Phosphorylation of p27Kip1 by JAK2 directly links cytokine receptor signaling to cell cycle control

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Abstract

Janus kinase 2 (JAK2) couples ligand activation of cell surface cytokine receptors to the regulation of cellular functions including cell cycle progression, differentiation and apoptosis. It thereby coordinates biological programs such as development and hematopoiesis. Unscheduled activation of JAK2 by point mutations or chromosomal translocations can induce hyperproliferation and hematological malignancies. Typical signal transduction by the JAK2 tyrosine kinase comprises phosphorylation of STAT transcription factors. In this study, we describe the identification of the cyclin-dependent kinase (CDK) inhibitor p27^{Kip1} as a novel JAK2 substrate. JAK2 can directly bind and phosphorylate p27^{Kip1}. Both, the JAK2 FERM domain and its kinase domain bind to p27^{Kip1}. JAK2 phosphorylates tyrosine residue 88 (Y88) of p27^{Kip1}. We previously reported that Y88 phosphorylation of p27^{Kip1} by oncogenic tyrosine kinases impairs p27^{Kip1}-mediated CDK inhibition, and initiates its ubiquitin-dependent proteasomal degradation. Consistently, we now find that active oncogenic JAK2V617F reduces p27^{Kip1} stability and protein levels in patient-derived cell lines harboring the mutant JAK2V617F allele. Moreover, tyrosine phosphorylation of p27^{Kip1} is impaired and p27^{Kip1} expression is restored upon JAK2V617F inactivation by small hairpin RNA-mediated knockdown or by the pyridone-containing tetracycle JAK inhibitor-I, indicating that direct phosphorylation of p27^{Kip1} can contribute to hyperproliferation of JAK2V617F-transformed cells. Activation of endogenous JAK2 by interleukin-3 (IL-3) induces Y88 phosphorylation of p27^{Kip1}, thus unveiling a novel link between cytokine signaling and cell cycle control in non-transformed cells. Oncogenic tyrosine kinases could use this novel pathway to promote hyperproliferation in tumor cells.

Keywords

cell cycle control; CDK inhibitors; p27^{Kip1}; tyrosine kinases; JAK2; JAK2V617F

Introduction

The decision between cell proliferation and cell cycle exit is orchestrated by signaling networks that control the activity of specific cyclin-dependent kinase (CDK) complexes (Morgan, 1997; Malumbres and Barbacid, 2009). Activation of G1-phase CDKs initiates cell cycle entry and promotes cell cycle progression towards S-phase. Misregulated CDKs induce unscheduled proliferation as well as genomic and chromosomal instability (Malumbres and Barbacid, 2009). The CDK inhibitor p27^{Kip1} has a key role in controlling

CDKs and cell proliferation in response to diverse mitogenic or antiproliferative stimuli (Chu *et al.*, 2008). Non-proliferating cells are frequently characterized by elevated p27^{Kip1} levels that decline upon mitogenic stimulation (Hengst and Reed, 1996; Chu *et al.*, 2008). Binding of p27^{Kip1} usually inhibits CDK activity. However, p27^{Kip1} was also found in active CDK complexes and surprisingly even contributes to CDK activation by promoting assembly of active cyclin D/CDK holoenzymes (LaBaer *et al.*, 1997; James *et al.*, 2008; Larrea *et al.*, 2008; Ray *et al.*, 2009). We recently identified a molecular switch that triggers the transition of p27^{Kip1} from a CDK inhibitor to a potential activator of specific CDKs, thus resulting in the conversion of a tumor suppressor into a potential oncogene. This mechanism is based on the phosphorylation of tyrosine residue 88 (Y88) of p27^{Kip1}, which results in ejection of an inhibitory 3_{10}-helix of p27^{Kip1} from the ATP-binding pocket of the CDK (Grimmler *et al.*, 2007). The induced conformational change permits ATP binding of the CDK and partially activates the p27^{Kip1}-bound kinase (Chu *et al.*, 2007, 2008; Grimmler *et al.*, 2007; James *et al.*, 2008; Larrea *et al.*, 2008; Ray *et al.*, 2009). One substrate of p27^{Kip1}-bound CDK2 is Y88-phosphorylated p27^{Kip1} itself. Activated CDK2 can now phosphorylate cyclin/CDK-bound Y88-phosphorylated p27^{Kip1} on threonine 187 (T187) (Grimmler *et al.*, 2007). Phosphorylated T187 is a pivotal core of the phosphodegron recognized by the SCF^{Skp2} complex, which initiates the ubiquitin-proteasome-dependent degradation of p27^{Kip1} (Grimmler *et al.*, 2007; Chu *et al.*, 2008; Frescas and Pagano, 2008). The p27^{Kip1}-Y88 phosphorylation is induced upon mitogen stimulation, suggesting that p27^{Kip1}-Y88 phosphorylation may serve as a potent mechanism of direct signal integration. Oncogenes like BCR-Abl use this mechanism to inactivate p27^{Kip1}; however, molecular pathways triggering this mechanism in normal cells remained undefined.

