results in a valine to phenylalanine substitution in the pseudokinase domain of Jak2 at amino acid position 617, generating a constitutively active protein. Since then, other constitutively active Jak2 mutations have been discovered in patients. These include the kinase domain mutation, T875N, found in acute megakaryoblastic leukemia (Mercher *et al.*, 2006) and a number of mutations and deletions in exon 12 in the pseudokinase domain (K539L, Δ(F537H538) + K539L, H538Q + K539L, Δ(N542E543)) in patients with PV and IMF (Scott *et al.*, 2007). A mutation, L611S, found in acute lymphoblastic leukemia (Kratz *et al.*, 2006) was not further characterized.

In murine bone marrow transfer models, introduction of the Jak2-V617F mutation is sufficient to induce a PV phenotype (Bumm *et al.*, 2006; Lacout *et al.*, 2006; Wernig *et al.*, 2006; Zaleskas *et al.*, 2006). Thus, the Jak2-V617F mutation seems to be very critical for disease development, but the evolution of the human disease and involvement of other factors is not fully understood.

In PV, activation of signal transducer and activator of transcription (STAT) 5 and STAT3 was described (Roder *et al.*, 2001; Garcon *et al.*, 2006). The constitutively active Jak2 mutants have been shown to lead to cytokine receptor-dependent constitutive activation of various signaling proteins, such as STATs, mitogen-activated protein kinases and phosphatidylinositol-3-kinase/AKT (James *et al.*, 2005; Lu *et al.*, 2005; Mercher *et al.*, 2006; Scott *et al.*, 2007).

SOCS proteins negatively regulate signal transduction in a classical feedback loop (Yoshimura *et al.*, 1995; Endo *et al.*, 1997; Naka *et al.*, 1997; Starr *et al.*, 1997). The members of this protein family contain a central SH2 domain, as well as a C-terminal domain called SOCS-Box, which is required for proteasomal degradation of SOCS-binding partners (De Sepulveda *et al.*, 2000; Frantsve *et al.*, 2001; Kamizono *et al.*, 2001; Rui *et al.*, 2002; Ungureanu *et al.*, 2002). Also, both SOCS1 and SOCS3 have been shown to interact directly with Jaks and thereby inhibit the phosphorylation of cytokine receptors, STATs and the Jaks themselves. Whereas SOCS3 is suppressing erythropoietin receptor (EpoR) signaling (Yoshimura *et al.*, 1995; Sasaki *et al.*, 2000), SOCS1 was shown to inhibit

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Tel-Jak2-mediated transformation of hematopoietic cells (Frantsve *et al*., 2001).

As more and more activating mutations in Jaks are discovered in patients affected by chronic myeloproliferative diseases and leukemia, the interest in understanding the molecular mechanisms of Jak action and inhibition rises. We compared three naturally occurring mutations of Jak2 and investigated the role of SOCS proteins in Jak2 mutant-mediated signaling. We found SOCS proteins to negatively modulate mutant Jak2 expression levels and showed that these mutants lead to constitutive activation of signaling proteins comparable with cytokine treatment if a certain expression threshold is overcome.

Results

Constitutively active Jak2 mutants show a reduced expression

Constitutively active Jaks are best studied in an inducible system. The activation of signaling pathways and protein fate can be monitored as a function of controlled protein expression, which is not the case for non-inducible systems. We generated stable inducible transfectants expressing Jak2-WT (wild type), Jak2-V617F, Jak2-T875N and Jak2-K539L (Figure 1a) using the HEK Flp-In-293 cell line in which the gene of interest can be inserted into a Flp recombinase target (FRT) site so that the different mutants are isogenically expressed from the same site in the genome. Induction by doxycycline (Dox) enables the expression of controlled levels of Jaks, depending on the Dox concentration used. Low concentrations of Dox (1–5 ng/ml) were used to avoid constitutive activation of Jak2-WT, an unphysiological effect often observed when Janus kinases are overexpressed. In our system, Jak2-WT autophosphorylation was not apparent up to 10 ng/ml Dox (highest concentration tested). We generated Flp-In-293 cells stably expressing or not expressing the EpoR, as it was proposed that the presence of a homodimeric type I cytokine receptor is important for V617F/STAT5-mediated transformation

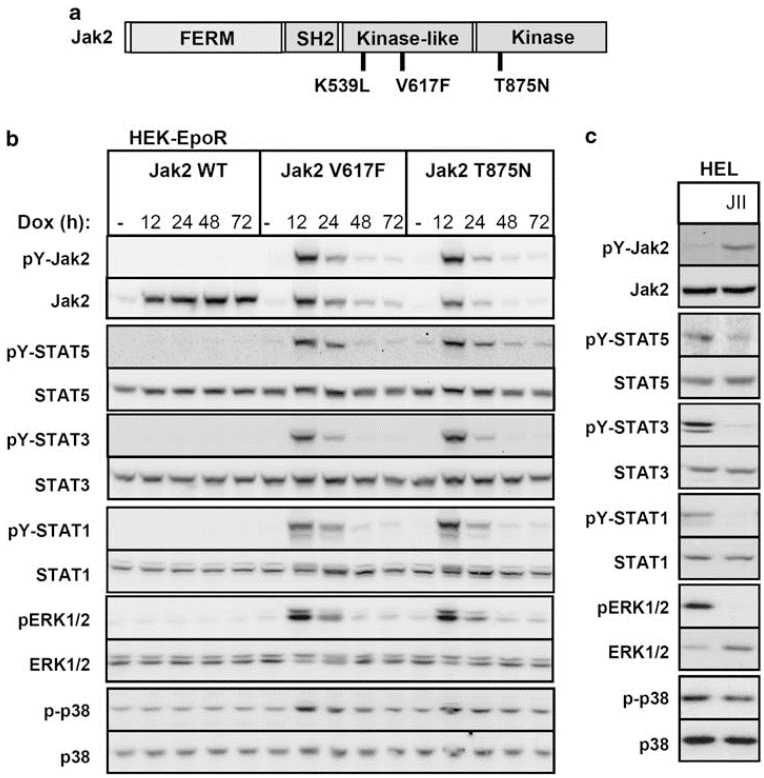


Figure 1 Constitutively active Jak2 mutants activate multiple signaling components and show a transient expression pattern. (**a**) Domain structure of Jak2 and location of the investigated Jak2 point mutations. (**b**) HEK-EpoR cells stably and inducibly expressing Jak2-WT, Jak2-V617F and Jak2-T875N were treated with 1 ng/ml of doxycycline (Dox) for the times indicated or were left untreated ( ). After lysis of the cells, the proteins were resolved by SDS polyacrylamide gel electrophoresis (PAGE) and subjected to western blot analysis. Phosphorylation of proteins was detected using phospho-specific antibodies directed against the different phospho-proteins. Equal loading and expression was checked by stripping the blot and reprobing it with antibodies directed against the various proteins. (**c**) HEL cells were treated with Jak Inhibitor I (JII; 0.5 μM) for 6 h or were left untreated. The lysates were analysed as described above.

(Lu *et al*., 2005). Expression of Jak2-WT, Jak2-V617F, Jak2-T875N or Jak2-K539L was induced in stable HEK-EpoR transfectants for different periods of time as indicated in Figure 1b and Supplementary Figure 1 (all data on the K539L mutant is provided in Supplementary Figure 1). The expression of the Jak2 mutants elicited constitutive signaling such as the phosphorylation of the mutated Jak2 proteins themselves, the STAT factors (STAT5, STAT3, STAT1) and phosphorylation of the mitogen-activated protein kinases (ERK1/2 and p38). In contrast, the expression of Jak2-WT did not lead to signaling events. HEK-V617F and HEK-T875N cells not expressing the EpoR also displayed constitutive signaling, but STAT5 was only weakly activated in comparison with HEK-EpoR cells (Supplementary Figure 2). This supports the data by Lu *et al*. showing that Jak2/STAT5 activation through homodimeric cytokine receptors is important for V617F-mediated transformation.

The most striking observation was that the expression levels of the mutant Jak2 proteins showed a maximum at 12 h of induction but then gradually decreased over time to reach a steady-state level (Figure 1b and Supplementary Figure 1a). Accordingly, the constitutive activation of signaling components was reduced to a steady-state level. This was not the case for Jak2-WT, which showed quite constant expression levels over time. Furthermore, this regulation was not dependent on EpoR expression, as it was also observed in HEK cells not expressing this receptor (Supplementary Figure 2).

In HEL cells, patient-derived leukemia cells naturally carrying the Jak2-V617F mutation, constitutive phosphorylation of STAT5, STAT3, STAT1, Erk1/2 and p38 can also be observed (Figure 1c). Treatment of these cells with Jak Inhibitor I (JII) reduced the phosphorylation of these signaling proteins. Thus, the pattern of signaling molecules activated in our model cell line HEK Flp-In-293 corresponds to those activated in patient-derived HEL cells. Phosphorylation of Jak2 increased upon treatment of the cells with JII, although downstream signaling was clearly inhibited.

Mutant Jak2 protein degradation is Jak2 activity-dependent and is mediated by the proteasome

To investigate whether the constitutive activity of Jak2 mutants is responsible for the transient expression kinetics, we investigated the effect of JII on the Dox-induced Jak2-V617F expression kinetics. We treated HEK-Jak2-V617F cells with JII or left them untreated and compared the expression of the mutant with cells expressing Jak2-WT (Figure 2a). In non-treated Jak2-V617F cells, the mutant protein was downregulated, whereas its expression was rescued and comparable with Jak2-WT protein expression upon addition of the Jak inhibitor. Thus, the activity of Jak2-V617F regulates its own protein stability. It can be noted that the phosphorylation of Jak2-V617F paralleled its expression kinetics and was not suppressed by treatment with JII.

To investigate Jak2-V617F expression levels in patient-derived cells, we studied the Jak2-V617F-positive leukemia cell lines, HEL (homozygous for V617F) and SET2 (heterozygous for V617F). JII was able to inhibit proliferation of HEL cells, as described before (Levine *et al*., 2005; Quentmeier *et al*., 2006; Walz *et al*., 2006) and of SET2 cells (data not shown). As protein downregulation was Jak activity-dependent in stable HEK cells, treatment of these leukemic cells with JII should result in an increase in Jak2 levels. Indeed, treatment of HEL and SET2 cells with JII (Figures 2b and c) showed an increase in endogenous Jak2-V617F levels compared with untreated cells.

To mimic the steady state of Jak2-V617F, Jak2-T875N and Jak2-K539L expression found in patients, we cultured the HEK and HEK-EpoR cell lines inducibly expressing Jak2-WT and the two constitutively active mutants in the presence of Dox for some passages, to allow for a steady-state protein level regulation. The cells then showed very low Jak2 mutant protein expression (as also observed in Figures 1b and 2a). The cells were then incubated with a Jak inhibitor for 48 h or were left untreated (Figure 2c and Supplementary Figure 1b). As observed for the patient-derived leukemic cells, HEL and SET2, the Jak2 levels are upregulated after a 48-h inhibitor treatment. Thus, the activity of Jak2-V617F, Jak2-T875N and Jak2-K539L regulates their protein levels.

To compare the protein stability of Jak2-WT with the one of Jak2-V617F and Jak2-T875N, we cultured HEK-Jak2 cells with Dox for 12 h in order to induce Jak2 expression and then exchanged the medium against the medium without Dox. Thereby, Jak2 gene expression stops and the protein levels of Jak2-WT and the Jak2 mutants can then be followed over time by western blot (Figure 2d). Jak2-WT degradation was much slower than the degradation of the two constitutively active mutants. Thus, we conclude that the protein stability of Jak2-WT and the constitutively active mutants is different and that the mutants have a reduced stability.

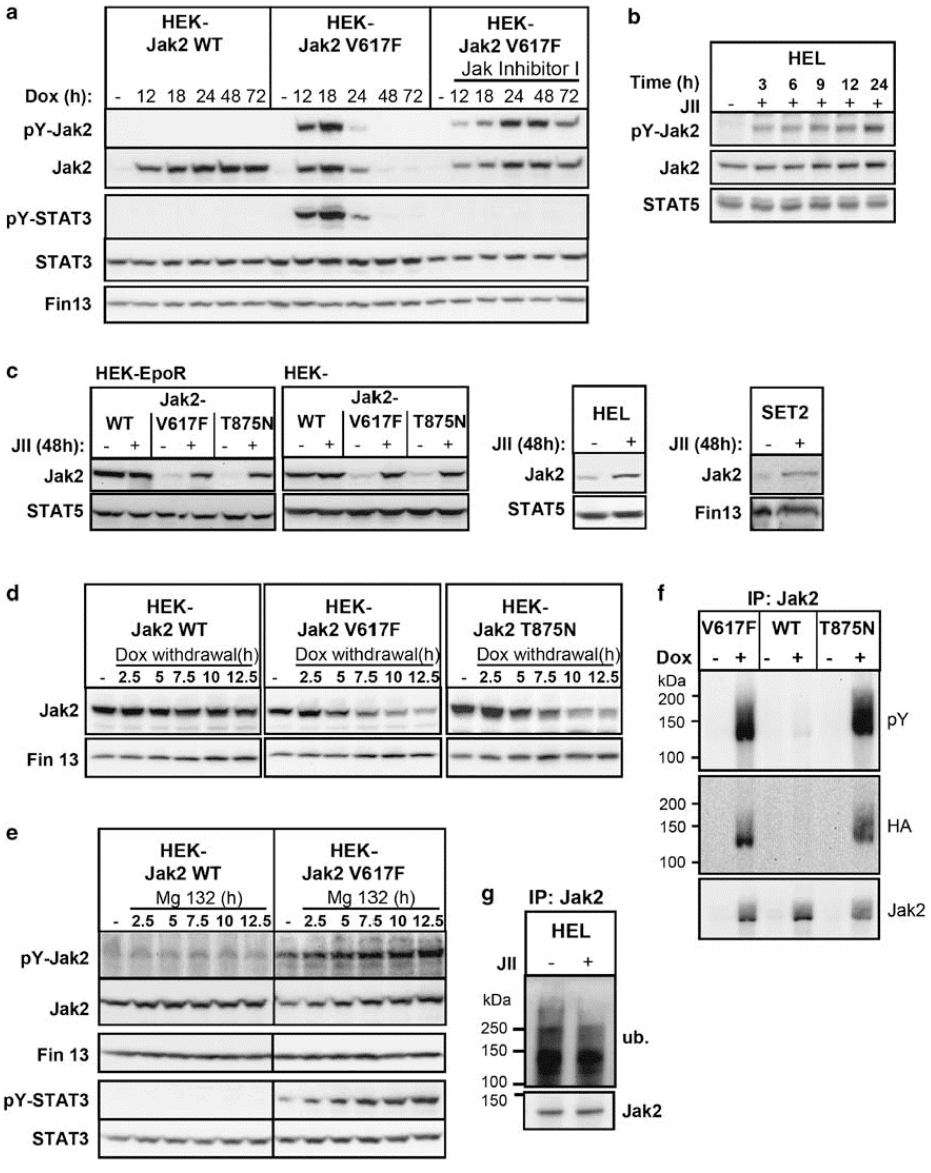
We next investigated whether Jak2-V617F is degraded by the proteasome. For this, we cultured HEK-Jak2-WT and HEK-Jak2-V617F cells with Dox for 12 h to induce Jak2 expression. Then the cells were incubated for different time periods with the proteasome inhibitor MG132. With increasing time of MG132 treatment, Jak2-V617F levels increased while the levels of Jak2-WT did not change (Figure 2e). The blot was reprobed with Fin13 to show that the protein loading was equal. We next studied whether the constitutively active Jaks could be ubiquitinated. HEK-Jak2-WT, HEK-Jak2-V617F and HEK-Jak2-T875N cells were transfected with an HA-ubiquitin construct. After induction of Jak2 proteins, we detected poly-ubiquitinated, phosphorylated Jak2-V617F and Jak2-T875N, but not Jak2-WT (Figure 2f). Finally, we detected reduced ubiquitination of Jak2-V617F in HEL cells treated with JII (Figure 2g). These data indicate that the activity-dependent downregulation of the protein levels of mutant Jak2 proteins occurs through the ubiquitin proteasome pathway.

Jak2-V617F and Jak2-T875N induce the expression of SOCS proteins

As SOCS proteins negatively regulate Jak activity, we investigated whether the constitutively active Jak2 mutants could induce SOCS protein expression and whether SOCS proteins would modulate the activity of the mutant Jak2 proteins. We focused on SOCS1 and SOCS3, as these proteins directly bind to Jak2 and modulate its activity (Yasukawa *et al*., 1999; Sasaki *et al*., 2000).

HEL or SET2 cells were treated for 6 h with JII, and mRNA levels of SOCS1 and SOCS3 were investigated by quantitative PCR analysis. Upon inhibitor treatment, the mRNA of both SOCS proteins was downregulated (Figure 3a), showing that in these patient-derived leukemic cells Jak2-V617F induces transcription of these SOCS genes.

To investigate SOCS expression in our inducible system, HEK-Jak2-WT and HEK-Jak2-V617F cells were induced with Dox for the times indicated, mRNA



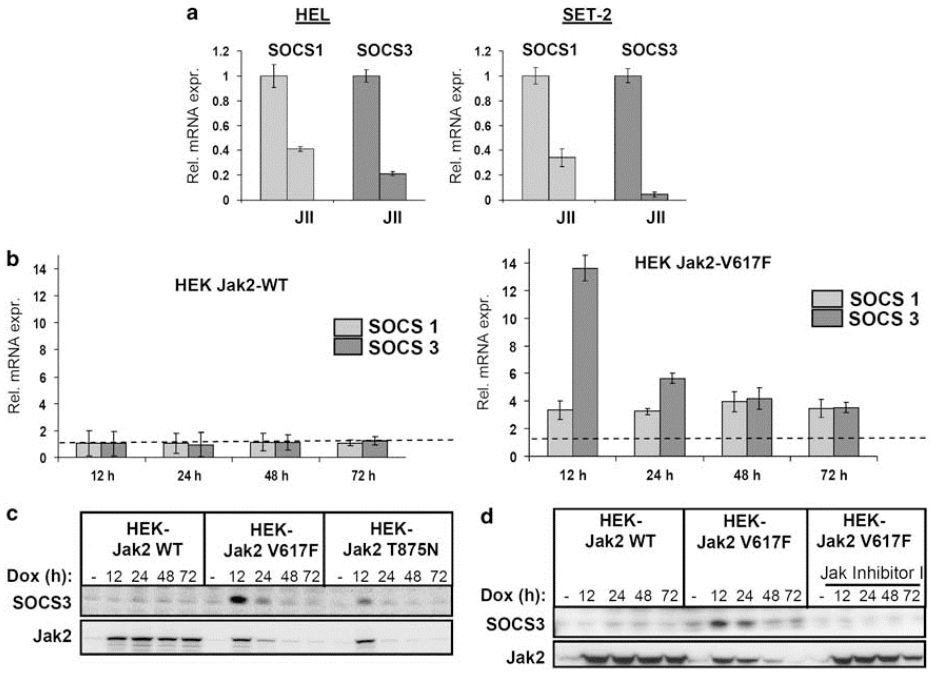


Figure 3 Jak2-V617F induces SOCS1 and SOCS3 expression. (**a**) HEL and SET2 cells were treated with Jak Inhibitor I (JII, 0.5 μM) or were left untreated. Total RNA was prepared and SOCS1 as well as SOCS3 expression was analysed by real-time quantitative PCR as described in the Materials and methods section. The experiment was carried out in triplicate and standard deviations are given. (**b**) HEK-Jak2-WT and HEK-Jak2-V617F cells were treated with 1 ng/ml of doxycycline (Dox) for the times indicated or were left untreated. SOCS1 and SOCS3 expression was monitored using real-time quantitative PCR. The expression relative to the non-treated cells is given. The experiment was carried out in triplicate and standard deviations are given. (**c**) HEK-Jak2-WT, HEK-Jak2-V617F and HEK-Jak2-T875N cells were treated with 1 ng/ml of Dox for the indicated times, and Jak2 levels as well as SOCS3 expression were monitored by western blot analysis of cell lysates. (**d**) HEK-Jak2-WT and HEK-Jak2-V617F were treated with 1 ng/ml of Dox for the indicated times. HEK-Jak2-V617F cells were also treated with JII (0.5 μM) as indicated. Jak2 levels and SOCS3 expression were studied by western blot analysis.