Janus kinase 2 (JAK2) is a non-receptor tyrosine kinase, which can bind with its FERM (band 4.1, ezrin, radixin, moesin) domain to the cytoplasmic tails of various transmembrane cytokine receptors (Parganas *et al.*, 1998). JAK2 activation by trans- or autophosphorylation leads to downstream signaling including activation of signal transducer and activator of transcription 5 (STAT5), phosphoinositol-3-kinase (PI-3 K), mitogen-activated protein kinase or Bcl-2 family members (Baker *et al.*, 2007). Phosphorylated STAT5 proteins enhance the transcription of apoptosis regulatory proteins like BCL-X$_L$ and cell cycle regulators including cyclins D$_1$, D$_2$, E and the CDK inhibitor p21^{Cip1} (Desrivieres *et al.*, 2006). JAK2 has a central role in hematopoiesis by regulating cell survival, proliferation and differentiation (Baker *et al.*, 2007). Constitutive activation of JAK2 can cause oncogenic transformation (Ihle and Gilliland, 2007). Mutation of valine 617 to phenylalanine (JAK2V617F) within its pseudokinase domain is one of the most common activating mutations of JAK2, and JAK2V617F is the most frequent mutation in BCR-Abl negative myeloproliferative disorders, with the highest incidence (95%) in polycythemia vera (PV) (James *et al.*, 2005; Kralovics *et al.*, 2005; Levine *et al.*, 2005; Zhao *et al.*, 2005). Hyperproliferation induced by JAK2V617F involves activation of STAT5 and its downstream targets (Walz *et al.*, 2006; Wernig *et al.*, 2008). It has been reported that transcriptional downregulation of cyclin D$_2$ and upregulation of p27^{Kip1} is a central mechanism of growth arrest upon JAK2V617F inhibition (Walz *et al.*, 2006). The decrease of p27^{Kip1} upon JAK2V617F expression correlated with STAT5-induced expression of Skp2, suggesting that the degradation of p27^{Kip1} could be a consequence of the overexpression of this p27^{Kip1}-directed ubiquitin ligase (Furuhata *et al.*, 2009).

In this study, we describe the identification of p27^{Kip1} as a novel JAK2 substrate. JAK2 binds to p27^{Kip1} through its FERM and kinase domains and phosphorylates Y88 of p27^{Kip1}. This leads to partial activation of p27^{Kip1}-bound CDK-cyclin complexes, and permits SCF^{Skp2}-dependent degradation of p27^{Kip1} (Grimmler *et al.*, 2007). The p27^{Kip1}-Y88 phosphorylation is induced upon JAK2 activation by interleukin-3 (IL-3), and decreased by JAK2 inactivation. Substantial Y88-phosphorylated p27^{Kip1} was detected in patient-derived

JAK2V617F positive hematopoietic cell lines. Inactivation of JAK2V617F reduced Y88-p27^{Kip1} phosphorylation and resulted in a concomitant increase of p27^{Kip1} protein and cell cycle arrest. These observations directly connect JAK2-mediated cytokine receptor signaling with the core cell cycle machinery, and uncover a novel pathway that can contribute to hyperproliferation induced by deregulated JAK2 activation.

Results

p27^{Kip1} becomes tyrosine phosphorylated upon IL-3 stimulation

We and others recently reported that serum stimulation can cause p27^{Kip1} tyrosine phosphorylation (Grimmler *et al.*, 2007; James *et al.*, 2008). Therefore, we aimed to determine mitogenic stimuli that induce this phosphorylation and to identify p27^{Kip1}-phosphorylating kinases. Cytokines are important positive and negative regulators of the cell cycle and can stimulate quiescent cells to proliferate. For example, IL-3 is required to maintain proliferation of different hematopoietic cell types, and its withdrawal leads to cell cycle arrest in G1-phase (Kelvin *et al.*, 1986). Investigating IL-3 induced p27^{Kip1} modifications in the IL-3-dependent murine pro-B cell line Ba/F3, we observed a strong induction of p27^{Kip1}-Y88 phosphorylation shortly after IL-3 stimulation, whereas no p27^{Kip1}-Y88 phosphorylation was detected upon deprivation of the cytokine (Figure 1a). The p27^{Kip1}-Y88 phosphorylation in response to IL-3 was also observed in IL-3 dependent murine myeloblast 32D cells (Supplementary Figure 1). IL-3 receptor signaling activates JAK2 (Parganas *et al.*, 1998), which was traced by its Y1007/1008 phosphorylation (Feng *et al.*, 1997). JAK2 activation as well as tyrosine phosphorylation of its substrate STAT5A/B was detected 5 min after IL-3 stimulation, slightly preceding the peak of p27^{Kip1}-Y88 phosphorylation (Figure 1a). Activation of Src family kinases, monitored by an antibody recognizing activated Src and related kinases, indicates no change in activity at time points investigated (Figure 1a). The coincidence of JAK2 activation and p27^{Kip1} tyrosine phosphorylation lead us to investigate whether JAK2 could initiate p27^{Kip1} phosphorylation. Incubation of Ba/F3 cells with the JAK kinase-specific pyridone-containing tetracycle 'JAK inhibitor-I' (Thompson *et al.*, 2002) before IL-3 stimulation, led to a decline in Y1007/1008-phosphorylated JAK2 as well as Y88-phosphorylated p27^{Kip1} (Figure 1b), indicating that JAK2 activation is a prerequisite for p27^{Kip1} Y88 phosphorylation.

JAK2 phosphorylates p27^{Kip1} on tyrosine 88

As p27^{Kip1}-Y88 phosphorylation shortly followed IL-3 stimulation and JAK2 activation (Figure 1a), we investigated whether activation of JAK2 was sufficient to phosphorylate p27^{Kip1}. We transfected 293T cells with p27^{Kip1} in the presence and absence of JAK2. Coexpression of JAK2 and p27^{Kip1} resulted in intense tyrosine phosphorylation of p27^{Kip1}, and treatment with the JAK inhibitor-I strongly reduced this phosphorylation (Figure 2a, Supplementary Figure 2) as well as JAK2-Y1007/1008 phosphorylation (Figure 2a). In addition to Y88, p27^{Kip1} comprises only two additional tyrosine residues, which are also located within its CDK-inhibitory domain. Using p27^{Kip1} phospho-Y88-specific antibodies, we identified Y88 as major phosphorylation site upon JAK2 expression (Figure 2a) and IL-3 stimulation (Figure 1). The p27^{Kip1}-Y88F and p27^{Kip1}-Y88/89F mutants were poor substrates for JAK2-induced phosphorylation (Figure 2b), indicating that JAK2 preferentially phosphorylates Y88. In all, 80% of p27^{Kip1} tyrosine phosphorylation is lost if Y88 is exchanged to phenylalanine (Figure 2b). The remaining signal was reduced to 3.4% by mutating Y89 in addition to Y88, indicating that Y89 might be a second, low-affinity phosphorylation site. To investigate if JAK2 can directly phosphorylate p27^{Kip1}, and to determine which tyrosines become phosphorylated, we incubated purified recombinant p27^{Kip1} with the purified JAK2 kinase domain. Direct phosphorylation of p27^{Kip1} by JAK2 was observed in kinase assays (Figure 2c). To identify tyrosine residues that can be

phosphorylated by JAK2 *in vitro*, we replaced one, combinations of two or all three tyrosines of p27^{Kip1} with phenylalanine. Efficient phosphorylation of p27^{Kip1} by JAK2 required the presence of Y88, whereas mutations of Y89 or Y74 to phenylalanine failed to reduce p27^{Kip1} phosphorylation (Figure 2c). These data suggest a direct phosphorylation of tyrosine residue 88 of p27^{Kip1} by JAK2.

p27^{Kip1} binds to the FERM and the kinase domains of JAK2

If JAK2 directly phosphorylates p27^{Kip1}, these proteins might associate in a stable complex. This could occur through direct binding or involve additional bridging proteins. To investigate direct binding of JAK2 to p27^{Kip1}, we first analyzed the interaction of purified recombinant His-p27^{Kip1} with recombinant GST-JAK2 *in vitro*. To exclude co-purification of interacting eukaryotic proteins, both recombinant proteins were isolated from *E.coli*. Pull-down of GST-JAK2 on glutathione beads co-precipitated p27^{Kip1}, indicating that both proteins can form a stable complex *in vitro* (Figure 3a). We next expressed p27^{Kip1} and JAK2 in 293T cells, where JAK2 co-precipitated with p27^{Kip1}, and p27^{Kip1} co-precipitated with JAK2 (Figure 3b). To test if the endogenous proteins interact under physiological conditions, we precipitated JAK2 from the mouse Ba/F3 pro-B cells and activated JAK2V617F from the human erythroleukemia cell line HEL. Importantly, endogenous p27^{Kip1} co-immunoprecipitates with JAK2 as well as with JAK2V617F (Figure 3c). These data support the hypothesis that p27^{Kip1} is a direct substrate of JAK2, which binds to p27^{Kip1} and phosphorylates Y88 of p27^{Kip1}. To further characterize the p27^{Kip1}-JAK2 interaction, we investigated binding of p27^{Kip1} to four domains of JAK2, representing the entire protein: the FERM-, SH2-like-, pseudokinase- and the kinase domain (Figure 3d, (Zhao *et al.*, 1995)). Full-length JAK2, the N-terminal FERM- and the C-terminal kinase domain of JAK2 precipitate p27^{Kip1} (Figure 3e). A weak interaction was observed with the pseudokinase domain. Immunoprecipitation of p27^{Kip1} and analysis of the co-precipitated JAK2 fragments confirmed this interaction (data not shown).