Figure 2 Reduction of mutant Jak2 levels is dependent on kinase activity and occurs by proteasomal degradation. (**a**) Stable, inducible HEK-Jak2-WT and HEK-Jak2-V617F cells were treated with 1 ng/ml doxycycline (Dox) for the indicated periods of time. Simultaneously, Jak2-V617F cells were also treated with Jak Inhibitor I (JII, 0.5 μM) as indicated. Western blots were analysed as described in Figure 1. Jak2 protein expression and STAT3 phosphorylation were assessed using specific antibodies. Staining of STAT3 and Fin13 expression was carried out to compare the loading of the samples. (**b**) HEL cells were treated with JII (0.5 μM) for 2 days or were left untreated. Viable cells were counted to adjust the amount of lysis buffer and the lysates were analysed by western blot using antibodies recognizing Jak2 and STAT5 (loading control). (**c**) Inducible HEK-EpoR-Jak2-WT, HEK-EpoR-Jak2-V617F and HEK-EpoR-Jak2-T875N cells as well as HEK-Jak2-WT, HEK-Jak2-V617F, and HEK-Jak2-T875N cells were treated with 1 ng/ml Dox for several passages to induce steady-state Jak2 levels. The cells were then treated with JII (0.5 μM) for 48 h or were left untreated. Patient-derived HEL and SET2 cells expressing Jak2-V617F were also treated with JII for 48 h. Viable cells were counted to adjust the amount of lysis buffer. Western blots were prepared from cell lysates, Jak2 levels were determined using a Jak2 antibody and STAT5 or Fin13 stainings are given as loading control. (**d**) HEK-Jak2-WT, -V617F and -T875N cells were treated with 1 ng/ml Dox for 12 h to induce Jak2 expression. Dox-mediated expression of Jak2 was then stopped by removal of the medium and exchange against the medium without Dox. Expression of Jak2 and Fin13 (loading control) was then monitored after the indicated times. (**e**) HEK-Jak2-WT and HEK-Jak2-V617F cells were treated with 1 ng/ml Dox for 12 h to induce Jak2 expression. The cells were then treated with the proteasome inhibitor, MG132 (10 μM), for the indicated time periods without the removal of Dox. The samples were processed as described above, and protein expression and phosphorylation were monitored using the corresponding antibodies. (**f**) HEK-Jak2-WT, HEK-Jak2-V617F and HEK-Jak2-T875N cells were transfected with a HA-ubiquitin expression plasmid (0.5 μg). At 24 h after transfection, Jak2 expression was induced with 1 ng/ml of Dox for 12 h. MG132 (10 μM) was added for 2 h before cell lysis. Jak2 protein was precipitated from the lysates and the precipitated proteins were subjected to western blot analysis. Poly-ubiquitinated Jak2 was detected using an HA antibody. Jak2 induction and phosphorylation were monitored with the respective antibodies. (**g**) HEL cells were treated with JII for 6 h or were left untreated. MG132 (10 μM) was added 2 h before cell lysis and Jak2 was precipitated from the lysates. The precipitated proteins were subjected to western blot analysis using antibodies recognizing ubiquitin (ub.) or Jak2.

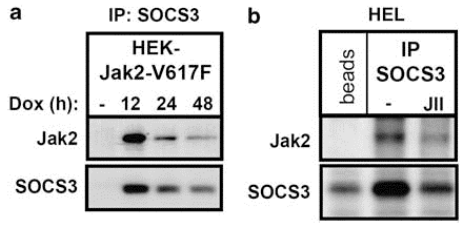


Figure 4 SOCS3 binds to Jak2-V617F. (**a**) HEK-Jak2-V617F cells were treated with 1 ng/ml of doxycycline (Dox) for the times indicated. MG132 was added 3 h before cell lysis. After lysis of the cells, SOCS3 was precipitated with a SOCS3 antibody. Co-precipitated Jak2 as well as SOCS3 was detected by western blot analysis. (**b**) HEL cells were treated with Jak Inhibitor I (JII) for 6 h or were left untreated. MG132 (3 h) was present in all samples. After cell lysis, SOCS3 was precipitated and co-precipitated Jak2 as well as SOCS3 were detected by western blot analysis. A sepharose control without antibody (beads) is also provided. The band observed in the beads control is due to non-specific binding of a HEL cell protein that co-migrates with SOCS3.

was isolated and analysed (Figure 3b). SOCS1 and SOCS3 mRNA levels were not upregulated by the induction of Jak2-WT expression compared with the non-induced cells. In HEK-Jak2-V617F, a different regulation is observed. SOCS1 is induced about three-fold over the whole period of Jak2-V617F induction compared with non-induced cells. The induction is rapid and constant over time. SOCS3 mRNA level shows kinetics that parallel Jak2-V617F protein expression. It is maximal at 12 h and decreases gradually over time to reach a three-fold induction (compared with the non-induced state) after 72 h of Dox treatment. Thus, the mRNA levels of SOCS1 and SOCS3 are upregulated in a Jak2-V617F-dependent manner and show a three- to four-fold elevated level at steady state compared with cells expressing Jak2-WT.

We then monitored SOCS3 protein expression in HEK-Jak2-V617F and HEK-Jak2-T875N cells. SOCS3 was upregulated in cells expressing Jak2-V617F and Jak2-T875N with kinetics matching the Jak2 mutant expression and activation levels (Figure 3c). In addition, the effect of JII on SOCS3 expression was investigated. If JII is administered to HEK-Jak2-V617F cells, no upregulation of SOCS3 can be observed (Figure 3d).

SOCS3 binds to Jak2-V617F

We carried out co-immunoprecipitations to investigate the binding of SOCS3 to Jak2-V617F. SOCS3 immunoprecipitation was carried out with lysates of HEK-Jak2-V617F cells induced for different times, as indicated (Figure 4a). The proteins were stabilized by treatment of the cells with the proteasome inhibitor MG132. As illustrated in Figure 4a, co-precipitation of Jak2-V617F is maximal at 12 h after Dox treatment and decreases according to the earlier observed transient expression pattern of Jak2-V617F. Co-precipitation of Jak2 with SOCS3 was also observed for Jak2-T875N and Jak2-K539L cells (Supplementary Figure 1d).

Similarly, Jak2-V617F can be co-precipitated with SOCS3 from lysates of HEL cells, as depicted in Figure 4b. In cells treated with JII, the precipitated SOCS3 levels were much lower, showing again that in this leukemia cell line SOCS3 is produced Jak2-V617F-dependently. Thus, in both of our experimental cell systems, we find an association between SOCS3 and Jak2-V617F.

Jak2-V617F and Jak2-T875N recruit SOCS1 and SOCS3 to the plasma membrane

HEK-yellow fluorescent protein (YFP)-Jak2-WT, HEK-YFP-Jak2-V617F and HEK-YFP-Jak2-T875N cells were generated to investigate the localization of the different Jak2 proteins. Jak2-WT and YFP-Jak2-WT were previously reported to be almost exclusively localized at the plasma membrane in living cells (Behrmann *et al*., 2004). YFP-Jak2-WT, YFP-Jak2-V617F and YFP-Jak2-T875N show an identical regulation of Jak activity, STAT3 activation as well as Jak2 protein stability compared with the non-YFP-tagged proteins (compare Figure 5a with Figure 1). Thus, the YFP-tagged Jak2 proteins behave in a biochemically identical way compared with the non-YFP-tagged Jak2. The localization of YFP-Jak2-WT and YFP-Jak2-V617F was investigated at 12 h after induction with Dox using live-cell confocal microscopy. The localization of Jak2-WT, Jak2-V617F and Jak2-T875N is identical and all proteins are localized predominantly at the plasma membrane with a slight cytoplasmic staining and an exclusion of Jak2 from the nucleus (Figure 5b), as described before for Jak2-WT (Behrmann *et al*., 2004).

To further investigate whether the constitutively active Jak2 mutants can recruit SOCS proteins, we expressed SOCS1-YFP and SOCS3-YFP in inducible HEK-Jak2-WT, HEK-Jak2-V617F and HEK-Jak2-T875N cells and observed their localization before and after Dox-dependent induction of the kinase (Figure 5c). Before induction of Jak expression, SOCS1-YFP showed a nuclear localization, as described before (Vuong *et al*., 2004), whereas SOCS3-YFP was evenly distributed throughout the cell. After the induction of Jak2-V617F and Jak2-T875N, SOCS1-YFP and SOCS3-YFP relocalized to the plasma membrane, whereas they retained their initial localization in HEK-Jak2-WT cells (Figure 5c). The recruitment of SOCS1-YFP and SOCS3-YFP to the plasma membrane by Jak2-V617F could be prevented by the addition of JII together with Dox (Figure 5d and data not shown). Furthermore, if HEK-Jak2-V617F cells in which SOCS1-YFP or SOCS3-YFP was localized at the membrane (after Dox treatment) were treated with JII, SOCS1-YFP and SOCS3-YFP regained their initial distribution (data not shown).

SOCS1 and SOCS3 downregulate Jak2-V617F activity and reduce Jak2-V617F protein levels

Both SOCS1 and SOCS3 were shown to negatively regulate Jak2-mediated signaling by binding to Jak2. To investigate whether SOCS3 can also inhibit the Jak2-V617F mutant, we co-transfected 293T cells with Jak2-WT or Jak2-V617F plasmids together with constructs

encoding different SOCS3 mutants. We chose SOCS3-WT as well as SOCS3 mutants in which the function of the kinase inhibitory region (KIR) (SOCS3-F25A point mutant), the SH2 domain (SOCS3-R71K point mutant) or the SOCS-Box (SOCS3-ΔBox deletion mutant) was knocked out. As expected, the expression of Jak2-V617F induced strong autophosphorylation, whereas Jak2-WT showed significantly lower levels of phosphorylation, although both proteins are expressed at similar levels (Supplementary Figure 3a). The experiment clearly shows that expression of both SOCS3-WT and SOCS3-ΔBox significantly reduced the phosphorylation of Jak2-WT as well as the one of the Jak2-V617F mutant, whereas SOCS3-F25A and SOCS3-R71K failed to inhibit both Jak2-WT and Jak2-V617F. These data indicate that SOCS3 inhibits Jak2-V617F and Jak2-WT, and in both cases direct inhibition of kinase activity involves the SH2 and KIR domains of SOCS3. To investigate whether the ΔBox mutant would fail to downregulate Jak2-V617F protein levels, we performed experiments where we transfected less Jak2. As depicted in Supplementary Figure 3b, SOCS3-WT reduced Jak2 levels, whereas the ΔBox mutant did not influence Jak2 expression. Furthermore, we studied whether SOCS1 and SOCS3 would dose dependently inhibit the Jak2-V617F mutant protein (Supplementary Figure 3c). We found both proteins to reduce the phosphorylation and protein levels of Jak2-V617F in a dose-dependent manner, indicating that Jak2-V617F is susceptible to negative regulation by these SOCS proteins.

Using an siRNA approach, we then investigated whether the suppression of SOCS1 or SOCS3 expression would affect Jak2-V617F levels. As HEK cells did not support well the siRNA transfection conditions over longer periods of time, we generated γ2A cells stably and inducibly expressing Jak2-V617F. As observed for the stable HEK cells, reconstitution of these Jak2-deficient cells with Jak2-WT or Jak2-V617F led to a downregulation of mutant Jak2 protein levels, which could be prevented by treatment with JII (Figure 6a). The γ2A-Jak2-V617F cells were then treated with Dox for 3 days to allow for steady-state protein expression and subsequently transfected with siRNAs for SOCS1 or SOCS3 (Figure 6b). Both siRNAs led to an increase in the Jak2-V617F protein expression, indicating that both SOCS proteins can participate in V617F degradation.

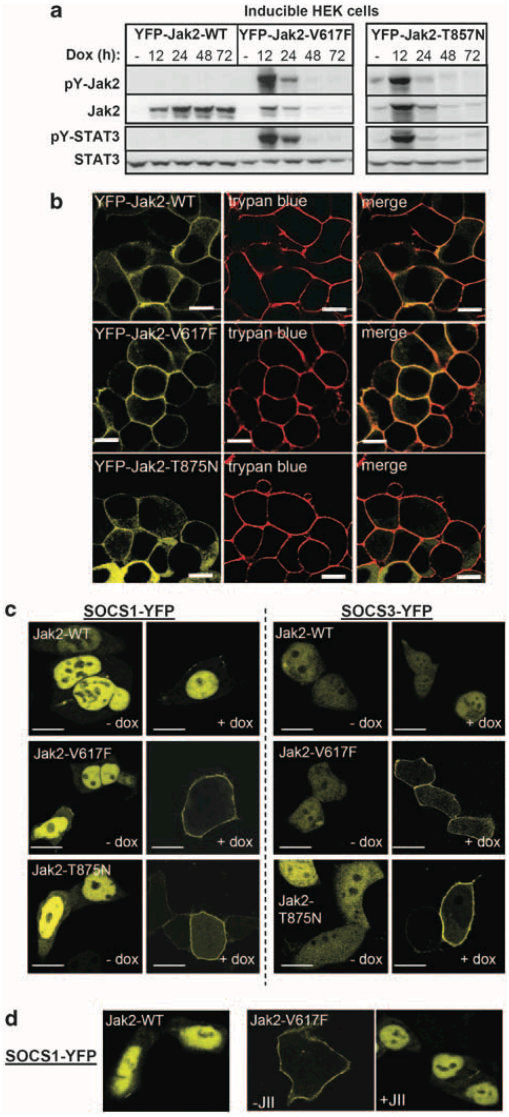


Figure 5 SOCS proteins colocalize with constitutively active Jak2 mutants at the membrane of living cells. (**a**) HEK-YFP-Jak2-WT, HEK-YFP-Jak2-V617F and HEK-YFP-Jak2-T875N cells were treated with 1 ng/ml of doxycycline (Dox) for the times indicated or were left untreated ( ). After lysis of the cells, the proteins were resolved by SDS polyacrylamide gel electrophoresis (PAGE), and Jak2 as well as STAT3 expression and phosphorylation were monitored by western blot analysis. (**b**) HEK-YFP-Jak2-WT, HEK-YFP-Jak2-V617F, and HEK-YFP-Jak2-T875N cells were treated with Dox for 12 h and subjected to confocal live-cell microscopy. The plasma membrane was stained with trypan blue Scale: 10 μm. (**c**) Stable, inducible HEK-Jak2-WT, HEK-Jak2-V617F and HEK-Jak2-T875N cells were transiently transfected with expression plasmids for SOCS1-YFP and SOCS3-YFP. Jak2 expression was induced with Dox for 12 h and the cells were subjected to confocal live-cell microscopy. (**d**) HEK-YFP-Jak2-WT and HEK-YFP-Jak2-V617F cells were treated as described in panel c. In addition, Jak Inhibitor I (JII) was given together with Dox (right panel).

Increasing levels of mutant Jak2 proteins favor cytokine independence

As illustrated in Figure 7a, the patient-derived HEL cell line showed constitutive activation of STAT5, which was inhibitable upon treatment with JII. Epo stimulation led to a clear increase in STAT5 activation. To investigate whether there is a threshold expression

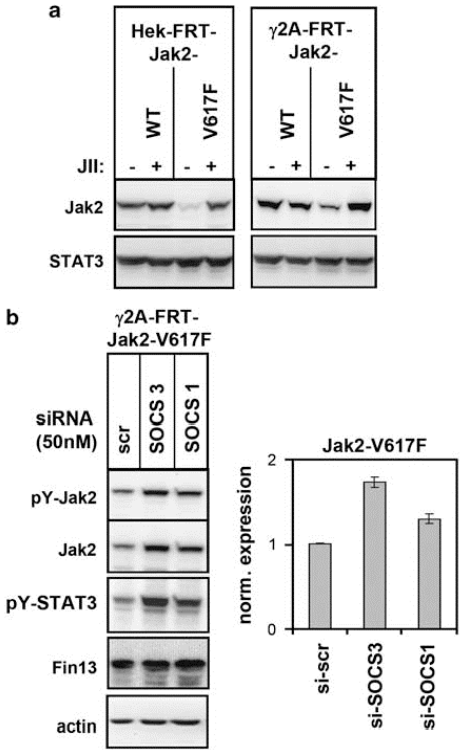


Figure 6 Treatment of cells with SOCS3 or SOCS1 siRNA increases Jak2-V617F protein expression. (**a**) γ2A-FRT cells stably and inducibly expressing Jak2-WT or Jak2-V617F were treated with 1 ng/ml of doxycycline (Dox) for several passages to induce steady-state Jak2 levels. The cells were then treated with Jak Inhibitor I (JII, 0.5 μM) for 48 h or were left untreated. Jak2 levels were monitored by western blot analysis using a Jak2 antibody and STAT3 expression levels are given as a loading control. (**b**) γ2A-FRT-Jak2-V617F cells were treated with Dox for 3 days to allow for steady-state protein expression and subsequently transfected with siRNAs for SOCS1 or SOCS3 using siRNA SMART pools. A scrambled control siRNA was transfected as a control. The bar diagram shows the normalized Jak2-V617F expression levels. Normalization was performed using the actin levels and the standard deviation is given for four different experiments.

level of mutant Jak2 protein at which constitutive activation in cells occurs, we cultured HEK-Jak2-V617F, HEK-Jak2-T875N and HEK-Jak2-K539L cells also stably expressing the EpoR with Dox for some passages to induce steady-state protein levels (Figure 7b and Supplementary Figure 1c). This was performed for different Dox concentrations (0.5, 1 and 5 ng/ml), resulting in different steady-state levels of the mutant Jak2 proteins.

At all concentrations of Dox, constitutive STAT5 activation was observed, whereas a cytokine-dependent increase could only be seen for lower Jak2 mutant expression levels (0.5 and 1 ng/ml Dox). In cells treated with 5 ng/ml Dox, Epo treatment did not lead to a further increase in STAT phosphorylation. In all cases, the constitutive signal could be inhibited by treatment of the cells with JII. Thus, although Jak2 mutants are downregulated by SOCS proteins, the resulting steady-state Jak2 expression levels induce constitutive signaling at every expression level and full cytokine-independent activation requires increased Jak2 steady-state levels.