Localization of JAK2, p27^{Kip1} and Y88-phosphorylated p27^{Kip1}

JAK2 is predominantly localized in the cytoplasm and frequently associated with the cytoplasmic tails of cytokine receptors. The p27^{Kip1} can shuttle between the nucleus and the cytoplasm (Connor *et al.*, 2003), and phosphorylation by several kinases including Akt/PKB leads to its translocation to the cytoplasm (Chu *et al.*, 2008). To investigate if the localization of p27^{Kip1} and JAK2 overlaps and to determine where Y88-phosphorylated p27^{Kip1} accumulates, we performed immunofluorescence experiments and biochemical fractionations. Transfection of U2OS and 293T cells with p27^{Kip1} and JAK2 or JAK2V617F revealed that Y88-phosphorylated p27^{Kip1} was highly abundant in the cytoplasm (Figure 4a, panels 1, 5, 9, 13), as seen for active JAK2 (Figure 4a, panel 6), or JAK2V617F (Figure 4a, panel 14) (Supplementary Figure 3). p27^{Kip1} could be detected in the nucleus and the cytoplasm (Figure 4a, panels 2, 10). Endogenous Y88-phosphorylated p27^{Kip1} of HEL erythroleukemia cells also localized predominantly within the cytoplasm (Figure 4b, panels 1, 3). To confirm the subcellular localization, we fractionated extracts from HEL- and Ba/F3 cells. In line with the immunofluorescence data, endogenous Y88-phosphorylated p27^{Kip1} was mainly present in the cytosolic fraction, together with p27^{Kip1} and JAK2V617F (HEL cells) or JAK2 (Ba/F3 cells) (Figures 4c and d).

Activated JAK2V617F of human leukemia cell lines phosphorylates p27^{Kip1} and decreases its stability

The JAK2V617F mutant transforms hematopoietic progenitors and is a common mutation in myeloproliferative disease (Levine and Gilliland, 2007). JAK2V617F permits IL-independent growth of the IL-3-dependent cell line Ba/F3 (Furuhata *et al.*, 2009). Inhibition of the activated kinase leads to increased p27^{Kip1} levels and decreased cyclin D$_2$ levels, and

arrests human erythroleukemia HEL cells in G1-phase (Walz *et al.*, 2006; Furuhata *et al.*, 2009). To ascertain Y88 phosphorylation of p27^{Kip1} by JAK2V617F *in vivo*, we investigated p27^{Kip1} regulation in two patient-derived leukemia cell lines naturally expressing JAK2V617F: the human erythroleukemia cell line HEL and the acute myeloid leukemia-derived cell line SET2. We observed that p27^{Kip1} is extensively phosphorylated on Y88 in both cell lines. Inactivation of JAK2 by JAK inhibitor-I strongly decreased p27^{Kip1}-Y88 phosphorylation (Figures 5a and b). Interestingly, an increase in p27^{Kip1} protein could be observed already 1 h after treatment (Figures 5a and b). We reported earlier that tyrosine-88 phosphorylation of p27^{Kip1} enhances its SCF^{Skp2}-mediated ubiquitin-proteasomal degradation and decreases its Cdk-cyclin inhibitory potential (Chu *et al.*, 2007, 2008; Grimmler *et al.*, 2007). Consistent with this observation, we now find that p27^{Kip1} stability is increased upon JAK2 inactivation (Figure 5c, Supplementary Figure 5), and that p27-Y88F leads to a stronger reduction of HEL cells in S-phase than wild-type p27^{Kip1} (Supplementary Figure 6). It has been proposed that p27^{Kip1} accumulation by JAK2 inhibition is due to decreased Skp2 expression (Agarwal *et al.*, 2008; Furuhata *et al.*, 2009). We observed that Skp2 levels remained unchanged at early time points of JAK2 inhibition, and only decreased after 4 h of JAK inhibitor-I treatment (Supplementary Figure 4). To exclude that JAK inhibitor-I might act on additional kinases, we used small hairpin RNA-mediated JAK2 knockdown using established small hairpin RNAs (Neilson *et al.*, 2007). Silencing of JAK2 expression resulted in a loss of p27^{Kip1}-Y88 phosphorylation and increased p27^{Kip1} levels, whereas Skp2 levels remained unchanged (Figure 5d). Consistent with earlier observations (Walz *et al.*, 2006), we observed an increase of cells in G0/G1-phase and a decrease of cells in S-phase, indicating that depletion of JAK2 delays or arrests cells in G0/G1 phase (Supplementary Figure 7). As Skp2 expression remained unchanged, despite the significant loss of p27^{Kip1} phosphorylation and cell cycle delay/arrest, these data support the model that inhibition of Skp2 expression is not essential for p27^{Kip1} stabilization after JAK2 inhibition.