Discussion

The most important findings of this study are (i) Jak2-V617F has a decreased stability which is due to its constitutive activity; (ii) Jak2-V617F activity is regulated by the feedback inhibitors SOCS3 and SOCS1; and (iii) a threshold level of Jak2-V617F is necessary for full cytokine-independent constitutive signaling, which could clearly explain selection for high-expressing cells in patients.

We generated HEK model cell lines in which we isogenically express Jak2-WT, Jak2-V617F, Jak2-T875N and Jak2-K539L in order to compare the signaling characteristics of the different mutants on an identical background (Figure 1b and Supplementary Figure 1). Induction of the expression of Jak2 mutant proteins by Dox led to their constitutive activation, whereas Jak2-WT was not active (up to a maximum of 10 ng/ml Dox tested), reflecting physiological conditions. The pattern of signaling molecules activated in these cells corresponded to those constitutively activated

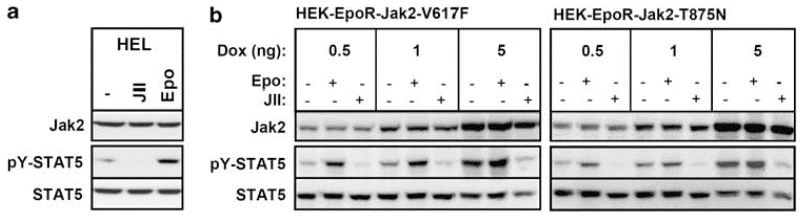


Figure 7 Constitutive activation of signaling pathways is dependent on mutant Jak2 levels. (**a**) HEL cells were left untreated or were treated with Jak Inhibitor I (JII) or Epo (10 ng/ml). After lysis of the cells, the proteins were resolved by SDS polyacrylamide gel electrophoresis (PAGE) and subjected to western blot analysis. STAT5 phosphorylation was assessed using a STAT5 phosphotyrosine-specific antibody. Equal loading and expression was checked by stripping the blot and reprobing it with a STAT5 antibody. (**b**) Inducible HEK-EpoR-Jak2-V617F and HEK-EpoR-Jak2-T875N cells were treated with 0.5, 1 and 5 ng/ml of doxycycline (Dox) for several passages to induce steady-state Jak2 levels. The cells were then stimulated with Epo (10 ng/ml for 30 min) or were treated with with JII for 3 h or were left untreated. Western blots were analysed as described in panel a.

in HEL leukemia cells naturally carrying the Jak2-V617F mutation (Figure 1c). The constitutively active mutants, V617F, T875N and K539L, behave similarly in the experiments shown in this study, indicating that the activity and the expression levels of these mutant kinases are regulated in a similar way.

Surprisingly, the expression of the constitutively active Jak2 mutants gradually decreased over time (Figure 1). The observed protein downregulation was dependent on the activity of the mutant kinase (Figure 2a). Interestingly, Jak2 phosphorylation is enhanced upon treatment with JII (although the kinase is inactive) (Figures 1c, 2a and b). This effect has previously been reported for another Jak inhibitor in the context of Jak2-V617F (Grandage *et al.*, 2006). Similarly, in the Jak2-V617F-expressing patient-derived cell lines, HEL and SET2, JII treatment led to the upregulation of Jak2 protein levels, indicating that also at the steady-state expression of Jak2-V617F its constitutive activity decreases protein stability (Figure 2c). We could re-create this situation in HEK-Jak2-V617F, HEK-Jak2-T875N and HEK-Jak2-K539L cells expressing steady-state levels of the mutants (Figure 2c and Supplementary Figure 1b). Whereas the inhibition of kinase activity increased protein expression, the withdrawal of Dox led to an accelerated decrease in protein levels (Figure 2d). Furthermore, we found the mutants to be poly-ubiquintinated (Figures 2f and g) and concluded that they are degraded by the proteasome in a Jak2 activity-dependent manner. This is in line with previous data reporting the poly-ubiquitination and degradation of phosphorylated Jak2-WT (Ungureanu *et al.*, 2002).

As SOCS proteins are known feedback inhibitors induced through the Jak/STAT pathway and negatively regulate Jak activity, we investigated whether they would modulate the activity of the mutant Jak2 proteins. We focused on SOCS1 and SOCS3 as these proteins directly bind to Jak2 and modulate its activity. Also, SOCS1 and SOCS3 mRNA upregulation has recently been reported in patients with Jak2-V617F-associated MPDs (Kralovics *et al.*, 2005b; Bock *et al.*, 2007). We found that SOCS1 and SOCS3 are regulated by Jak2-V617F in HEL and SET2 cells as well as in stably transfected HEK cells (Figure 3), that endogenous SOCS3 binds to the mutant Jak2 proteins (Figure 4 and Supplementary Figure 1d) and that both YFP-SOCS1 and YFP-SOCS3 relocalize to the membrane in living cells upon induction of constitutively active Jak2 mutants (Figure 5). Moreover, SOCS1 and SOCS3 dose dependently reduce Jak2-V617F phosphorylation and protein levels (Supplementary Figure 3c). Our data are in contrast to the results of a previous study reporting that SOCS3 fails to inhibit Jak2-V617F and potentiates both its phosphorylation and expression levels (Hookham *et al.*, 2007). We therefore tested the specificity of the observed inhibition of Jak2-V617F by SOCS3. The direct inhibition of Jak2 kinase activity by SOCS3 requires both the KIR and the SH2 domains of SOCS3 but not the SOCS-Box (Sasaki *et al.*, 1999). We found that similar to Jak2-WT, Jak2-V617F is efficiently inhibited by SOCS3 and SOCS3-ΔBox, a mutant in which the SOCS-Box has been deleted (Supplementary Figure 3a). On the other hand, mutants of SOCS3 in which the function of the KIR (SOCS3-F25A) or the SH2 domain (SOCS3-R71K) were knocked out were not able to inhibit Jak2-WT or Jak2-V617F activity. Thus, the specific pattern of the observed inhibition strongly suggests that Jak2-V617F is still sensitive to SOCS3-mediated inhibition. This also shows that the inhibitory mechanism is identical for Jak2-WT and Jak2-V617F, and corresponds to the mechanism extensively characterized by Sasaki *et al.* (1999). Finally, using an siRNA approach, we found that both SOCS1 and SOCS3 can regulate Jak2-V617F expression levels (Figure 6). Taken together, our results suggest that constitutively active Jak2 mutants are regulated by SOCS proteins at the level of kinase inhibition and by regulation of the expression levels of the mutant kinases. A similar mechanism was proposed for TEL-Jak2, for which a SOCS1-dependent inhibition of kinase activity and proteasomal degradation was reported (Frantsve *et al.*, 2001; Kamizono *et al.*, 2001).

To investigate whether there is a threshold expression level of mutant Jak2 proteins at which full cytokine-independent constitutive activation occurs, we cultured HEK-Jak2-V617F, HEK-Jak2-T875N and HEK-Jak2-K539L cells, also stably expressing the EpoR, with different Dox concentrations, in order to obtain different steady-state expression levels of mutant Jak2 protein (Figure 7b and Supplementary Figure 1c). We observed that increasing levels of constitutively active Jak2 mutants lead to increasing levels of activated signaling proteins (STAT5) and ultimately reach a level at which cytokine stimulation does not further enhance signaling. It can be noted that in HEK-Jak2-V617F and HEK-Jak2-T875N cells, which do not express the EpoR, no STAT5 activation could be detected at steady-state Jak2 levels (data not shown and Supplementary Figure 2; 24 72 (h)). Thus, the presence of the EpoR is necessary for constitutive Jak2/STAT5 signaling at these lower steady-state Jak2-V617F levels. In line with this observation, it was recently reported that the expression of the homodimeric type I cytokine receptors, EpoR, thrombopoietin receptor or G-CSFR, are required for Jak2-V617F/STAT5-mediated transformation (Lu *et al.*, 2005). A schematic representation of our model of mutant Jak2 regulation is provided and discussed in Figure 8.

In line with such a model, homozygous patients with MPD were described to have higher hemoglobin level, higher rate of fibrotic transformation and higher risk of cardiovascular events (Tefferi *et al.*, 2006; Vannucchi *et al.*, 2007). Animal studies also support the hypothesis that higher levels of Jak2-V617F lead from a thrombocytic to an erythrocytic and a fibrotic phenotype (Bock *et al.*, 2006; Lacout *et al.*, 2006). Interestingly, a subset of patients with PV and IMF show duplication of the mutated allele (Andrieux and Demory, 2005; Bacher *et al.*, 2005; Reilly, 2005). A recent study in transgenic mice indicates that the expression level of Jak2-V617F determines the MPD phenotype: a low transgene expression leads to an essential thrombocythemia

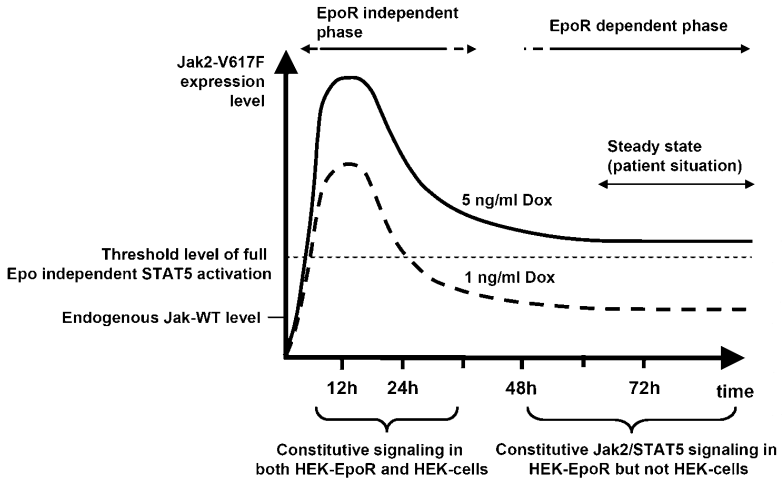


Figure 8 Schematic representation of the regulation of Jak2-V617F levels. Upon expression of the Jak2 mutants, their levels reach a maximum (broken and solid lines represent the situations of doxycycline (Dox) induction with 1 and 5 ng/ml, respectively) and then SOCS-dependently decrease to reach a specific steady-state level. In the maximum of expression where the levels of Jak2 are considerably higher than endogenous, constitutive activation of STATs is observed independent of the presence of the EpoR. This suggests that Jak2-V617F can also elicit signals through other Jak2 binding receptors. Such receptors are present in our model cell line as well as in myeloid cells. This corresponds to the findings of Lu *et al.* (2008), who also showed that V617F does not absolutely require the expression of the Jak2 activating homodimeric receptors. However, in the patient-mimicking steady state (which represents the equilibrium phase of Jak2-V617F expression and SOCS-mediated downregulation), the EpoR is crucially required for constitutive Jak2/STAT5 activation, stressing again the importance of homodimeric receptors in the context of Jak2-V617F. Finally, the two curves illustrate how increasing levels of steady-state Jak2-V617F will lead to the transition of a constitutively active situation which is still Epo-sensitive (broken line; 1 ng/ml docyxycline) to full constitutive activation which is Epo-independent (solid line; 1 ng/ml of docyxycline induction) as shown in Figure 7b.

phenotype, whereas a higher transgene expression mediated a PV-like phenotype. A similar correlation was also found in patients with MPD, in whom the expression level reflects the allele load (Tiedt *et al.*, 2008); in patients with PV and IMF, the Jak2-V617F mutation frequently progresses to homozygosity through mitotic recombination, which is less frequently observed in patients with essential thrombocythemia (Kralovics *et al.*, 2005a).

Our study provides evidence that the constitutively active Jak2 mutants have decreased stability, that they are degraded by the proteasome and that they are susceptible to negative regulation by SOCS proteins. Thus, mechanisms interfering with this regulation could considerably contribute to the development and progression of MPD and Jak2-V617F-positive leukemia by increasing the levels of constitutively active Jak2 mutants. For example, mechanisms that were reported to interfere with SOCS function are methylation (Yoshikawa *et al.*, 2001; Galm *et al.*, 2003; Ekmekci *et al.*, 2004), mutations (Melzner *et al.*, 2005) and deletions (Melzner *et al.*, 2006) of SOCS genes. It is important that epigenetic silencing of SOCS3 and SOCS1 was recently reported in ~40% of patients with Ph-negative chronic myeloid disorders (Capello *et al.*, 2008; Teofili *et al.*, 2008). The mechanism of mutant Jak2 regulation that we report strongly suggests that the regulation and potential mutation of SOCS proteins could be important clinical parameters in patients carrying constitutively active Jak2 proteins. It may also be important to extend such studies to other proteins involved in this proteasomal degradation such as the yet-to-be-identified E2-ubiquitinating enzymes recruited to the SOCS-containing E3 ligase complexes.

Materials and methods

Information on constructs, materials, cell culture and transfection as well as on cell lysis, immunoprecipitation and western blot analysis is provided as Supplementary Information.

Quantitative PCR

Total RNA from HEL, SET2 and HEK Flp-In-293 cells expressing Jak2-WT or Jak2-V617F was extracted using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) following the tissue protocol of the manufacturer's instructions. The real-time quantitative PCR was carried out in an I-Cycler (Bio-Rad, Nazareth, Belgium). Quantitative PCR results were normalized to TBP (TATA-binding protein). Relative fold changes were calculated by the comparative threshold cycles (C_T) method, $2^{-\Delta\Delta CT}$. Details are provided as Supplementary Information.

Confocal live cell microscopy

HEK Flp-In-293 cells stably expressing Jak2 proteins were seeded on poly-L-lysine-coated coverslips 36 h before microscopy. Cells were seeded 60 h before microscopy in case of

an additional transient expression of SOCS1 or SOCS3 N-terminally tagged with YFP. Transfection was carried out on the next day. Induction of protein expression with Dox was started 12 h before microscopy. Confocal live-cell imaging (at 37 °C in 5% CO_2 in a phenol red-free medium) was carried out using a Zeiss LSM510 invert laser scanning microscope (Zeiss, Sliedrecht, Netherlands). YFP was detected with $\lambda_{excitation} = 488$ nm and $\lambda_{emission} = 515-550$ nm, cell surface was stained with 0.05% trypan blue (Schulein *et al*., 1998) and recorded with $\lambda_{excitation} = 543$ nm and $\lambda_{emission} > 590$ nm.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

Supplementary Information

Supplemental materials and methods

Materials, cell culture and transfection

Epo was obtained from Peprotech. Janus Kinase Inhibitor 1 and the proteasome inhibitor MG132 (all from Calbiochem) were dissolved in DMSO. Doxycycline (Sigma) was usually used at concentrations of 1 ng/mL unless stated differently in the Figure legends. HEL (DSMZ) SET2 (DSMZ) and HEK Flp-In-293 cells (Invitrogen) were maintained in RPMI and Dulbecco´s modified Eagle´s medium, respectively with 10 to 20% fetal calf serum, 100 mg/L streptomycin and 60 mg/L penicillin. The HEK Flp-In stable transfectants were generated using the Flp-In™ System from Invitrogen and cultured with 50 µg/mL hygromycin. Cells were transfected using the Fugene reagent (Roche) or Dharmafect (Dharmacon) according to the manufacturer's protocol.

Constructs and siRNA

The HA-tagged EpoR expression construct was a kind gift of Dr. Stefan N. Constantinescu (Brussels, Belgium). Flag-tagged pCMV-SOCS3 constructs and Flag-tagged pEF-SOCS1 and pEF-SOCS3 constructs were generously provided by Dr. Akihiko Yoshimura (Fukuoka, Japan) and Dr. Douglas Hilton (Victoria, Australia), respectively.

Standard cloning procedures were performed throughout this study. Mutations were introduced using the Quick Change Kit from Stratagene into pcDNA5/FRT/TO-Jak2 (Haan et al, 2008). For the generation of pcDNA5/FRT/TO-YFP-Jak2, YFP-Jak2 was subcloned from pEF-YFP-Jak2 (Behrmann et al, 2004) using AgeI and NheI sites introduced between AflII and ApaI of pcDNA5/FRT/TO (Invitrogen). pcDNA5/FRT/TO-YFP-Jak2-V617F and -T875N were generated by exchanging HindIII/NheI-digested sequences between correspondingly mutated pcDNA5/FRT/TO-Jak2 derivatives and pcDNA5/FRT/TO-YFP-Jak2.

For siRNA-experiments, the SOCS1, SOCS3 and scrambled control siRNA SMART-pools® from Dharmacon were used at a concentration of 50 to 100 nM.

Cell lysis, immunoprecipitation and Western blot analysis

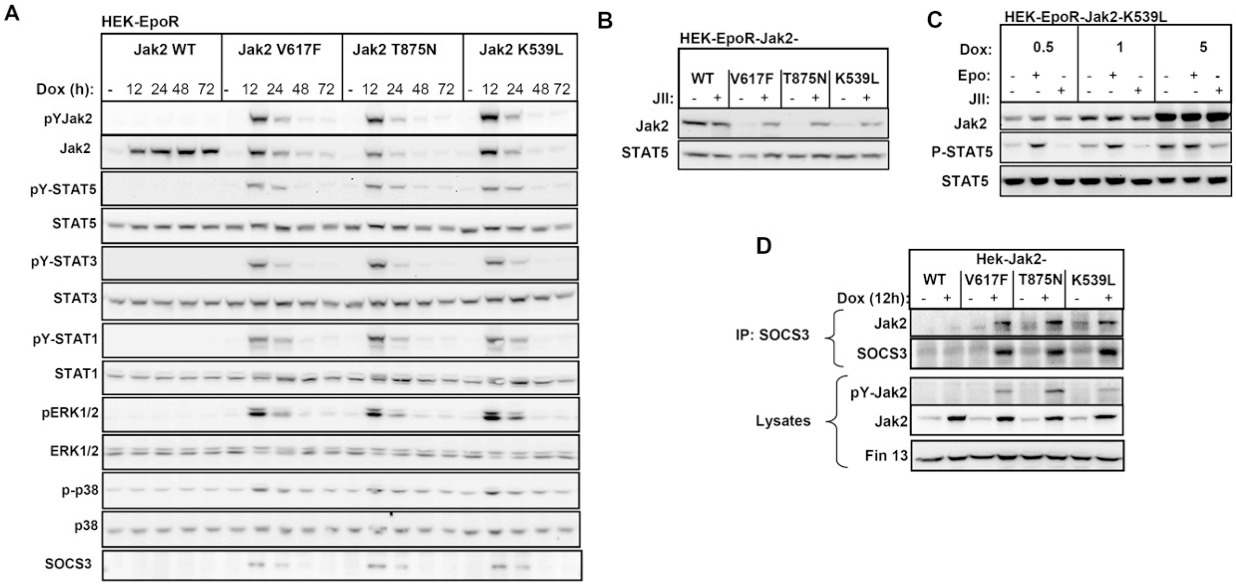
All steps of cell lysis and immunoprecipitation were performed at 4°C using ice cold buffers. Cells were lysed on the dish with lysis buffer containing 0.5% IGEPAL-CA630 or 1% Triton-X-100, 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10 mM NaF, 1 mM sodium vanadate, 10 mM PMSF, 1 mM benzamidine, 5 µg/mL aprotinin, 3 µg/mL pepstatin, 5 µg/mL leupeptin and 1 mM EDTA. After incubation of the cleared lysates with antibodies, the immunoprecipitates were collected with protein A-sepharose for 1 hour, washed several times and analyzed further by SDS-PAGE. The

proteins were transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech) and probed with the respective antibodies. Anti-Jak2 (Cell Signaling or Upstate) and anti-SOCS3 (IBL) were used for immunoprecipitation. Anti-phosphotyrosine and anti-SOCS3 were from Santa Cruz Biotech. The anti-Jak2, anti-phospho-Jak2, anti-phospho-STAT3, anti-Erk1/2, anti-phospho-Erk1/2, anti-Calnexin and anti-HA antibodies were purchased from Cell Signaling. Anti-STAT3 and anti-Fin13 were from Transduction Laboratories while anti-GFP was from Rockland. The horseradish peroxidase-conjugated secondary antibodies were purchased from Dako. Signals were detected using an ECL solution containing 2.5 mM luminol, 2.6 mM hydrogenperoxide, 100 mM Tris/HCl pH 8.8 and 0.2 mM para-coumaric acid.

Quantitative PCR

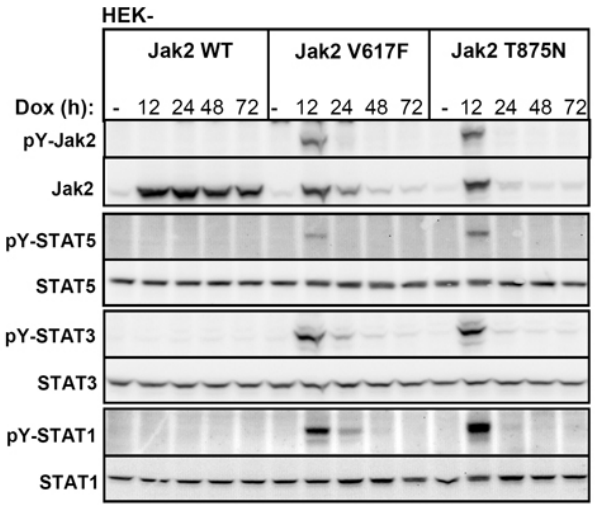
Total RNA from HEK Flp-In-293 cells expressing Jak2-WT or Jak2-V617F was extracted using the RNeasy Mini Kit (Qiagen) following the tissue protocol of the manufacturer`s instructions. The concentration of isolated RNA was measured using a NanoDrop ND1000 spectrophotometer. Constant amounts of 1 µg of total RNA were reversely transcribed with the ThermoScript RT-PCR System (Invitrogen) in a volume of 20 µL, using random hexamer primers according to the manufacturer`s instructions. DNA contaminations were removed using the RNAse-free DNAse Set (Qiagen). All investigated samples were transcribed in parallel. The real-time PCR mastermix contained: 10.5 µL of water, 12.5 µL of SYBR Green, 1 µL (12.5 ng) of template and 0.5 µL (10 µM) forward primer and 0.5 µL (10 µM) reverse primer. 25 µl of master-mix was filled into the wells of a 96-well qPCR plate (BioRad). The plate was covered by microseal film (BioRad Laboratories), centrifuged and placed into the I-Cycler. The following I-Cycler experimental protocol was used: denaturation program (95ºC for 3 min), amplification and quantification program (95ºC for 10 sec., 60ºC for 30 sec.), repeated 40 times. Quantitative PCR results were normalized to TBP (TATA-binding protein). Relative -fold changes were calculated by the comparative threshold cycles (C_T) method, $2^{-\Delta\Delta CT}$. Gene-specific primers for qPCR analysis were purchased from Eurogenetc: SOCS1 forward: 5`-AGCTCCTTCCCCTTCCAG-3`; reverse: 5`-CAAAATAACACGGCATCCC-3`; SOCS3 forward: 5`- ATGAGAACTGC-CGGGAATC-3`; reverse: 5`-CCCAGGCTCCACAACCA-3`; TBP forward: 5`-ACCCA-GCAGCATCACTGTT-3`; reverse: 5`-CGCTGGAACTCGTCTCACTA-3`. The specificity of qPCR products was documented by I-Cycler melting curve analysis (denaturation program: 95ºC for 1 min, amplification program: 55ºC for 90 s, repeated 81 times), which resulted in single product-specific melting temperatures (SOCS1: 84.5ºC, SOCS3: 81ºC).

Supplementary figures



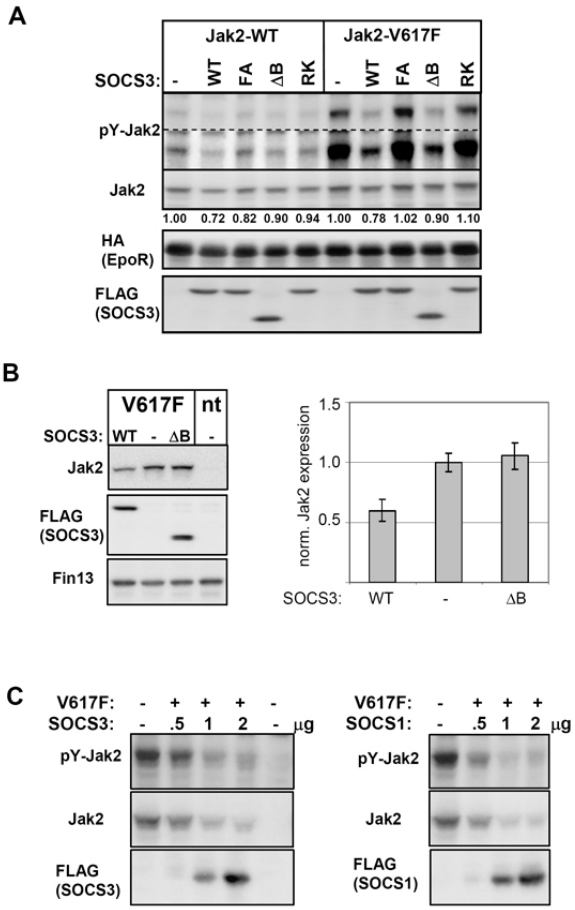
Supplemental figure 1: The constitutively active mutant Jak2-K539L behaves similarly to Jak2-V617F and Jak2-T875N.

A: Jak2-K539L is constitutively active and shows reduced protein expression. HEK-EpoR cells stably and inducibly expressing Jak2-WT, -V617F, -T875N or -K539L were treated with 1 ng/mL doxycycline for the times indicated or were left untreated (-). After lysis of the cells, the proteins were resolved by SDS-PAGE and subjected to Western blot analysis. Phosphorylation of proteins was detected using phospho-specific antibodies directed against the different phospho-proteins. Equal loading and expression was checked by stripping the blot and reprobing it with antibodies directed against the various proteins. **B:** Treatment of HEK-EpoR-Jak2-K539L cells with Jak Inhibitor I increases the steady state expression levels of the mutant protein (as observed for Jak2-V617F and -T875N) Inducible HEK-EpoR-Jak2-WT, -V617F, -T875N and -K539L cells were treated with 1 ng/mL doxycycline for several passages to induce steady state Jak2 levels. The cells were then treated with Jak Inhibitor I (0.5 μM) for 48 h or were left untreated. Western blots were prepared from cell lysates, Jak2 levels were determined using a Jak2 antibody and STAT5 staining is given as loading control. **C:** Cytokine-dependent signaling is lost at higher steady state expression levels of Jak2-K539L. Inducible HEK-EpoR-Jak2-K539L cells were treated with 0.5, 1 and 5 ng/mL doxycycline for several passages to induce steady state Jak2 levels. The cells were then stimulated with Epo (10 ng/ml for 30 min), were treated with with Jak Inhibitor I (JII) for 3 h or were left untreated. Western blots were analyzed as described in A. **D:** Mutant Jak2 can be coprecipitated with SOCS3. HEK-Jak2-WT, -V617F, -T875N or -K539L cells were treated with 1ng/mL doxycycline for 12 h and MG132 was added 3 h prior to cell lysis. SOCS3 was then precipitated with a SOCS3 antibody. Co-precipitated Jak2 as well as SOCS3 was detected by Western blot analysis. Phosphorylation and expression of Jak2 proteins as well as Fin13 expression was detected from the lysates.



Supplemental figure 2: Constitutively active Jak2 mutants activate multiple signaling components and show reduced expression levels in HEK cells.

HEK- cells stably and inducibly expressing Jak2-WT, -V617F and -T875N were treated with 1 ng/mL doxycycline for the times indicated or were left untreated (-). After lysis of the cells, the proteins were resolved by SDS-PAGE and subjected to Western blot analysis. Phosphorylation of proteins was detected using phospho-specific antibodies directed against the different phospho-proteins. Equal loading and expression was checked by stripping the blot and reprobing it with antibodies directed against the various proteins.



Supplemental figure 3: SOCS1 and SOCS3 inhibit Jak2-V617F activity.

A: 293T cells were transfected with 0.5 µg Jak2-WT or Jak2-V617F plasmid together with 0.5 µg HA-tagged EpoR construct and 0.5 µg of the different FLAG-tagged pCMV-SOCS3-constructs (SOCS3-WT, SOCS3-F25A, SOCS3-R71K and SOCS3-ΔBox). 48 h post-transfection, the cells were lysed and the lysates were subjected to Western blot analysis. The detections were performed using antibodies directed against phosphotyrosine, Jak2 as well as FLAG- and HA-tags. For clarity, two exposures of the phosphotyrosine-Jak2 detection are shown (separated by a dashed line). Different exposures of the same detection are separated by a dotted line. A quantification of the Jak2 expression is provided below the Jak2 blot with Jak2-WT or –V617F levels in non-SOCS3 expressing cells set to 1.0. **B:** 293T cells were transfected with 0.25 µg Jak2-V617F plasmid together with 0.5 µg HA-tagged EpoR construct and 0.5 µg of FLAG-tagged pCMV-SOCS3-WT or –Δbox constructs. A non-transfected control is given (nt). 48 h post-transfection, the cells were lysed and the lysates were subjected to Western blot analysis. The detections were performed using antibodies directed against Jak2 and FLAG- tag. Fin13 levels are given as loading control. The bar diagram represents a quantification of Western blot analyses from four experiments. Jak2-V617F expression was normalized using the Fin13 levels and the standard deviation for four experiments are given. **C:** SOCS1 and SOCS3 dose-dependently inhibit Jak2-V617F. 293T cells were transfected with 0.5 µg HA-EpoR, 0.5 µg Jak2-V617F and increasing amounts pEF-FLAG-SOCS1 or pEF-FLAG-SOCS3 and analyzed by Western blot analysis.

5 DISCUSSION AND PERSPECTIVES

Most signal transduction pathways rely on protein kinase activity, which is therefore an attractive target for small molecule inhibition. Many protein kinase inhibitors have been developed to study specific functions of kinases in signaling pathways and as potential therapeutic agents (202). The human kinome contains more than 500 proteins and all kinases share the common fold of the kinase domain. The design of a specific inhibitor for a kinase of interest has been proven difficult because most inhibitors target the common ATP-binding site and today only a small percentage of kinases can be inhibited with high specificity.

The enzymatic activity of a kinase can be pharmacologically inhibited in two different ways: by competing with ATP for binding to the catalytic cleft of the kinase or by allosteric inhibition. In the non-ATP competitive (allosteric) inhibition, the inhibitor binds to a loop or a pocket outside the ATP-binding cleft of the kinase, preferably blocking the binding and/or phosphorylation of the substrate or directly inhibiting the kinase activation. It is assumed that this approach would result in high selectivity and potency, mainly because such inhibitors would compete with only nM of protein substrates, rather than μM or mM levels of ATP. The design of allosteric kinase inhibitors is particularly challenging because the exact binding mode of the targeted interaction has to be known, which is seldom the case. In contrast to the enormous variety in potential allosteric binding sites, the active sites are highly conserved in kinases. The majority of kinase inhibitors are based on ATP-competitive inhibition interfering with the substrate binding. The ATP-binding pocket is highly conserved among kinases, thus most ATP-competitive kinase inhibitors target more than one kinase and off-target effects are common. The activity of kinase inhibitors may be significantly influenced by multiple factors, such as cell permeability, drug efflux, their stability, toxicity and solubility. However, the remarkable success and the relative high selectivity of Gleevec (imatinib mesylate) in the treatment of chronic myelogenous leukemia has proven the concept of ATP-competitive inhibitors to be very useful. Currently there are eight small molecule inhibitors targeting protein kinases approved by the FDA for clinical use in humans. Importantly, protein kinase inhibitors are valuable not just for the treatment of diseases, but also as reagents to help us to understand more about the physiological roles of protein kinases. The specificity of inhibitors was thought to be of critical importance for its clinical utility. However, the approval of Sunitinib, a multi-kinase inhibitor targeting many kinases at low nanomolar range for the treatment of renal cell carcinoma and Gleevec-resistant

gastrointestinal stromal tumor, has obviously changed this view. The required specificity of kinase inhibitors depends on the type of diseases they are used to treat. In case of cancers, where treatment time is limited and the danger to life is iminent, specificity may not be the major concern as long as the disease is driven into remission. However, strong side effects usually accompany low specificity, which is an important issue in case of diseases that require constant treatment over prolonged time. Many inhibitors that cannot be used as drugs for reasons of e.g. toxicity represent extremely useful tools for research purposes. In research, specificity is one of the most important parameter that the kinase inhibitor should fulfil.

TARGETING JAKs WITH KINASE INHIBITORS

Protein kinase inhibitors have been used to better understand the role of Janus kinases in many signal transduction pathways. Noteworthy, most inhibitors targeting the catalytic cleft of Janus kinases showed only micromolar activity. AG490 and WHI-P131 represent good examples of small molecule inhibitors initially reported to specifically inhibit Jak2 and Jak3, respectively, and they have been extensively used to study Jak-mediated signaling responses. However, both of these compounds were also shown to suppress other kinases, e.g. EGFR, in much lower concentrations, what consequently makes the direct attribution of the biochemical and physiological effects of these inhibitors to the Jaks not possible. Recently inhibitors of Jaks have been reported, which possess nanomolar activity in cellular assays (14). However, most of the potent Jak inhibitors show activity against several Jak members and also target other kinases. The sequential similarity of the Jak kinase domains is quite high, which increases the challenge to generate specific compounds that do not inhibit other Jaks.

Alternative approaches are available to examine the involvement of a given kinase in signal transduction. Genetic techniques, such as “knockouts”, have provided important insights into the roles of specific proteins. However, it is known that possible compensatory mechanisms can occur, what masks the real function of the targeted kinase. Moreover, knockout mice may not be viable at all, e.g. Jak2 knockout mouse die during embryogenesis due to the lack of erythropoiesis. The siRNA-based approach represents a potential solution by offering a temporal or stable suppression of the expression of the gene of interest. However, this approach may be incomplete in terms of the efficiency of inhibition, and moreover it could evoke double-stranded RNA-

mediated side effects. Furthermore, targeting the kinase of interest by genetic or siRNA approaches might have effects, which cannot be attributed to the enzymatic activity of the kinase but e.g. to its potential structural role. To overcome this problem, kinase-inactive mutants can be introduced into kinase-deficient cells.

However, a way to combine mutational and pharmacological approaches is to use a mutation to confer selectivity for a small molecule, i.e. the chemical genetics approach. A genetically modified kinase and a chemically modified small molecule inhibitor are the key components of this approach. The kinase of interest has to be mutated in a way so that it can accommodate the modified inhibitor. The so-called “gatekeeper” residue, which limits the size of the ATP-binding pocket in PTKs, is conserved as a large- to medium-sized residue. It shows variation between kinases and by this also controls the sensitivity towards different ATP-competitive inhibitors. Mutation of this residue to a smaller one like glycine or alanine creates a space, which is not found in any other kinase. This novel pocket can then be uniquely accessed by the modified inhibitor analogue, which is a known kinase inhibitor with a bulky modification that occupies the extra space generated by the mutation of the gatekeeper.

We have generated analogue-sensitive mutants of Jak1 and Jak2, whose activity could be specifically suppressed by an inhibitor analogue. All Janus kinases, but especially Jak1, are involved in many cytokine signaling pathways. We investigated how specific inhibition of either Jak1 or Jak2 by a small molecule inhibitor would affect the IFNγ-mediated response and we could demonstrate that inhibition of either Jak1 or Jak2 strongly reduces STAT1 phosphorylation, which in turn has a significant repercussion on IFNγ-mediated target gene expression. The vast majority of genes induced by IFNγ including those involved in the antiviral response were strongly down-regulated if Jak1 or Jak2 enzymatic activity was inhibited. Surprisingly, expression of two major players in IFN signalling, IRF1 and STAT1, was dependent on Jak2 but not Jak1. How relevant this finding is in terms of late IFNγ–mediated effects has to be further determined. It has been shown that STAT1 in its unphosphorylated state is transcriptionally active and initiates or sustains IFNγ-induced gene transcription at the late phase of the response (118, 203, 204), which would most likely be affected by specific Jak2 inhibitors. Our data further suggest that the selective inhibition of either Jak1 or Jak2 would impair the antiviral responses as well as the immune system, since the IFNγ-induced expression of many genes involved in these responses was strongly downregulated.

This study was based on the inhibitor analogue 1NM-PP1 that suppresses activity of analogue-sensitive kinases bearing small residues at the "gatekeeper" position. Ideally, only the analogue-sensitive kinases should be sensitive to this inhibitor. However, there are several naturally occurring kinases, such as Src family members, which possess a threonine residue at the "gatekeeper" site, which is also relatively small. It is known that these kinases are also sensitive to some inhibitor analogues (214, 215), thus, caution is needed in interpreting experiments performed using cells expressing the analogue-sensitive mutants instead of the wild-type kinases. Although we have performed experiments showing that the wild-type Jak1 and Jak2 were not suppressed by the inhibitor analogue 1NM-PP1 it cannot be excluded that other kinases i.e. Src were not affected. It has to be taken into account that kinase inhibitors may also bind to non-kinase proteins. 1NM-PP1 had to be used at relatively high concentration what increases the probability of non-kinase off-target effects (Fig. 17).

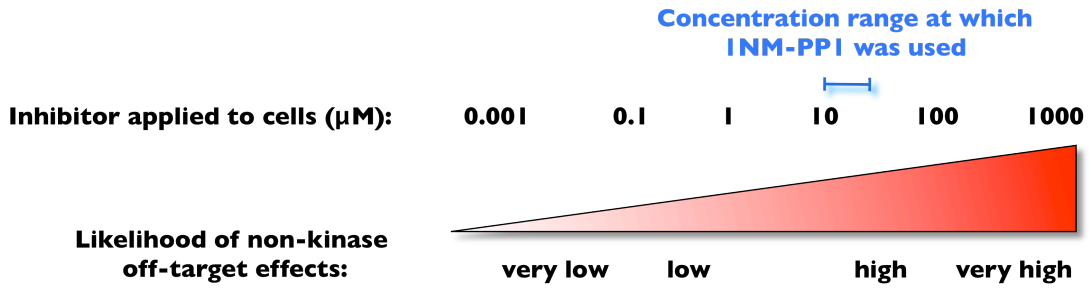


Figure 17

Estimated likelihood of non-kinase off-target effects at different concentration of inhibitor applied to cells. The fraction of small molecules that bind to a protein in vitro is usually low at nanomolar concentrations but it increases if used at μM range. Compounds used at concentrations above 100 μM are rather unlikely to have any significant selectivity. Adapted from (205). Concentration range at which the analogue inhibitor 1NM-PP1 was used to inhibit the analogue-sensitive Jak1 and Jak2 mutants is indicated.