Discussion

We have recently identified p27^{Kip1} tyrosine phosphorylation as an important molecular mechanism that impairs function and stability of the CDK inhibitor p27^{Kip1} in the presence of mitogens, leading to partial CDK activation and cell cycle progression (Grimmler *et al.*, 2007; Chu *et al.*, 2008; Ray *et al.*, 2009). Oncogenic tyrosine kinases like BCR-Abl can phosphorylate and inactivate p27^{Kip1}; however, specific kinases phosphorylating p27^{Kip1} during mitogen-induced cell cycle entry remained unknown. In this study, we describe that cytokine-induced JAK2 activation triggers Y88–phosphorylation of p27^{Kip1}. JAK2 can directly bind and phosphorylate p27^{Kip1}. Thus, the identification of JAK2 as a p27^{Kip1}-Y88-phosphorylating kinase now provides a novel and direct link between cytokine signaling and cell cycle control in untransformed cells.

The p27^{Kip1} can form a complex with JAK2 that is stable enough to be immunoprecipitated (Figure 3). The FERM domain and the kinase domain of JAK2 bind to p27^{Kip1}. As full-length JAK2 bound p27^{Kip1} more efficiently than single domains (Figure 3e), both domains seem to cooperate for efficient binding. Activation of JAK2 can trigger diverse biological responses including cell proliferation, differentiation or apoptosis. JAK2 links cytokine stimulation to p27^{Kip1} phosphorylation, leading to its inactivation and elimination. However, ligand binding to cytokine receptors does not always promote cell proliferation. Depending on the cell type, cytokines like oncostatin M can even prevent cell proliferation and induce p27^{Kip1} (Halfter *et al.*, 2006). Therefore, activated JAK2 may not always phosphorylate and inactivate p27^{Kip1}. It is tempting to speculate that the ability of JAK2 to bind and inactivate p27^{Kip1} might be cell type specific and regulated. For example, the p27^{Kip1}–JAK2 interaction may be inhibited by modifications such as phosphorylation or by competing

binding partners including various cytokine receptors. Finally, regulation of the subcellular localization of p27^{Kip1} or active JAK2 may determine the efficiency of p27^{Kip1} phosphorylation and inactivation by JAK2.

JAK2 associates with the cytoplasmic tail of cytokine receptors, but also with receptor tyrosine kinases and seven transmembrane-spanning receptors (Wallace and Sayeski, 2006). In addition, active JAK2V617F was recently detected in the nucleus (Dawson *et al.*, 2009). Even though Y88-phosphorylated p27^{Kip1} accumulated in the cytosol under the conditions here, it remains to be determined where JAK2 can phosphorylate p27^{Kip1}. Depending on its cellular context and mode of activation, JAK2 may phosphorylate p27^{Kip1} in different cellular compartments. Previously, tyrosine-phosphorylated p27^{Kip1} was detected in the nucleus and it was speculated that tyrosine phosphorylation leads to nuclear translocation of p27^{Kip1} (Kardinal *et al.*, 2006; Tossidou *et al.*, 2008; Ray *et al.*, 2009). We now observe that endogenous Y88-phosphorylated p27^{Kip1} in the hematopoietic cell lines HEL and Ba/F3, as well as overexpressed p27^{Kip1} phosphorylated by JAK2 in U2OS and 293T cells is predominantly cytoplasmic (Figure 4, Supplementary Figure 3), indicating that Y88 phosphorylation alone is insufficient to restrain p27^{Kip1} to the nucleus.