By performing DNA microarray analysis we aimed at identification of genes, whose IFNγ-induced expression is either Jak1- or Jak2-dependent. Since we could not exclude the possibility that by treating the cells with the 1NM-PP1 inhibitor we target not only the analogue-sensitive Jak1 or Jak2 variant, we focused specifically on genes known to be IFNγ- and Jak-dependent. Experiments performed with cells expressing wild-type Jak1 or Jak2 served as controls confirming that the effects seen in cells expressing the analogue-sensitive Jaks upon 1NM-PP1 treatment are really due to suppression of AS-

Jaks and not to off-target effects of the inhibitor. Certainly, it would be of great interest to develop a bulky analogue of a Jak-specific inhibitor. Such Jak inhibitor-analogue could be used at lower concentration without compromising the efficiency of inhibition of the analogue-sensitive kinase what was an issue in case of 1NM-PP1.

The fact that a whole variety of cytokine receptors uses only four Jaks means that even a specific Jak inhibitor targets multiple cytokine pathways. Specific inhibitors for Jak1 or Jak2 to target e.g. leukemic cells expressing Jak1 or Jak2 gain-of-function mutants, respectively, will not confine their effects to the oncogenic pathways but would also have repercussion on receptors activating e.g. mainly Jak1 (IL-6-type cytokines) or only Jak2 (EpoR, TpoR). The question is, however, how a specific Jak1 inhibitor would affect other signaling pathways, which in addition to Jak1 require Jak2, Tyk2 or Jak3. We could demonstrate that the overall response to IFNγ is significantly decreased if either Jak1 or Jak2 was specifically inhibited. It would be of interest to apply an analogous approach to other signaling pathways, e.g. to IFNα/β- or IL-2-mediated responses, which signal via the combination of Jak1/Tyk2 and Jak1/Jak3, respectively.

KINASE INHIBITORS IN THE CLINIC

Regardless of the above-mentioned unspecificity presented by most inhibitors, several of them have successfully been approved for patient treatment (i.g. Sunitinib or Sorafenib target multiple kinases (Table 1)). Moreover, the case of Imatinib (Gleevec), an inhibitor of Abl kinase (206), shows that sometimes inhibiting more than one protein target increases the efficacy of treatment with great benefit for patients suffering from chronic myeloid leukemia (CML). In addition to Abl kinase, Imatinib has been identified as inhibitor of RTKs PDGFR (platelet derived growth factor receptor) and KIT (the receptor for SCF) (207). By applying a chemical genetics approach an analogue-sensitive allele of Bcr-Abl has been generated (35). Wong *et al.* have shown that specific inhibition of that mutant by the inhibitor analogue was sufficient to suppress cell proliferation in murine CML lacking the RTK KIT. Very surprisingly, in cells expressing KIT, single inhibition of Bcr-Abl had no physiologic effect. Combinatorial inhibition by Imatinib of both Bcr-Abl and KIT was necessary for a comparable obstruction of cell proliferation. These results argue that the efficacy of Imatinib in CML is not exclusively owing to its ability to block Bcr-Abl but, additionally, to some unspecificity towards KIT.

Moreover, suppression of Abl kinase activity by Imatinib was shown to induce ER stress (208), a mechanism that shuts down general protein translation and up-regulates the expression of specific protective stress response genes as well as apoptotic genes. Depending on the severity of the different responsive pathways, the cell is either protected or undergoes apoptosis, which is the case if ER stress is prolonged.

ER stress is induced by several drugs, e.g. the proteasome inhibitor Bortezomib used in cancer therapy (193). ER stress is associated with several signal transduction events, which play roles in different physiological effects. Global reduction of mRNA translation is achieved by phosphorylation and inactivation of eIF2α what leads to reduced protein synthesis. Interestingly, certain mRNAs gain a selective advantage for translation under these conditions, including the mRNA encoding transcription factor ATF4, which regulates the promotors of several genes implicated in the unfolded protein response. Depending on the activation intensity and the cellular context, eIF2α phosphorylation by upstream kinases, like PERK, can induce survival, growth arrest and/or apoptosis in response to ER stress. Our studies show for the first time that the protein kinase inhibitor Erlotinib induces activation of the phospho-eIF2α–ATF4 pathway. We showed that phosphorylation of eIF2α was linked to the induction of cell growth arrest in leukemic cells expressing Jak2 or Jak3 gain-of-function mutants. Noteworthy, none of these cells express EGFR, which represents the main target of Erlotinib, thus unravelling an interesting off-target effect of a compound that was believed to specifically target EGFR. Previously, other research groups have shown that Erlotinib inhibits the growth of leukemic cell lines and myeloblasts of patients with myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). However, this off-target effect was explained by the inhibitory action on Jak2 (192, 209-211). In our hands Erlotinib was not able to suppress the activity of Jaks, neither of the wild-type nor of mutants, and in *in-vitro* kinase assays it showed only micromolar potency (data not shown), which excludes it from being a “Jak inhibitor”. Molecular modelling indicated that the methionine gatekeeper of Jak2 precludes its binding to Erlotinib.

The rationale for combination treatment is to use drugs that work by different mechanisms of action, thereby decreasing the likelihood that resistant cancer cells will develop. Resistance to Imatinib treatment has been reported in patients with CML due to Bcr-Abl gene amplification or acquired point mutations of Bcr-Abl. Since there is no specific Jak inhibitor used right now in the clinic to treat patients suffering from any haematological neoplasm, it is difficult to predict what would be the influence of

prolonged administration of a drug targeting an oncogene, such as Jak2-V617F. Currently, a Jak2 inhibitor TG101348 is expected to enter phase II of clinical trails for treatment of myelofibrosis (*www.targegen.com*), which belongs to the group of myeloproliferative neoplasms where the Jak2-V617F mutation is found with high incidence. Because of its clinical relevance, TG101348 was included into our studies, showing strong inhibitory effect on the growth of leukemic cells. If used in combination with Erlotinib, the concentration of both inhibitors, TG101348 and Erlotinib, could be reduced without compromising the efficacy of growth suppression.

JAK2-V617F AND ITS REGULATION

Any therapy with the goal to treat cancer must show a differential toxicity towards tumour cells relative to healthy cells. Cancer cells usually show some unique properties not shared by normal cells, e.g. oncogene addiction and/or an altered metabolism. Cells expressing Jak gain-of-function mutants are “addicted” to their constitutive activity. On the other hand the tumorigenic properties of Jak mutants depend on the normal cellular functions of certain genes that are themselves not oncogenic. It has been shown that STAT5 together with the homodimeric type I receptors EpoR, TpoR, or G-CSFR are critical for Jak2-V617F-mediated transformation (172, 178). By this rationale, cancer cells are addicted to oncogenes, such as Jak2-V617F, and non-oncogenes like STAT5 or EpoR.

The level of activation of signaling proteins like STAT5 strongly depends on the level of Jak2-V617F mutant and on the overall induction of feedback regulators like SOCS proteins. We found that the Jak2-V617F protein, if catalytically active, is targeted to degradation in the proteasome. When we increase Jak2-V617F levels in cells, the level of phosphorylated STAT5 increases concomitantly, ultimately reaching a point at which cytokine stimulation does not further enhance signaling. Interestingly, 25-30% of patients with PV are characterized by Jak2-V617F homozygosity and display a significantly higher haemoglobin level as well as a higher rate of fibrotic transformation (212, 213). Using an animal model where expression of Jak2-V617F could be regulated it has been hypothesized that the ratio of mutant to wild-type Jak2 is critical for the phenotypic manifestation (201). If expression of Jak2-V617F was lower than the endogenous wild-type Jak2, mice developed a phenotype resembling ET (essential thrombocythemia). Conversely, higher levels of Jak2-V617F resulted in a PV-like phenotype.

Moreover, in addition to the V617F mutation in Jak2, several other alterations in the Jak genes have been identified leading to constitutively activated kinases. What is really interesting is the fact, that different mutational hotspots could be identified in Jak2 mutants that were found in patients suffering from different disease i.e. different types of leukemia (T-ALL, B-ALL, AML) and MPNs (PV, ET or IMF) (79). As mentioned-above, the MPN phenotype strongly depends on the level of Jak2-V617F, which regulates activation of STAT5. However, little is still known about the pathogenesis of leukemia with the exception of chronic myeloid leukemia, which in majority is caused by the BCR-ABL fusion protein. Why are some of the Jak mutants found in leukemia and others in MPNs? One could speculate that these Jak mutants might have different substrate specificities possibly due to binding to different cytokine receptor chains or cross-activating different Jaks bound to the second chain of the receptor complexes. Proteomic analysis would certainly be of use to generate protein expression and more importantly protein phosphorylation profiles for each Jak mutant. Substrate identification could employ the chemical genetics approach, as analogue-sensitive mutants allow for specific labeling of the direct substrates of the investigated kinases. Moreover, it would certainly be of interest to compare different Jak mutants with respect to their phosphorylation sites that in the end could determine direct interacting proteins of the Jak mutants. Identified substrates if involved directly in the tumorigenic cell transforming potency could be considered as potential targets for inhibitors.

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7 LIST OF ABBREVIATIONS

ABL	Abelson proto-oncogene
ALL	Acute Lymphoblastic Leukemia
AMKL	Acute Megakaryoblastic Leukemia
AML	Acute Myeloid Leukemia
AS	Analogue-Sensitive
ATF	Activating Transcription Factor
ATP	Adenosine Triphosphate
BCR	Break-point Cluster Region
CIS	Cytokine-inducible SH2 containing protein
CML	Chronic Myeloid Leukemia
CREB	cAMP Responsive Element Binding Protein
EGF	Epithelial Growth Factor
EPO	Erythropoietin
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-regulated Kinase
ET	Essential Thrombocythemia
FACS	Fluorescence-Activated Cell Sorting
FDA	Food and Drug Administration
FERM	4.1, ezrin, radixin, moesin
FRT	Flipase Recognition Target
GAS	Gamma-Activated Sequence
GIST	Gastrointestinal Stromal Tumor
gp130	Glycoprotein 130
GH	Growth Hormone
GRB-2	Growth Receptor Binding Protein 2
HAT	Histone Acetyltransferase
IFN	Interferon
IGF-1	Insulin-like Growth Factor 1
IL	Interleukin
IRE1	Inositol-requiring Enzyme 1
IRF	Interferon Regulated Factor
IRK	Insulin Receptor Kinase
JAK	Janus Kinase
JH	Jak Homology
JNK	Jun N-terminal Kinase

KIR	Kinase Inhibitory Region
MAPK	Mitogen-activated Protein Kinase
MPN	Myeloproliferative Neoplasm
NCBI	National Center for Biotechnology Information
NK	Natural Killer
NSCLC	Non-small Cell Lung Cancer
OSM	Oncostatin M
PIAS	Protein Inhibitor of Activated STAT
PDB	Protein Data Base
PH	Pleckstrin Homology
PI3-K	Phosphoinositide 3-kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PKI	Protein Kinase Inhibitor
PMF	Primary Myelofibrosis
PTP	Protein Tyrosine Phosphatase
PV	Polycythemia Vera
qPCR	Quantitative Polymerase Chain Reaction
RCC	Renal Cell Carcinoma
RTK	Receptor Tyrosine Kinase
SCID	Severe Combined Immunodeficiency
SH	Src Homology
SHP	SH2 domain-containing Phosphatase
STAT	Signal Transducer and Activator of Transcription
SOCS	Suppressor of Cytokine Signaling
TCPTP	T-Cell Protein Tyrosine Phosphatase
TPO	Thrombopoietin
TR	Tet Repressor
UPR	Unfolded Protein Response
XBP-1	X-box Binding Protein 1
YFP	Yellow Fluorescent Protein
ZAP-70	Zeta-chain-associated Kinase 70

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I'd like to acknowledge all the people from the ReceptEUR network. Particularly, big thanks to Arturo Sanz Sanz (Erasmus MC) for being of great help in the protein array experiments.

Anita, for the very special person she is and for the incredible amount of patience she had with me in the last 3 years. I think it's time now to start on that list of things to do "yes, after my thesis pumpkin" (*smile*).

Lastly, I offer my regards to all of those who supported me in any respect during the completion of my PhD.

9 APPENDIX

Hypoxia-Inducible Factor 1α Is Up-Regulated by Oncostatin M and Participates in Oncostatin M Signaling

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The interleukin-6–type cytokine oncostatin M (OSM) acts via the Janus kinase/signal transducer and activator of transcription pathway as well as via activation of mitogen-activated protein kinases and is known to critically regulate processes such as liver development and regeneration, hematopoiesis, and angiogenesis, which are also determined by hypoxia with the hypoxia-inducible factor 1α (HIF1α) as a key component. Here we show that treatment of hepatocytes and hepatoma cells with OSM leads to an increased protein level of HIF1α under normoxic and hypoxic conditions. Furthermore, the OSM-dependent HIF1α increase is mediated via Janus kinase/signal transducer and activator of transcription 3 and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase 1/2 pathways. OSM-mediated HIF1α up-regulation did not result from an increase in HIF1α protein stability but from increased transcription from the *HIF1α* gene. In addition, we show that the OSM-induced *HIF1α* gene transcription and the resulting enhanced HIF1α protein levels are important for the OSM-dependent vascular endothelial growth factor and plasminogen activator inhibitor 1 gene induction associated with several diseases. *Conclusion:* HIF1α levels increase significantly after treatment of hepatocytes and hepatoma cells with OSM, and HIF1α contributes to OSM downstream signaling events, pointing to a cross-talk between cytokine and hypoxia signaling in processes such as liver development and regeneration. (HEPATOLOGY 2009;50:253-260.)

Oncostatin M (OSM) is an interleukin (IL)-6–type cytokine produced by monocytes and macrophages, T cells, and several other cell types. OSM receptors are widely expressed and are composed of the common signal transducer gp130 in complex with the LIFR or the OSMR. OSM has pleiotropic effects that in part overlap with those of other IL-6–type cytokines; examples include inflammation, neurogenesis, regulation of cell proliferation, and fibrosis.[1-3] In addition, OSM plays a crucial role in the orchestration of hematopoiesis and liver development.[3]

Upon OSM-induced receptor clustering, Janus kinases—mainly Janus kinase 1—are activated, phosphorylate tyrosines within the receptor that recruit other signaling proteins with matching SH2 domains such as signal transducers and activators of transcription (STATs) or adapter proteins for the mitogen-activated protein kinases to the receptor. The major signaling cascades activated by OSM include STAT3 as well as the extracellular signal-regulated kinase (Erk) 1/2 and p38 pathways.[2-4]

Several processes such as hematopoiesis, angiogenesis, liver development, metabolism, inflammation, and tu-

Abbreviations: Erk, extracellular signal-regulated kinase; HIF, hypoxia-inducible factor; HIF1α, hypoxia-inducible factor 1α; HRE, hypoxia response element; IL, interleukin; mRNA, messenger RNA; OSM, oncostatin M; PAI1, plasminogen activator inhibitor 1; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; siRNA, small interfering RNA; STAT, signal transducer and activator of transcription; TNF-α, tumor necrosis factor α; VEGF, vascular endothelial growth factor.

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morigenesis are also crucially influenced by the ambient oxygen tension of the tissue. Hypoxia-inducible factors (HIFs) act as master regulators for the expression of genes essential in a hypoxic microenvironment. The best characterized factor is HIF1, which regulates more than 100 genes. HIF1 is formed by dimerization of the oxygen-sensitive hypoxia-inducible factor 1α (HIF1α) subunit with the constitutively expressed beta-subunit, which is also known as ARNT. Under normoxic conditions, the HIF1α subunit is hydroxylated and quickly degraded via the proteasome. Under hypoxic conditions, the activity of the hydroxylases is reduced, and HIF1α protein is stabilized and can bind to hypoxia-response elements (HREs) within the regulatory areas of HIF target genes and efficiently recruit cofactors.[5]

In addition to hypoxia, HIF1α has also been shown to be up-regulated under normoxia in response to growth factors, thrombin, lipopolysaccharide, angiotensin II, insulin, or the cytokines IL-1 and tumor necrosis factor α (TNF-α).[6] Although several details have been unraveled regarding the ability of hypoxia to stabilize HIF1α, the mechanisms by which those factors (especially cytokines) induce HIF1α have not been fully elucidated.

Interestingly, OSM has been described to orchestrate the hypoxia-influenced processes of hematopoiesis, angiogenesis, liver development, and regeneration.[3,7] This suggests a possible cross-talk of the OSM and hypoxia signaling pathways. The expression of the HIF1 target genes vascular endothelial growth factor (VEGF) and plasminogen activator inhibitor 1 (PAI1)—which are crucial for angiogenesis and tissue remodeling, respectively—can also be up-regulated by the cytokine OSM.[8-12] OSM supports *in vitro* differentiation of fetal hepatic cells into liver-like structures, which is paralleled by enhanced VEGF expression.[11] Moreover, OSM mediates differentiation of oval cells into hepatocytes.[13] OSM plays a crucial, nonredundant role in liver regeneration, as shown for OSM receptor knock-out mice after partial hepatectomy or CCl_4 treatment,[14] and OSM gene therapy attenuates liver damage induced by dimethylnitrosamine.[15] Interestingly, HIF1α is also expressed during liver regeneration,[16] and HIF1α supports the growth of hepatoma cells *in vivo* and *in vitro*.[17,18]

Because both OSM and HIF1α play a pivotal role in liver-related processes (development, regeneration, carcinogenesis), the aim of the present study was to investigate whether the OSM signaling pathway has an impact on the HIF1 system in hepatoma cells and hepatocytes.

Materials and Methods

Cell Culture and Reagents. HepG2 hepatoma cells were maintained in DMEM/NUT-MIX-F12 medium (Lonza) supplemented with 10% fetal bovine serum (PAA), 100 mg/L streptomycin, and 60 mg/L penicillin (Cytogen). The human hepatocyte cell line PH5CH8 has been described.[19] Cells were grown at 37°C in a water-saturated atmosphere at 5% CO_2. Hypoxia treatment was performed at 37°C in a water-saturated atmosphere at 5% CO_2 and 6% oxygen. HepG2 cells were transfected using the Fugene reagent (Roche) according to the manufacturer's recommendations. Cotransfections of small interfering RNA (siRNA) and reporter gene constructs are described in the Supporting Information. Human recombinant OSM was obtained from Peprotech. Actinomycin D and cycloheximide were obtained from Calbiochem. Stattic[20] was from Sigma.

Western Blot Analysis and Antibodies. All steps of cell lysis and immunoprecipitation were performed at 4°C using ice cold buffers. Cells were lysed on a dish with lysis buffer containing 30 mM Tris/HCl (pH 6.7), 5% glycerol, 2.5% mercaptoethanol, and 1% sodium dodecyl sulfate. The lysates were further analyzed via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. Antibodies against HIF1α, STAT3, STAT1, and Fin13 were obtained from BD Transduction Laboratories. Antibodies against phospho-STAT3, phospho-STAT1, Erk1/2, phospho-Erk1/2, p38, and phospho-p38 were obtained from Cell Signaling. ECL signals were detected as described.[21] Before reprobing, blots were stripped as described.[4]

Reporter Gene Assays. HepG2 cells were transfected with 1 μg of the β-galactosidase control plasmid (pCH110, Amersham Biosciences) and 1.5 μg of the respective reporter gene construct. Twenty-four hours after transfection, the cells were treated with the different stimuli as described in the figure legends. Cell lysis and luciferase assays were performed using the Promega luciferase assay system (Promega, Madison, WI) (see Supporting Information for further details). All experiments were performed at least in triplicate, and biological triplicates were also performed within one experiment. Luciferase activity values were normalized to transfection efficiency monitored by the cotransfected β-galactosidase expression vector. For some experiments the luciferase activity values after OSM stimulation were additionally normalized to values from control unstimulated cells.

Quantitative Real-Time Polymerase Chain Reaction. The exact protocol is described in the Supporting Information. Total RNA was extracted using the RNeasy Mini Kit (Macherey Nagel) according to the manufacturer's instructions. The concentration of isolated RNA was measured using a NanoDrop spectrophotometer. One microgram of total RNA was reverse-transcribed with a ThermoScript RT-PCR System (Invitrogen). Quantita-

tive real-time polymerase chain reaction (PCR) was performed on an iQ5 Real-Time PCR detection system (Bio-Rad Laboratories). Standard curves using four 10-fold dilutions (1×, 0.1×, 0.01×, 0.001×) were produced to ensure that the amplification efficiencies were similar and in the range of 95% to 105%. The messenger RNA (mRNA) level of each target gene was normalized to the relative amount of the housekeeping gene TBP. The comparative threshold cycles (C_T) method, $2^{-\Delta CT}$, was used to calculate the changes in gene expression for each target gene.

Statistical Analysis. Each experiment was performed at least three times. Representative data are shown and are expressed as the mean ± standard deviation. Depending on datasets, statistical analysis was performed using a *t* test, Mann-Whitney test, or analysis of variance. *P* values of <0.05 were considered significant.

Results

OSM Increases Expression of Functional HIF1α Under Normoxic Conditions. Exposure of HepG2 hepatoma cells to OSM for different times led to a profound and transient increase in HIF1α protein levels that lasted up to 24 hours. The up-regulation reached a maximum after about 6 hours of stimulation. As expected, OSM induced STAT3, Erk1/2, and p38 phosphorylation (Fig. 1A). HIF1α was also induced upon stimulation of the human hepatocyte cell line PH5CH8 with OSM (Fig. 1B). Quantitation of western blots showed that upon OSM stimulation, the HIF1α protein is up-regulated by factors of 2.66 ± 0.3 in PH5CH8 cells and 2.7 ± 0.9 in HepG2 cells.

To investigate whether OSM-induced HIF1α is functional, we tested the effect of OSM on HepG2 cells transfected with a HIF1-responsive luciferase reporter gene construct. OSM treatment increased luciferase activity three-fold (Fig. 1C). Thus, OSM-induced HIF1α is transcriptionally active even under normoxic conditions.

OSM Augments Hypoxia-Dependent HIF1α Induction and Hypoxia-Mediated Target Gene Expression. Next, we compared the induction of HIF1α by OSM under normoxia with that induced by hypoxia. In addition, we examined whether OSM may affect the hypoxia-dependent induction of HIF1α. We found that HIF1α levels induced by OSM under normoxia were slightly lower than those induced by hypoxia after 4 and 6 hours of induction, respectively. Interestingly, when cells were treated with OSM under hypoxia, the increase in HIF1α protein levels was higher than under each treatment alone (Fig. 2A). Quantitation of western blots showed that the HIF1α protein is up-regulated by factors of 3.6 ± 0.19 upon hypoxia, 2.7 ± 0.9 upon OSM, and 6.4 ± 1.8 upon combined treatment with hypoxia and OSM.

We then investigated HIF1α mRNA expression under the same conditions and found that OSM induces HIF1α mRNA levels stronger (3.4-fold) than hypoxia (2.3-fold). Combined treatment with hypoxia and OSM led to an

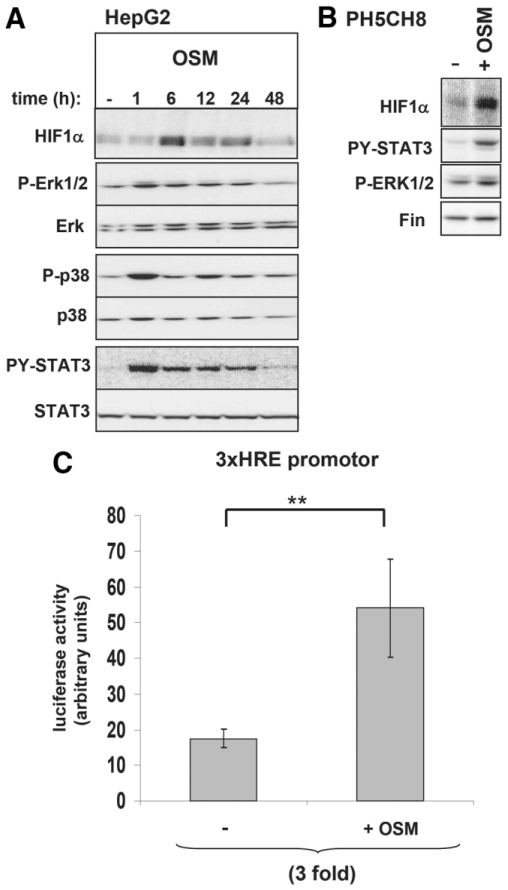

Fig. 1. OSM induces HIF1α protein levels over an extended period, and the induced HIF1α is transcriptionally active. (A) HepG2 cells were treated for the indicated periods with OSM (10 ng/mL). Lysates of the cells were separated via SDS-PAGE, and western blots of the membranes were detected with HIF1α, phospho-STAT3, phospho-Erk1/2, phospho-p38, STAT3, Erk1/2, and p38 antibodies. (B) PH5CH8 cells were stimulated for 4 hours with OSM (10 ng/mL) or left untreated. Western blots of the membranes were detected as described in (A). (C) HepG2 cells were transfected with the luciferase reporter gene plasmids pGL3-EPO-HRE-Luc and the β-galactosidase expression vector pCH110. Twenty-four hours after transfection, the medium was exchanged and the cells were treated for an additional 16 hours with OSM (10 ng/mL) before lysates were prepared, and the reporter gene activity was measured as described in Experimental Procedures. $^{**}P < 0.01$.

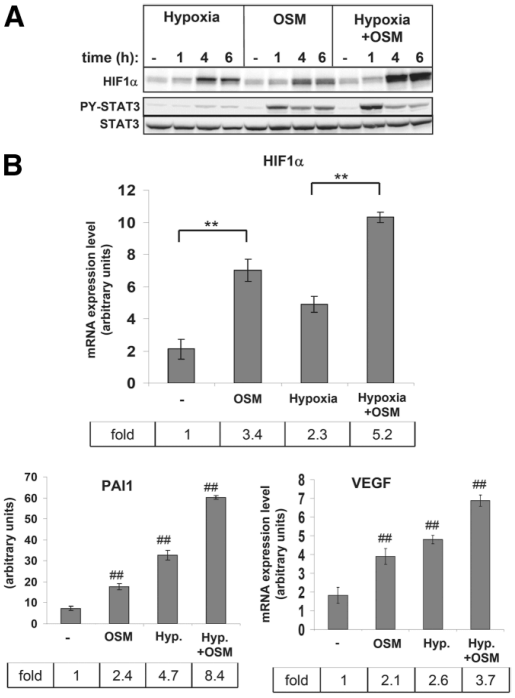

Fig. 2. OSM enhances hypoxia-induced HIF1α, PAI1, and VEGF expression. (A) HepG2 cells were treated for the indicated periods with hypoxic conditions and/or OSM (10 ng/mL). Lysates of the cells were separated via SDS-PAGE, and western blots of the membranes were detected with antibodies directed against HIF1α, phospho-STAT3, and STAT3. (B) HepG2 cells were stimulated for 4 hours with hypoxic conditions and/or OSM (10 ng/mL). RNA was prepared, and HIF1α mRNA levels were analyzed via quantitative PCR. $^{**}P < 0.01$. (C) HepG2 cells were treated as described in (B) and VEGF and PAI1 mRNA levels were analyzed via quantitative PCR. $^{\#\#}P < 0.01$ versus untreated controls.

even higher induction of HIF1α mRNA (5.2-fold) (Fig. 2B). Thus OSM significantly increases HIF1α mRNA under normoxia and hypoxia, which matches with the protein up-regulation seen in the western blots (Fig. 2A). Two important target genes of the hypoxic response, PAI1 and VEGF, were also significantly up-regulated by OSM, and a combined treatment with hypoxia and OSM led to an even stronger induction of PAI1 and VEGF mRNA (Fig. 2C).

OSM-Mediated Up-Regulation of HIF1α Protein Levels Is Due to De Novo Transcription but not Regulation of Protein Stability. To find out whether OSM influences HIF1α protein stability, we aimed to measure HIF1α protein half-life. Therefore, we stimulated HepG2 cells with OSM or $CoCl_2$ (a hypoxia mimetic) for 6 hours to induce a robust HIF1α expression before the translation inhibitor cycloheximide was added for different periods. We found that the OSM-induced HIF1α protein disappeared completely after a 10-minute treatment with cycloheximide (Fig. 3A, right panel). In contrast, $CoCl_2$-induced HIF1α was still well detectable after 1 hour; it disappeared after 3 hours of cycloheximide treatment (Fig. 3A, left panel). There was no difference in HIF1α protein stability between $CoCl_2$ and the combined treatment with OSM and $CoCl_2$. These data show that the OSM-mediated HIF1α up-regulation is not due to an enhanced stability of the protein.

In contrast, experiments with the transcription inhibitor actinomycin D showed that less HIF1α protein was detectable when cells were treated with OSM in the presence of actinomycin D relative to cells treated with OSM alone (Fig. 3B). This was even more remarkable because actinomycin D treatment prevents the STAT3-induced up-regulation of the feedback inhibitor SOCS3, which suppresses STAT3 activation upon OSM. Despite the higher STAT3 activation observed in actinomycin D–treated cells, no HIF1α expression could be observed. Actinomycin D did not affect HIF1α expression in cells exposed to hypoxia, which was expected because hypoxia essentially increases protein stability. Here, the HIF1α

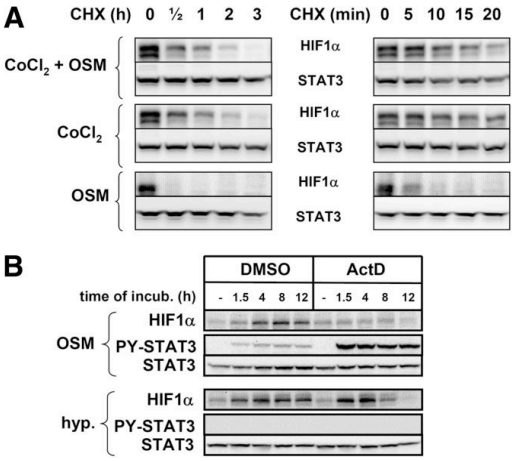

Fig. 3. OSM increases transcription of HIF1α but does not affect HIF1α protein stability. (A) HepG2 cells were stimulated for 6 hours with $CoCl_2$ (50 μM) and/or OSM (10 ng/mL). Cycloheximide (10 μg/mL) was then added for the indicated periods before lysates were prepared. Western blots were detected with HIF1α and STAT3 antibodies. For HIF1α detection, the western blots were exposed so that the band intensity of the untreated lane for all treatments was comparable. (B) HepG2 cells were treated for the indicated periods with OSM (10 ng/mL) or hypoxia (hyp.) in the presence of dimethyl sulfoxide alone or actinomycin D (5 μg/mL). Western blots of lysates separated via SDS-PAGE were detected with HIF1α, phospho-STAT3, and STAT3 antibodies.

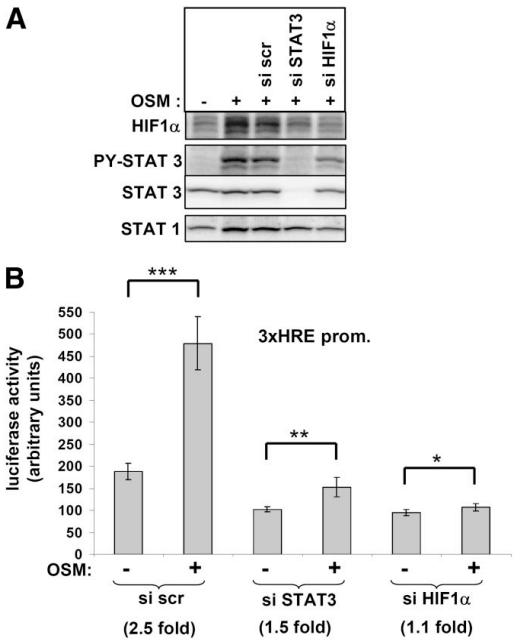

Fig. 4. Knockdown of STAT3 by siRNA decreases HIF1α protein levels and HIF1α-responsive reporter gene activity upon OSM treatment. (A) HepG2 cells were transfected with siRNAs as indicated. Forty-eight hours after transfection, cells were treated for 4 hours with OSM (10 ng/mL). Lysates of the cells were separated via SDS-PAGE, and western blots of the membranes were detected with HIF1α, phospho-STAT3, STAT3 and STAT1. (B) HepG2 cells were transfected with siRNAs as indicated, the luciferase reporter gene plasmids pGL3-EPO-HRE-Luc and the β-galactosidase expression vector pCH110. The cells were treated for additional 16 hours with OSM (10 ng/mL) before lysates were prepared. $***P < 0.001$. $**P < 0.01$. $*P < 0.05$.

levels decreased only after 8 hours, in accordance with the normal half-life of HIF1α under hypoxia (8-10 hours).[22]

In addition, our data in the Supporting Information show that OSM, in contrast to hypoxia, does not affect hydroxylation and ubiquitination (Supporting Fig. 2). Together, these data provide evidence that OSM enhances HIF1α via transcriptional regulation.

STAT3 Protein Is Crucial for OSM-Induced Up-Regulation of HIF1α. After having shown that OSM-mediated up-regulation involves transcriptional regulation, we addressed the role of STAT3, playing a pivotal role in signaling of IL-6–type cytokines. Suppression of STAT3 by siRNA led to a loss of HIF1α expression upon OSM treatment, similar to the HIF1α siRNA used as a positive control (Fig. 4A). The unspecific control siRNA did not show these pronounced effects. Upon transfection of STAT3 or HIF1α siRNAs together with the 3xHRE reporter gene construct, OSM only weakly induced the reporter gene activity, whereas OSM induced reporter gene activity 2.5-fold when the unspecific control siRNA was transfected (Fig. 4B). In addition, the STAT3 inhibitor Stattic or dominant negative STAT3 also inhibited OSM-dependent effects (increase of HIF1α protein and mRNA levels, HRE promotor activity, or target gene induction) (Supporting Figs. 3-5). Furthermore, we provide evidence for the relevance of Erk signaling in OSM-mediated induction of HIF1α protein and activity (Supporting Figs. 3-5).

OSM-Induced HIF1α Is Crucially Involved in the Transcriptional Regulation of the Genes for VEGF and PAI1. To investigate the relevance of HIF1α expression in OSM signal transduction, we examined the effects of HIF1α suppression on the target genes PAI1 and VEGF. We found that the OSM-dependent induction of both the VEGF and PAI1 mRNA were decreased upon HIF1α suppression (Supporting Fig. 6). In addition, the OSM-mediated induction of the VEGF and PAI1 promotor was down-regulated by HIF1α siRNA and by STAT3 siRNA (Fig. 5A), while a control siRNA had no effect. Consistent with this finding, we found that the OSM-dependent induction of the VEGF promoter was reduced by about 50% when a VEGF promoter construct mutated at the HRE was used for transfection (Fig. 5B). Because the robust induction of the VEGF promoter by hypoxia requires the integrity of the HRE and the AP1 site, we also used a construct where the AP1 site was mutated; the OSM-dependent induction of this promoter was reduced by about 25% (Fig. 5B). Importantly, mutation of the previously described STAT3 binding site within the VEGF promoter had no effect on the OSM-dependent induction of reporter gene activity (Fig. 5B). Thus, OSM-induced VEGF transcription seems to be mediated via HIF1 and AP1, rather than by STAT3.

In addition, HIF1 seems to play a crucial role in OSM-mediated activation of the PAI1 promotor, for which a reduction of about 40% was observed when the HRE was mutated (Fig. 5C).

Together, these data indicate that *de novo*–transcribed HIF1α importantly contributes to the OSM-induced VEGF and PAI1 transcription.

Discussion

The first major finding of the present study is that the cytokine OSM, which activates the STAT3, Erk1/2, and p38 signaling pathways, can induce a robust up-regulation of HIF1α protein levels in hepatocytes and hepatoma cells under normoxic conditions and leads to the formation of transcriptionally active HIF1 complexes. OSM-induced HIF1α protein up-regulation was stronger compared with other cytokines (IL-6, IL-1β, TNF-α, interferon-γ), some of which have been implicated in

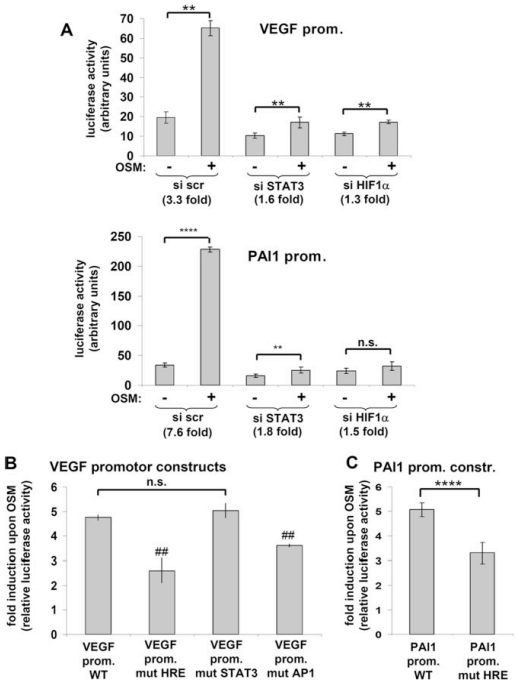

Fig. 5. Regulation of the VEGF and PAI1 gene promotors by OSM. (A) HepG2 cells were transfected with siRNAs as indicated, the luciferase reporter gene plasmids pGL3-VEGF-Luc or pGL3-hPAI1-796 and the β-galactosidase expression vector pCH110, and processed as described in Fig. 4B. $^{****}P < 0.0001$. $^{**}P < 0.01$. n. s., not significant. (B) HepG2 cells were transfected with the luciferase reporter gene plasmids pGL3-VEGF-Luc or the following mutants thereof: pGL3-VEGFmutHRE-Luc (containing a mutated HIF1α binding site), pGL3-VEGFmutSTAT3-Luc (containing a mutated STAT3 binding site), or pGL3-VEGFmutAP1-Luc (containing a mutated AP1 binding site). The β-galactosidase expression vector pCH110 was cotransfected. Twenty-four hours after transfection, the medium was exchanged, and the cells were treated for an additional 16 hours with OSM (10 ng/mL) before lysates were prepared. Values for luciferase activity (relative to β-galactosidase activity) are shown as fold induction compared with untreated samples. $^{\#\#}P < 0.01$ versus wild-type construct. (C) HepG2 cells were transfected with pGL3-hPAI1-796 or with pGL3-hPAI1-796-M2 containing a mutation in the HRE and processed as described in (B). $^{****}P < 0.0001$.