The p27^{Kip1} is Y88-phosphorylated in myeloid cell lines expressing the active JAK2V617F variant (Figures 5a and b). Inactivation of JAK2V617F reduces Y88 phosphorylation of p27^{Kip1} and leads to its concomitant increase and enhanced stability. Our recent observation that Y88 phosphorylation by oncogenic tyrosine kinase BCR-Abl triggers premature Skp2-dependent p27^{Kip1} proteosomal degradation (Grimmler *et al.*, 2007), is well in line with our observation here that inhibition of oncogenic JAK2V617F can reestablish p27^{Kip1} stability and expression. In both JAK2V617F-expressing cell lines investigated, increased p27^{Kip1} protein could already be detected 1 h after JAK inhibitor-I addition, indicating that p27^{Kip1} stabilization is an immediate consequence of JAK2V617F inactivation. Immediately after IL-3 stimulation (15 and 30 min), we detected an increase of Y88 phosphorylation without a significant decline of p27^{Kip1} levels (Figure 1). As the half-life of p27^{Kip1} is significantly longer than these short time points, effects on overall protein level would need more time to build up. Recent reports proposed that JAK2V617F activity leads to decreased p27^{Kip1} stability by transcriptional induction of Skp2 involving STAT5 (Agarwal *et al.*, 2008; Furuhata *et al.*, 2009). The increase of p27^{Kip1} after 24-h JAK inhibitor-I treatment correlated with decreased Skp2 expression (Agarwal *et al.*, 2008). In HEL cells, we observed no decline in Skp2 levels for up to 8 h of JAK inhibitor-I treatment (Supplementary Figure 4a), and importantly, p27^{Kip1} protein levels increase already at earlier time points, when Skp2 level remain unchanged (Figures 5a and b). Skp2 expression itself is regulated during cell cycle progression and the protein is degraded in early G1-phase after ubiquitination by APC^{Cdh1} (Wei *et al.*, 2004; Frescas and Pagano, 2008). As other cell cycle-regulated proteins including cyclin E and cyclin D$_2$ also decline after extended JAK Inhibitor-I incubation (16 h, Supplementary Figure 4b), it cannot be excluded that this decline of Skp2 level might also result from cell cycle synchrony in early G1-phase. In addition, knockdown of JAK2 expression by RNA interference abolished p27^{Kip1}-Y88 phosphorylation and led to accumulation of p27^{Kip1}, with no change in Skp2 protein level (Figure 5d). Together, these data indicate that p27^{Kip1} regulation by JAK2 can occur independently from Skp2 expression. However, it is clear that Y88-triggered p27^{Kip1} degradation at the G1/S transition requires Skp2 expression as well as sufficient SCF^{Skp2} E3 ubiquitin ligase activity (Chu *et al.*, 2008; Frescas and Pagano, 2008). Cells expressing insufficient Skp2 protein, therefore, may require additional induction of Skp2 in order to overcome a p27^{Kip1}-imposed cell cycle arrest and to proliferate.

The p27^{Kip1} is a negative regulator of hematopoietic progenitor proliferation. JAK2V617F transforms hematopoietic progenitors in PV patients, causing increased proliferation and

maturation (Levine and Gilliland, 2007; Bruchova *et al.*, 2009). *Ex vivo* culture of primary erythroid progenitors recently revealed that $p27^{Kip1}$ was increased at certain stages of erythroid differentiation in cells from healthy subjects, whereas $p27^{Kip1}$ levels remained low in erythroid cells from PV patients. Low $p27^{Kip1}$ in erythroid PV cells correlated with high proliferation (Bruchova *et al.*, 2009). It is therefore tempting to speculate that $p27^{Kip1}$-Y88 phosphorylation by JAK2V617F might also have a role in PV development. Initial data indicate that $p27^{Kip1}$ is extensively phosphorylated on Y88 in PV patients with high levels of active JAK2. Skp2 expression neither correlated with active JAK2 nor with $p27^{Kip1}$ levels in these primary cells (H Jäkel, D Wolf, L Hengst; unpublished results).

JAK2 inhibitors prove to be effective in controlling hyperproliferation and symptom reduction in PV, although they merely eliminate mutant clones (Verstovsek, 2009). Y88 phosphorylation of $p27^{Kip1}$ could contribute to JAK2V617F-dependent hyperproliferation and transformation in this and other hematological malignancies. JAK inhibitors may reduce hyperproliferation in part by preventing $p27^{Kip1}$-Y88 phosphorylation-induced inactivation and degradation.

The phosphorylation of $p27^{Kip1}$ by JAK2 provides a novel exciting mechanism, by which JAK2 can directly impinge on cell cycle progression in normal and transformed cells. The identification of $p27^{Kip1}$ as target of JAK2 and JAK2V617F could open new avenues for diagnosis, as well as targeted therapeutic intervention in JAK2-dependent myeloproliferative diseases.

Materials and methods

Cell culture, transfection and cell lysis

HEL and Ba/F3 cells were obtained from American Type Culture Collection (ATCC) and propagated as recommended. The 293T cells were transfected using calcium phosphate. For a 6 cm dish, 2 µg HA-p27 PCDNA3.1 plasmid and 3 µg of JAK2 pCMX plasmid were transfected. For immunoprecipitation experiments, HA-p27 plasmid and Flag-JAK2 plasmid or indicated Flag-JAK2 domains were transfected. 40 h after transfection, cells were washed in ice-cold phosphate buffered saline containing 1 mM pervanadate and lysed in 0.5% NP-40 lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% NP-40, phosphatase inhibitors (Phosphostop, Roche, Mannheim, Germany) and protease inhibitor cocktail (Sigma Aldrich, St Louis, MO, USA)). For immunoblot analysis of $p27^{Kip1}$ and $p27^{Kip1}$-Y88 phosphorylation, protein extracts were boiled at 65 °C for 10 min, and the heat labile proteins were precipitated by centrifugation at 16 000 r.p.m. for 15 min. The cleared supernatant containing heat stable proteins such as $p27^{Kip1}$ (Hengst *et al.*, 1994) was separated by SDS–PAGE. The subsequent immunoblot analyses were performed by using ECL (Pierce, Thermo Fisher Scientific, Waltham, MA, USA) or Odyssey infrared fluorescence detection (Li-Cor, Bad Homburg, Germany) technologies.