HIF1α up-regulation before (IL-6, IL-1β, TNF-α) (Supporting Fig. 1). Moreover, the OSM-increased HIF1α protein was shown to be involved in the enhanced expression of the HIF1 target genes PAI1 and VEGF.

Our study also shows that OSM and hypoxia differ in their mechanism of HIF1α up-regulation. Regulation of HIF1α activity is complex and under normoxic conditions, HIF1α has an extremely short half-life because it is continuously degraded due to the initial hydroxylation at two proline residues and transcriptional activity is reduced due to hydroxylation of asparagine 803. Hypoxia reduces the activity of the oxygen-utilizing hydroxylases, thereby stabilizing the protein and increasing its transactivity.[5] However, our data provide evidence that OSM does not contribute to an increased stability or increased transactivity as shown in the experiments with the Gal-HIF1α-TADN or TADC gene constructs (Supporting Fig. 2C). Furthermore, we could show that OSM-induced HIF1α was clearly ubiquitinated, which was less the case for hypoxia-stabilized HIF1α (Supporting Fig. 2A,B). We propose that transcriptional mechanisms are responsible for the OSM-mediated HIF1α protein up-regulation because it was inhibitable by the transcriptional inhibitor actinomycin D, whereas this was not the case for hypoxic treatment. The importance of HIF1α regulation at the mRNA level is further supported by findings showing that hepatocyte growth factor, angiotensin-II, lipopolysaccharide, IL-1, thrombin, or hypoxia also enhance HIF1α mRNA levels in different cell types (see Bonello et al.[23] and references therein).

Moreover, our tests with inhibitors for Janus kinases, STAT3, and mitogen-activated protein kinase kinase indicate that these pathways play an important role in OSM-dependent induction of HIF1α mRNA and protein expression (Supporting Fig. 3), HIF1α-dependent reporter gene activity (3x HRE promotor) (Supporting Fig. 4A), as well as in the regulation of the target genes VEGF and PAI1 (Supporting Fig. 5). In contrast, inhibitor tests suggested that p38 mitogen-activated protein kinases or the PI3K/Akt pathway are not involved in OSM-induced HIF1α expression (data not shown).

Experiments with siRNA further revealed that STAT3 plays a crucial role in OSM-regulation of HIF1α protein levels and HIF1α-dependent transcriptional activity. Moreover, dominant negative STAT3 (STAT3-DN) also led to a down-regulation of HIF1α-dependent transcriptional activity (Supporting Fig. 4B). It has been shown that HIF1α transcription is regulated by SP-1[24] and NF-κB transcription factors.[23] In addition to that, the present study indicates that the transcriptional regulator STAT3 appears to be a key player for HIF1α transcription in response to OSM. STAT3 was also found to be involved in HIF1α mRNA expression in tumor cells and tumor-associated myeloid cells.[25]

Our study provides evidence that HIF1α is important for OSM signal transduction. Experiments with HIF1α siRNA showed that the expression of the OSM target genes VEGF and PAI1 involves regulation by HIF1α. OSM and other IL-6–type cytokines have been shown to induce expression of the HIF1 target gene VEGF.[10,12,26-29] The VEGF gene is also considered a STAT3 target gene, because a dominant negative STAT3 reduced[10,27,30,31] and constitutively active forms of

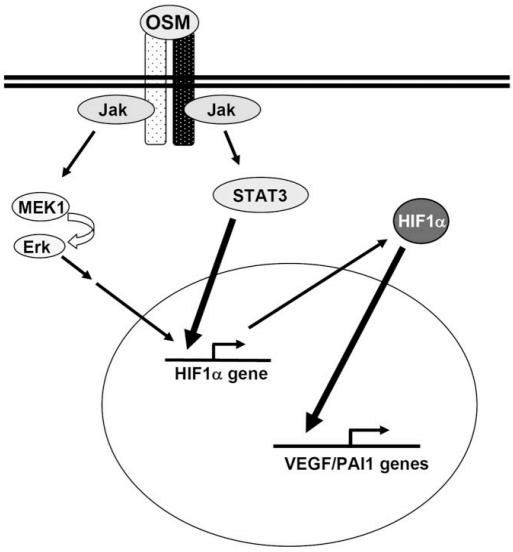

Fig. 6. Schematic representation of VEGF gene regulation by OSM.

STAT3 induced the VEGF promoter,[27,30,31] and a STAT3 binding element was detected at site −848.[30,31] We found that mutation of the described STAT3 binding site in the VEGF promoter at −848 did not affect OSM-dependent induction of reporter gene activity. These data are consistent with those of another study in which deletion of this site also did not affect IL-6–induced reporter gene activity.[27] Instead, our results with the VEGF reporter gene constructs clearly demonstrate a relevance of the HIF1 and AP1 binding elements for OSM-mediated regulation, because the constructs mutated at the HRE and the AP1 sites displayed significantly reduced induction in response to OSM. Thus, we conclude from our data that STAT3 regulation of the VEGF gene may rather be mediated indirectly via Erk1/2 and STAT3-dependent induction of HIF1α transcription. HIF1α then regulates VEGF and PAI1 transcription (Fig. 6), which leads to increased secretion of VEGF and PAI1 proteins (Supporting Fig. 7).

Although the present study was performed with hepatoma cells and nontransformed hepatocytes, it will be interesting to further clarify the role of HIF1α in OSM-mediated signal transduction and regulation of secreted factors involved in tissue remodeling (such as VEGF and PAI1) in processes such as liver development, regeneration, and inflammation.

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Supplemental information:

Supplemental experimental procedures

Reporter gene constructs

The pGL3-EPO-HRE-Luc containing three HRE elements preceding the firefly luciferase gene was described before (1). The pGL3-VEGF-Luc contains the *Kpn*I-*Nhe*I fragment of the human VEGF promoter region from -2.28 kb to +55 bp relative to the transcriptional start site (2) upstream of the firefly luciferase gene. Mutations were introduced into the published binding sequences for HIF1 at -978 (ACG → CGA) (3), for STAT3 at -848 (TTCC→GCGT) (4), or for AP1 at -1129 (TCA→GCT) (5). The pGL-hPAI-796 plasmid containing the human PAI1 promoter 5'-flanking region from -796 bp to +13 bp and the pGL3-hPAI-796-M2 construct with a mutation in the HRE were described (6). The constructs for pG5E1B-LUC (7), pSG424 (8) and for Gal4-HIF1α-TADN, Gal4-HIF1α-TADNM (9), Gal4-HIF1α-TADC and Gal4-HIF1α-TADCM (9) were already described. In Gal4-HIF1α-TADNM and Gal4-HIF1α-TADCM proline P564 or asparagine N803 were mutated to alanine, respectively.

Detailed description of the quantitative PCR procedure

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The concentration of isolated RNA was measured using a NanoDrop spectrophotometer. 1 µg of total RNA was reverse-transcribed with the ThermoScript RT-PCR System (Invitrogen) in a volume of 20 µL, using 50 mM oligo$(dT)_{20}$ according to the manufacturer's instructions. The cDNA was additionally treated with 2 units RNase H at 37°C for 20 min.

Quantitative real time PCR (qPCR) was carried out on an iQ5 Real-Time PCR detection system (Bio-Rad Laboratories). The reaction was performed in a total volume of 20 µL containing cDNA corresponding to 12.5 ng RNA template, 10 pmol of each forward and reverse primer and 10 µL of 2x Absolute QPCR SYBR Green Fluorescein Mix (ThermoScientific). Thermal cycling conditions for all qPCR assays consisted of an initial enzyme activation step at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C and annealing and elongation at 60°C for 30 sec. The housekeeping gene TBP (TATA-binding protein) and the target genes were assayed in parallel for each sample. All samples were run in triplicates.

Standard curves using four 10-fold dilutions (1x, 0.1x, 0.01x, 0.001x) were produced to ensure that the amplification efficiencies were similar and in the range of 95-105%. The exact efficiencies were 103.9% for TBP, 98% for HIF1a, 102.4 % for VEGF and 98.5% for PAI1. The specificity of real-time PCR was verified by single product-specific melting curves. The melting curve analysis was carried out as follows: 95ºC for 1 min, 60ºC for 1 min and 60ºC for 30 sec followed by steps of increases of 0.5ºC for 30 sec until 95ºC is reached. Gene-specific primers used in qPCR

analysis were purchased from Eurogentec (Belgium), as follows: TBP forward 5`-ACCCAGCAGCATCACTGTT-3`, reverse 5`- CGCTGGAACTCGTCTCACTA-3`; HIF1α forward 5`-CGTTCCTTCGATCAGTTGTC-3`, reverse 5`-TCAGTGGTGGCAGTGGTAGT-3`; VEGF forward 5`-TTCTCACACCATTGAAACCA-3`, reverse 5`-GATCCTGCCCTGTCTCTCTG-3`; PAI1 forward 5`-TTCAGAGGTGGAGAGAGCC-3`, reverse 5`-TTGTCCACGGCTCCTTTC-3`.

siRNA transfections

2.5 x 10^5 HepG2 cells were transfected with either STAT3 or HIF1 validated Stealth Duo Pak siRNA or Stealth RNAi negative control using 3 ml Lipofectamine RNAiMAX per well (all from Invitrogen) following the reverse transfection protocol. The final concentration of each duplex was 30 nM. 48 h after transfection the cells were used for qPCR or Western blot analysis.

For cotransfection of siRNAs and reporter gene constructs, transfection mixtures included 30 nM siRNA and additionally 500 ng of the β-galactosidase control plasmid and 500 ng of the reporter gene construct. 5 ml Lipofectamine RNAiMAX were used per well according to the reverse transfection protocol.

ELISA

5 x 10^5 HepG2 cells per well were seeded in 1.5 mL FCS-free DMEM/F-12. Cells were treated with 10 ng/mL OSM for 12h before the supernatants were harvested. ELISA for VEGF and PAI1 (both R&D Systems) were used according the manufacturer's protocol to analyze these proteins in the supernatants.

Other reagents

pCAGGS or pCAGGS-STAT3DN (gifts from Koichi Nakajima and Toshio Hirano, Osaka, Japan). Jak inhibitor I, U0126, PD98059, SB202190, SB203590, Wortmannin, LY294002 and MG132 were from Calbiochem. Stattic was from Sigma. Antibodies against IκB and phospho-IκB used for Western blot detections were from New England Biolabs. The g-fibrinogen antibody was from Upstate. Human recombinant IFNg, IL-6, TNFα and IL1β were from Peprotec. The VEGF and PAI1 ELISA kits were from R&D.

Supplemental results

OSM induces HIF1α more efficiently than other cytokines.

As IL1β and TNFα, both activating the NFκB pathway, have previously been described to lead to an increased HIF1a expression (10), we compared the effect of OSM versus the effects of these inflammatory cytokines in HepG2 cells. Whereas IL1β clearly leads to a transient increase of HIF1α expression after 4 and 6 hours, TNFa-induced expression was only marginal, although the cells were responsive to TNFα and IL1β, as phosphorylation of IkB could readily be observed (Suppl. Fig. 1A). The increase of HIF1α by OSM was stronger than for the two other stimuli. Costimulation of OSM and IL1b enhanced the HIF1α levels even more (data not shown).

IL6, which induces STAT3 as does OSM, also led to an upregulation of HIF1α but OSM had a much stronger effect than IL6 (Suppl. Fig. 1B). However, IFNγ, which induces a strong STAT1 but not a STAT3 signal, did not affect HIF1α expression significantly. Thus, compared to other cytokines, OSM seems to be a particularly potent inducer of HIF1α.

OSM and hypoxia differentially affect HIF1α expression and activity.

Under normoxic conditions the HIF1α subunit is hydroxylated, polyubiquitinated and quickly degraded via the proteasome. Under hypoxic conditions the activity of the hydroxylases is reduced, HIF1α protein is stabilized and can bind to hypoxia-responsive elements (HREs) within the regulatory areas of HIF target genes and efficiently recruit cofactors.

To compare the ubiquitination status of HIF1α, we incubated cells treated with OSM, hypoxia and OSM plus hypoxia with the proteasome inhibitor MG132. We observed that upon addition of MG132 a broad HIF1α band pattern of weaker intensity appeared at higher molecular masses on the Western blot as observed by others (11, 12). This band pattern represents the polyubiquitinated HIF1α. Two exposures of the HIF1α detection are shown since the intensity of the HIF1α and ubiquitinated- HIF1α bands was different. In the lower exposure one can see the similar induction of HIF1α by OSM and hypoxia and the even higher induction by the combined treatment (see also printed text). Upon OSM treatment the polyubiquitinated HIF1α band increases, thus showing that a polyubiquitination readily occurs upon OSM-treatment (compare lanes 3 and 4 in Suppl. Fig. 2A). This observation is in line with the observed quick degradation of OSM-induced HIF1α (Fig. 3A)). Upon stimulation by hypoxia alone or OSM and hypoxia combined the polyubiquitinated HIF1α band decreases in intenstiy compared to the control (lane 3)(compare lanes 3, 7 and 8 in Suppl. Fig. 2A), although the HIF1α levels are much elevated. This corresponds to a reduction of ubiquitination compared to the relative HIF1α expression. Thus, our results show that OSM-stimulated HIF1α is clearly ubiquitinated while hypoxic conditions interfere with the ubiquitination as described previously (13).

In addition, we performed an ubiquitination assay based on transfection of both HIF1α and ubiquitin. HepG2 cells, transfected with vectors for V5-tagged HIF1α and HA-ubiquitin, were treated with OSM or incubated under hypoxia for 6 h. Western blots of the immunoprecipitates (with the V5-tag antibody) were detected with a ubiquitin antibody. We found that HIF1a was strongly ubiquitinated under normoxia while ubiquitination occurred to a lesser extend under hypoxia. Treatment of cells with OSM did not affect ubiquitination when compared to the normoxic control. Thus, these data indicate that OSM does not inhibit ubiquitination of HIF1α.

We next compared the ability of OSM and hypoxia to interfere with the hydroxylation of the proline and asparagine residues involved in protein stabilization and transactivation. These residues are hydroxylated by corresponding hydroxylases under normoxia but not under hypoxia. They are located in two transactivation domains (TADs) present in HIF1α and referred to as amino-terminal (TADN) (contains proline) and carboxy-terminal (TADC) (contains asparagine) TAD. HepG2 cells were cotransfected with the luciferase reporter construct pG5-E1B-Luc that contains 5 copies of a Gal4 response element and vectors allowing expression of fusion proteins consisting of the Gal4-DNA binding domain (Gal4) and either HIF1a TADN or TADC. If these TADs are not hydroxylated the reporter gene activity increases and an increase in luciferase activity is measured. As shown in Suppl. Fig. 2C, HIF1α TADN and TADC transactivity were induced by hypoxia in line with previous studies whereas treatment with OSM had no effect. It has been well demonstrated that the key amino acid proline 564 in the TADN plays a crucial role in HIF1α stabilization. The mutation of the critical amino acid proline (P 564) in the construct Gal4-HIF1aTADNM led to a hydroxylation-resistant fusion protein and caused an increase in transactivity; but OSM did not acuse any effect. Furthermore, asparagine (N 803) in HIF1a TADC can also be hydroxylated by another hydroxylase named FIH and thus block the interaction of HIF1α and p300/CBP. While this mutation of asparagine 803 to alanine enhanced transactivation it also abolished hypoxic induction and again OSM had no effect. Thus, OSM does not affect protein stability by influencing hydroxylases, which is in line with our cycloheximide data on the protein stability of OSM-induced HIF1α.

Together these data indicate that OSM does not affect hydroxylation and ubiquitination of HIF1α and support the data indicating that OSM induces HIF1α via a transcriptional mechanism.

Involvement of Jaks, Erks, and STAT3 in OSM-mediated upregulation of HIF1α protein and mRNA levels.

To identify signaling components involved in OSM-mediated HIF1α expression, inhibitor experiments were performed. As shown in Suppl. Fig. 3A, inhibition of Jaks by Jak inhibitor I almost completely abrogated OSM-mediated STAT3 and Erk phosphorylation and HIF1α expression. Inhibition of the Erk pathway with U0126 and PD98059 also partly diminished OSM-mediated HIF1α induction. Comparable observations as in HepG2 hepatoma cells were made for

the hepatocyte cell line PH5CH8 (Suppl. Fig. 3C). Again, the Jak inhibitor abrogated the OSM-induced HIF1α upregulation while treatment with U0126 showed a partial downregulation of HIF1α protein.

To investigate the influence of STAT3 in the OSM-mediated HIF1a expression HepG2 cells were pretreated with the STAT3 inhibitor Stattic (14) before OSM stimulation. STAT3 inhibition by Stattic also led to a loss of HIF1α expression (Suppl. Fig. 3B). Expression of the STAT3-dependent acute phase protein γ-fibrinogen, monitored as a positive control, was also lost upon treatment with Stattic. Taken together, these data show that STAT3 and the MEK/Erk pathway are involved in OSM-mediated HIF1α protein expression.

To clarify the role of the MEK/Erk and the Jak/STAT pathway in the OSM-mediated expression of HIF1a mRNA, HepG2 cells were pretreated with Jak Inhibitor I, U0126 or Stattic before stimulation with OSM. After 4 h the RNA was isolated und analyzed by qPCR. Upon OSM stimulation the HIF1a mRNA level was induced 2.5-fold whereas the Jak inhibitor I abrogated this effect (Suppl. Fig. 3D). The inhibition of the MEK/Erk cascade resulted in a 1.5-fold induction of the HIF1α mRNA level. Stattic totally inhibits the HIF1a mRNA induction by OSM. These data indicate that the Jak/STAT and to a lesser extent the MEK/Erk pathway are both important for the OSM-mediated HIF1α induction on the mRNA level.

Although OSM leads to p38 phosphorylation, treatment with two different p38 inhibitors, SB202190 and SB203590, had no effect on OSM-mediated HIF1α expression (data not shown). We did not observe activation of the PI3 kinase pathway by OSM, and application of the PI3 kinase inhibitors Wortmannin and LY294002 did not affect HIF1α expression (data not shown).

<u>Jaks, Erks, and STAT3 are involved in the OSM-mediated upregulation of the HIF1α-dependent 3xHRE promotor gene construct.</u>

To investigate the effects of the different inhibitors on HRE promoter activity, HepG2 cells were transfected with the luciferase reporter gene plasmids pGL3-EPO-HRE-Luc and the b-galactosidase expression vector pCH110 and 3xHRE-dependent luciferase activity was measured in the presence or absence of the different inhibitors (Suppl. Fig. 4A). Jak inhibitor I and the MEK inhibitor led to a down-regulation of the OSM-induced reporter gene activity. Although these results correlate well with the observed reduction of HIF1a mRNA and protein levels (Suppl. Fig. 3), we cannot exclude that MEK/Erk inhibition by U0126 additionally affected, directly or indirectly, HIF1α transactivational activity (15-17).

As an alternative to Stattic (which is toxic upon prolonged treatment) in studying the function of STAT3 in OSM-mediated HIF1α induction, reporter gene assays with the HIF-responsive luciferase construct were performed upon co-expression of dominant negative STAT3. As shown in Suppl. Fig. 4B, induction of luciferase activity was reduced when an expression construct for dominant negative STAT3 was cotransfected. Dominant negative STAT3 did not, however, affect

increased luciferase activity observed upon treatment with $CoCl_2$ (data not shown). Thus, STAT3 plays an important role for the increase of HIF1α activity upon OSM-treatment. Taken together, these data show again that STAT3 and the MEK/Erk pathway are involved in OSM-mediated induction of HIF1α activity.

Inhibitors of Jaks, Erks, and STAT3 abrogate the OSM-mediated upregulation of VEGF and PAI1 mRNA levels.

The VEGF and the PAI1 are both known targets of HIF1α and OSM. By inhibition of the MEK1/Erk cascade and the Jak/STAT signaling with specific inhibitors we could show that both pathways are important for the OSM-mediated upregulation of the VEGF and PAI1 mRNA (Suppl. Fig. 5): Jak inhibitor I, the MEK1 inhibitor U0126, and the STAT3 inhibitor Stattic abrogated the induction of the respective mRNAs upon OSM treatment.

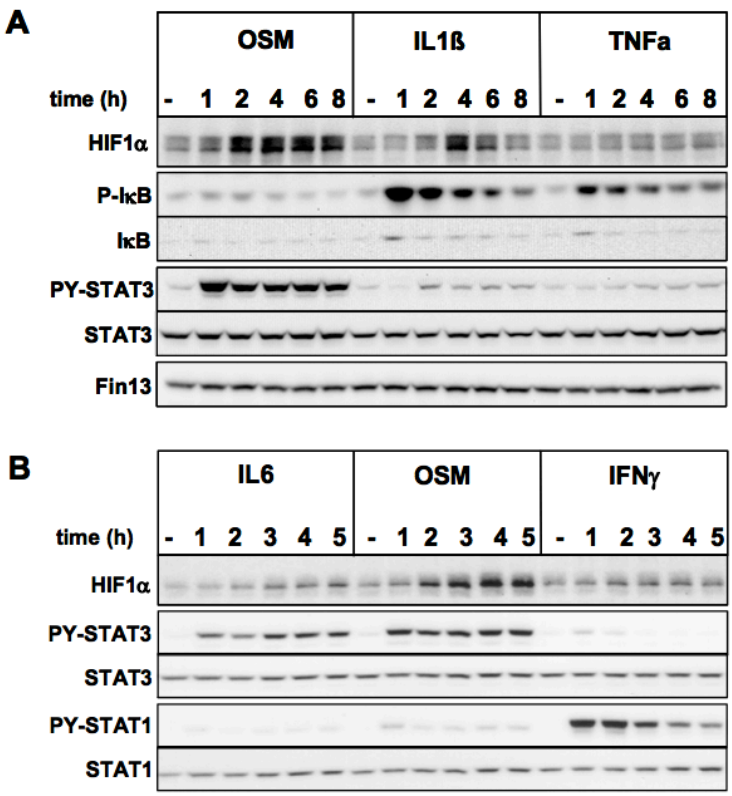
OSM-induced HIF1α is crucially involved in the transcriptional regulation of the genes for VEGF and PAI1.

Hypoxia-induced HIF1α and OSM are described to induce the expression of VEGF and PAI1. To investigate the relevance of HIF1α expression in the OSM signal transduction, we examined the effects of HIF1α suppression by siRNA on the target genes PAI1 and VEGF upon OSM stimulation. VEGF and PAI1 mRNA levels were downregulated by HIF1α siRNA compared to control siRNA (Suppl. Fig. 6).

OSM-induced VEGF and PAI1 at the protein level.

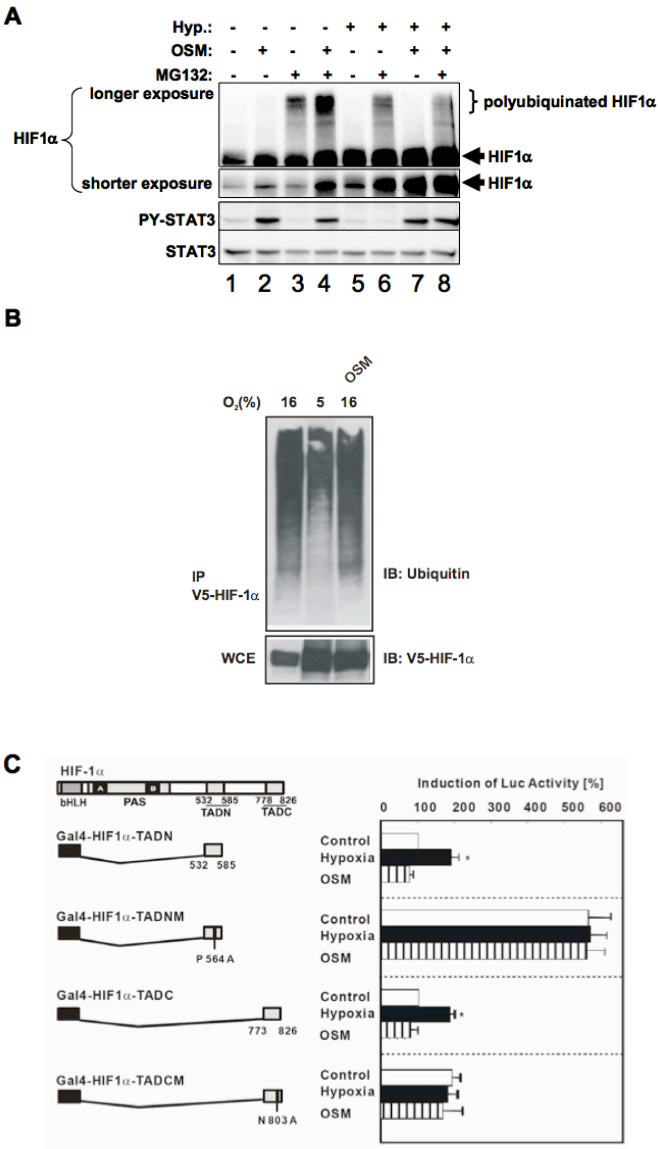
To investigate the effects of OSM on VEGF and PAI1 protein level expression, HepG2 cells were stimulated with OSM for 12 h to induce the expression of VEGF and PAI1. The protein concentrations in the supernatant were determined by ELISA. As shown in Suppl. Fig. 7, we found that OSM upregulates VEGF and PAI1 also at the protein level.

Supplemental Figures



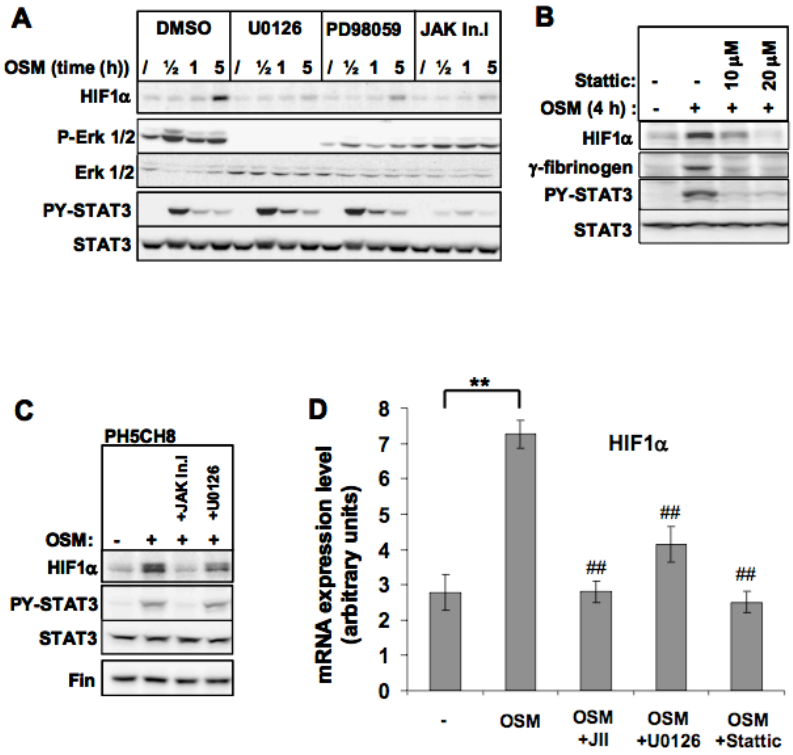
Supplemental figure 1: OSM induces HIF1α protein levels more efficiently than other cytokines.

(A) HepG2 cells were treated for the indicated periods of time with OSM, IL1b or TNFα (10 ng/mL). Lysates of the cells were separated by SDS-PAGE, and Western blots of the membranes were detected with antibodies directed against HIF1α, phospho-IkB, phospho-STAT3, IkB, STAT3, and Fin13. (B) HepG2 cells were treated for the indicated periods of time with OSM, IL6 or IFNg (10 ng/mL). Lysates of the cells were separated by SDS-PAGE, and Western blots of the membranes were detected with antibodies directed against HIF1α, phospho-STAT3, phospho-STAT1, STAT3, and STAT1.



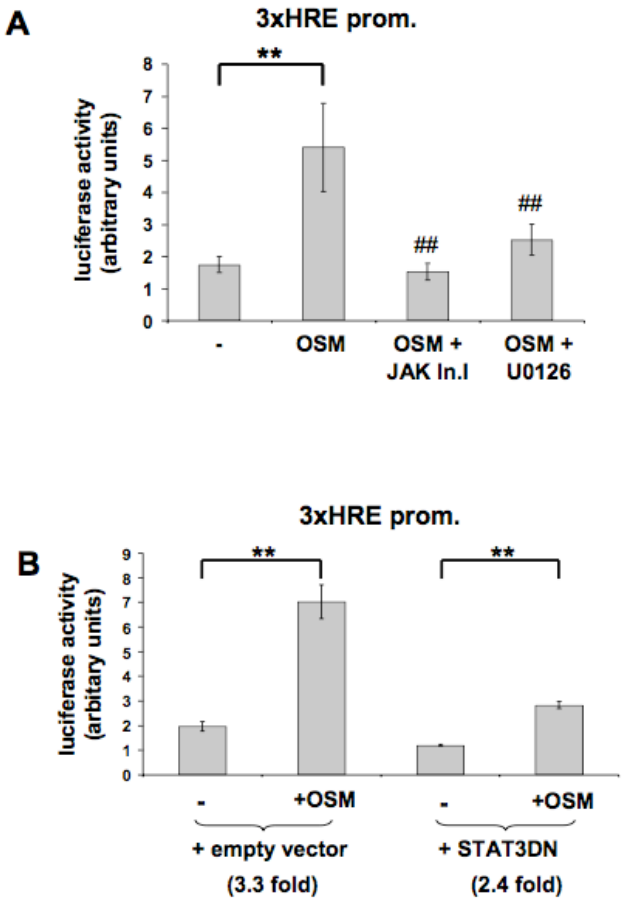
Supplemental figure 2: The regulation of HIF1α by OSM is independent of the hydroxylation sites and does not affect ubiquitination of HIF1α

(A) Where indicated, HepG2 cells were pre-incubated for 3h with MG132 (10 mM) before treatment with hypoxia and/or OSM (10 ng/mL) for additional 4h. Lysates and Western blots were prepared and the membranes were detected with an HIF1α, phosphospecific STAT3- and STAT3-antibody (B) HepG2 cells transfected with expression vectors for V5-tagged HIF1a and HA-tagged ubiquitin were cultured under normoxia (16% O_2), hypoxia (5% O_2) and under normoxia in the presence of OSM for 6 h were immunoprecipitated (IP) with anti-V5 antibodies and analyzed with ubiquitin antibodies. Whole-cell extracts (WCE) of HepG2 cells were probed with V5 antibodies. (C) HepG2 cells were cotransfected with a luciferase reporter construct pG5-E1B-LUC and different fusion gene constructs in which the Gal4 DNA-binding domain was fused to either the HIF1α region from amino acid 532-585 containing TADN or 727-826 containing TADC, respectively, as shown on the left. The mutations in the constructs are indicated. After 24 h the transfected cells were treated with OSM or hypoxia for 6 h. Values are means ± SEM of three independent culture experiments. *: $P \leq 0.05$ vs. control in the same group.



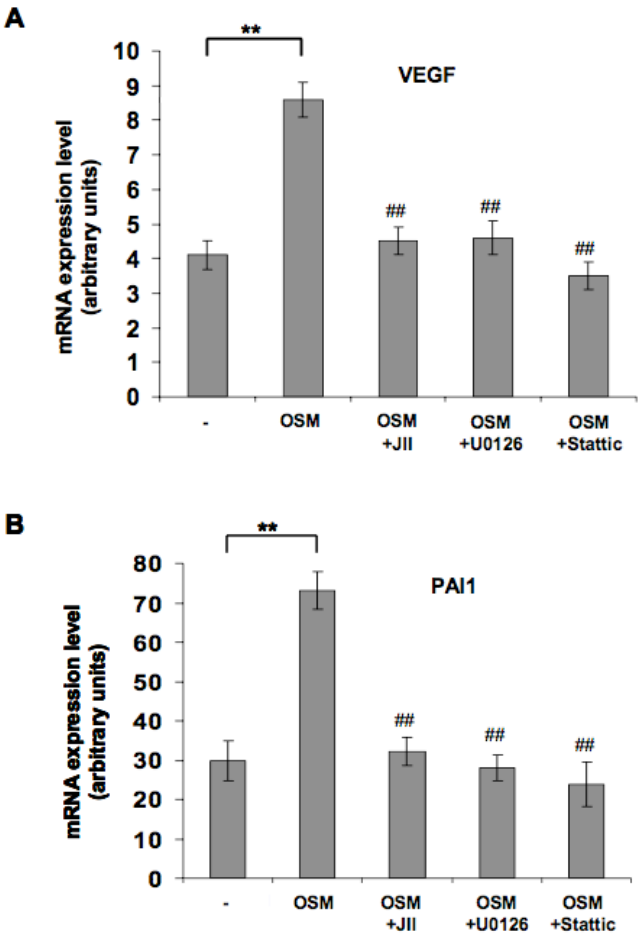
Supplemental figure 3: Jaks, STAT3, and Erks mediate OSM-induced HIF1α upregulation.

(A) HepG2 cells were pretreated for 30 min with DMSO alone, U0126 (2.5 mM), PD98059 (10 mM) or Jak inhibitor I (200 nM). Cells were then stimulated for the indicated periods of time with OSM (10 ng/mL). The lysates were used to prepare Western blots, which were detected with antibodies directed against HIF1α, phospho-Erk1/2, phospho-STAT3, STAT3, and Erk1/2. (B) HepG2 cells were pretreated for 30 min with DMSO alone or Stattic (10 and 20 mM) and then processed as described in (A). C) PH5CH8 cells were pretreated for 30 min with DMSO alone, U0126 or Jak inhibitor I as described in (A). D) HepG2 cells were pretreated for 0.5 h with Jak Inhibitor 1 (1 mM), U0126 (5 mM) or Stattic (20 mM). After 4 h stimulation with OSM, RNA was extracted and HIF1α mRNA level was measured by quantitative PCR. **: $P < 0.01$, ##: statistically significant difference with respect to OSM-treated cells ($P < 0.01$).



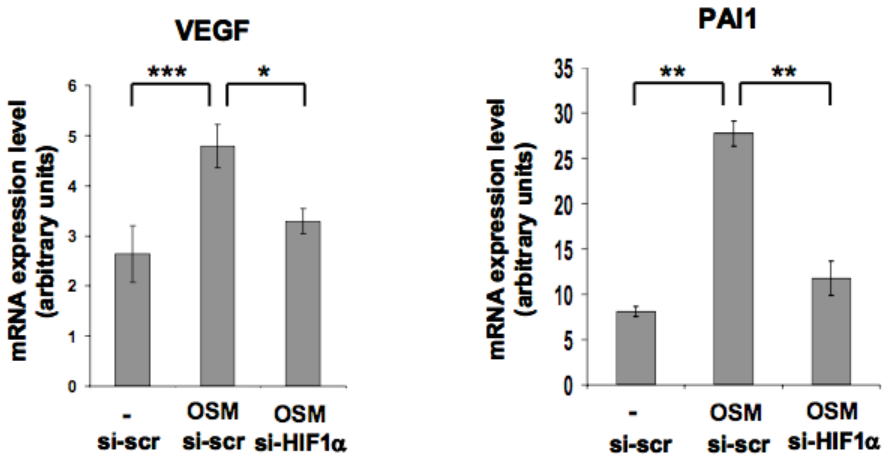
Supplemental figure 4: Jaks, Erks, and STAT3 are involved in the OSM-mediated upregulation of the HIF1α-dependent 3xHRE promotor gene construct.

A) HepG2 cells were transfected with the luciferase reporter gene plasmids pGL3-EPO-HRE-Luc and the b-galactosidase expression vector pCH110 and the cells were processed as described in Fig. 1C. The different inhibitors were added together with OSM. **: P < 0.01, # #: statistically significant difference with respect to OSM-treated cells (P < 0.01). B) HepG2 cells were transfected with the luciferase reporter gene plasmids pGL3-EPO-HRE-Luc and the b-galactosidase expression vector pCH110 in the absence or presence of an expression vector for dominant negative STAT3 and the cells were processed as described in Fig. 1C. **: P< 0.01.



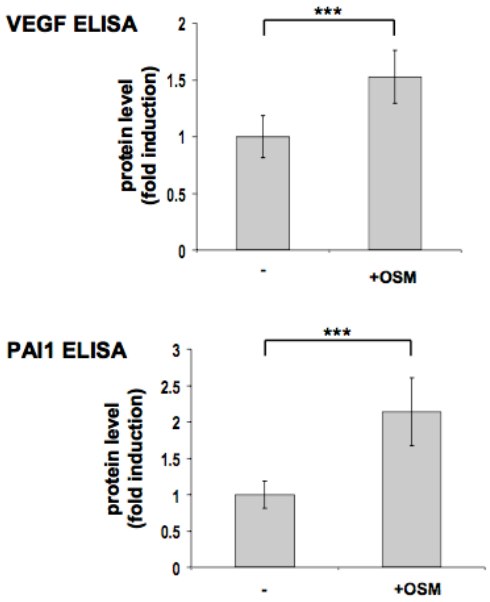
Supplemental figure 5: Inhibitors of Jaks, Erks, and STAT3 abrogate the OSM-mediated upregulation of VEGF and PAI1 mRNA levels.

HepG2 cells were pretreated for 0.5 h with Jak Inhibitor 1 (1 mM), U0126 (5 mM) or Stattic (20 mM). Afterwards the cells were stimulated with OSM for 4 h followed by the extraction of the RNA. The VEGF and PAI1 mRNA level was measured by quantitative PCR. **: $P < 0.01$, ##: statistically significant difference with respect to OSM-treated cells ($P < 0.01$).



Supplemental figure 6: HIF1a knockdown by siRNA reduces OSM-induced mRNA levels of VEGF and PAI1.

HepG2 cells were transfected with siRNA against HIF1α or scrambled negative control siRNA. 48h after transfection the cells were stimulated with OSM for 4 h followed by the extraction of the RNA. The VEGF and PAI1 mRNA levels were measured by quantitative PCR. ***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$.



Supplemental figure 7: OSM upregulates VEGF and PAI1 at the protein level.

HepG2 cells were treated with OSM (10 ng/mL) for 12h. The supernatants were harvested and protein concentrations of VEGF and PAI1 were analyzed by ELISA. ***: P<0.001

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