Plasmids and RNA interference

Mammalian HA-$p27^{Kip1}$ and its tyrosine mutants in the pcDNA3.1 vector as well as bacterial His-$p27^{Kip1}$ and its tyrosine mutants in the pET28a vector were described previously (Grimmler *et al.*, 2007). N- and C-terminus of JAK2 cDNA (a kind gift from W Doppler) was amplified by a two-step PCR and recombined into pDONR207. To obtain the full-length JAK2 the 1.6 kb *Xho*I-*Nde*I fragment of the JAK2 cDNA was inserted via ligation into the pDONR-JAK2 plasmid (pENTR-JAK2). To generate expression vectors containing JAK2 fragments, entry vectors were generated via PCR with specific primers and recombination with pDONR207 was performed. The JAK2V617F construct was cloned by a two-step PCR. The PCR fragment and the pENTR-JAK2 plasmid were digested with

*Bsp*119I and *Xba*I, and the PCR product was ligated into the truncated pENTR-JAK2 plasmid backbone. All expression vectors were generated via recombination with the appropriate destination vector (Gateway technology, Invitrogen, Carlsbad, CA, USA), and verified by sequencing. All primers used for cloning are listed in Supplementary Table 1.

HEL cells were electroporated with control (scramble) pLKO1.puro (Addgene (Cambridge, MA, USA) plasmid #1864 (Sarbassov *et al.*, 2005)) or JAK2-specific small hairpin RNAs pLKO1.puro plasmids (Sigma, TRCN0000003180, as described (Neilson *et al.*, 2007)) with AMAXA Nucleofector (Lonza, Cologne, Germany) according to the manufacturer's recommendations and incubated for 68 h after electroporation.

Antibodies and reagents

Following antibodies were used: mouse anti-p27^{Kip1}-horseradish peroxidase-coupled antibodies (clone 57, BD Bioscience, Franklin Lakes, NJ, USA), anti-p27^{Kip1}-C19 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-pY1007/1008-JAK2 (Millipore, Billerica, MA, USA), mouse anti-JAK2 (clone 691R5, Invitrogen), rabbit anti-JAK2 antibodies coupled to agarose (Millipore), mouse anti-pY694/699-STAT5A/B (clone 8-5-2, Millipore), rabbit anti-phospho-Src family (Y416) antibody (Cell signaling, Danvers, MA, USA), mouse anti-phosphotyrosine antibodies (clone 4G10, Upstate, Millipore), mouse anti-pY88-p27^{Kip1} (Grimmler *et al.*, 2007), mouse anti-DDDDK (anti-Flag octapeptide, clone M2, Sigma), mouse monoclonal anti-HA tag antibody (clone 12CA5, Abcam, Cambridge, MA, USA), mouse anti-Skp2 (clone A-2, Santa Cruz), rabbit anti-Cyclin D$_2$ (Santa Cruz), mouse anti-Cyclin E (clone HE12, Santa Cruz), mouse anti-GAPDH (Sigma), mouse anti-α-tubulin (Sigma), mouse anti-LaminA/C (Upstate), rabbit anti-Sp1 (H-225, Santa Cruz) and rabbit anti-IGF-IRβ (C-20, Santa Cruz). Recombinant mouse IL-3 (rIL-3) was purchased from PeproTech (Rocky Hill, NJ, USA) and 'JAK inhibitor-I' from Merck (Darmstadt, Germany).

Western blot, co-immunoprecipitation and cell fractionation

For co-precipitation experiments, cells were lysed in immunoprecipitation buffer (50 m$_M$ (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) pH 7.5, 150 m$_M$ NaCl, 1 m$_M$ ethylenediaminetetraacetic acid (EDTA), 1 m$_M$ EGTA, 10% glycerol, 1% Triton X100, Phosphostop (Roche) and protease inhibitor cocktail (Sigma)) and centrifugated at 16 000 r.p.m. for 20 min. Transfected HA-p27^{Kip1} was immunoprecipitated with anti-HA antibodies covalently linked to protein A-Sepharose (Immunosorb A; EC Diagnostics AB, Uppsala, Sweden), and Flag-JAK2 was immunoprecipitated with anti-Flag antibodies. After SDS–PAGE, western blot analysis was performed with anti-Flag- and mouse anti-p27^{Kip1}-horseradish peroxidase-coupled antibodies. For co-immunoprecipitation analysis of endogenous proteins, JAK2 was immunoprecipitated with anti-JAK2 agarose beads. Immunoblot analyses were performed with mouse anti-JAK2 antibodies and mouse anti-p27^{Kip1}-horseradish peroxidase-coupled antibody. For subcellular fractionation, protein extracts were separated into nuclear, cytoplasmic and membrane compartments (Behrmann *et al.*, 2004). Briefly, cells were incubated in hypotonic lysis buffer and homogenized using a Dounce homogenizer. The nuclei were precipitated by centrifugation at 1000 *g*, 10 min, and washed twice with lysis buffer. The nuclei were lysed in a detergent-containing buffer (20 m$_M$ Tris/HCl pH 7.5, 1% Triton ×100, 280 m$_M$ NaCl, 1 m$_M$ dithiothreitol, 1m$_M$ ethylenediaminetetraacetic acid, protease- and phosphatase inhibitors), and insoluble proteins were precipitated by centrifugation. The crude cytoplasmic fraction was centrifuged at 20 000 *g* and the pellet (membranes) was solubilized in membrane lysis buffer (20 m$_M$ Tris–HCl pH 7.5, 120 m$_M$ NaCl, 1% Triton ×100, 1 m$_M$ ethylenediaminetetraacetic acid (EDTA), 1m$_M$ dithiothreitol , protease and phosphatase inhibitors). The supernatant was once more centrifuged (100 000 *g*) to obtain the cytosolic fraction.

GST-pulldown assay

Recombinant GST-JAK2 fusion protein was expressed in *E.coli*, isolated in lysis buffer (137 m$_M$ NaCl, 2.7 m$_M$ KCl, 1.47 m$_M$ KH$_2$PO$_4$, 4.3 m$_M$ Na$_2$HPO$_4$ and 0.5% Triton ×100) and purified by binding to gluthathione Sepharose (GE Healthcare, Little Chalfont, UK). GST-JAK2 was incubated with recombinant purified His-p27^{Kip1} for 90 min. After repeated washing in lysis buffer, bound proteins were eluted in SDS–PAGE at 95 °C and analyzed by western blot.

In vitro kinase assays

Kinase reactions were performed by incubating purified recombinant His-p27^{Kip1} or His-p27^{Kip1} tyrosine mutants with the GST-JAK2 kinase domain (amino acid 752–1129 of mouse JAK2; ProQinase, Freiburg, Germany) in kinase buffer (5 m$_M$ MgCl2, 2 m$_M$ MnCl2, 150 m$_M$ NaCl, 20 m$_M$ (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)/KOH pH 7.5, 0.05% NP-40, 1 m$_M$ dithiothreitol, 625 μ_M Na-ortho-vanadate, 50 μm 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), 1 μg/μl aprotinin/leupeptin, 100 μ_M ATP and 10 μCi γ-^{32}P-ATP (NEN)) for 30 min at 35 °C. Proteins were separated by SDS–PAGE, stained with Coomassie brilliant blue and exposed to a Storage Phosphor Screen (GE Healthcare). Incorporation of radioactive phosphate into p27^{Kip1} was quantified with a Typhoon 9410 scanner (GE Healthcare) and ImageQuant software (GE Healthcare).

Immunofluorescence

U2OS cells were transfected with HA-p27^{Kip1} or HA-p27^{Kip1}-Y88F and Flag-JAK2 or Flag-JAK2V617F and seeded on glass bottom plates. Cells were fixed with 4% para-formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 and incubated overnight with the primary antibodies (rabbit anti-p27^{Kip1} C19 (Santa Cruz), rabbit anti-pY1007/1008-JAK2 (Millipore) and mouse anti-pY88-p27^{Kip1} (Grimmler *et al.*, 2007)). After washing, cells were incubated with fluorescence (Alexa Fluor, Invitrogen) labeled secondary antibodies for 1 h, washed again and finally DNA was detected with Hoechst stain. Confocal image analysis was performed using a TCS SP5 Confocal Laser Scanning Microscope (Leica, Wetzlar, Germany).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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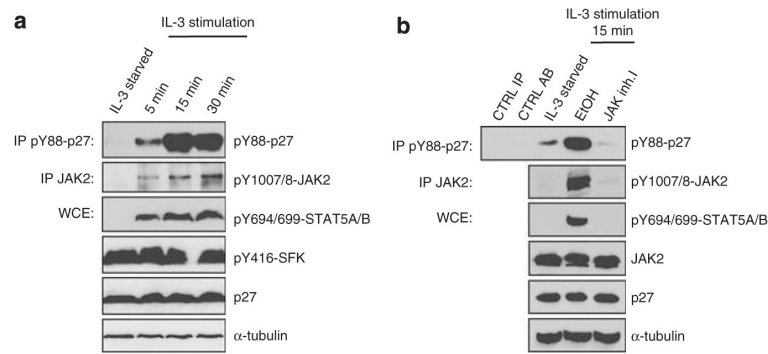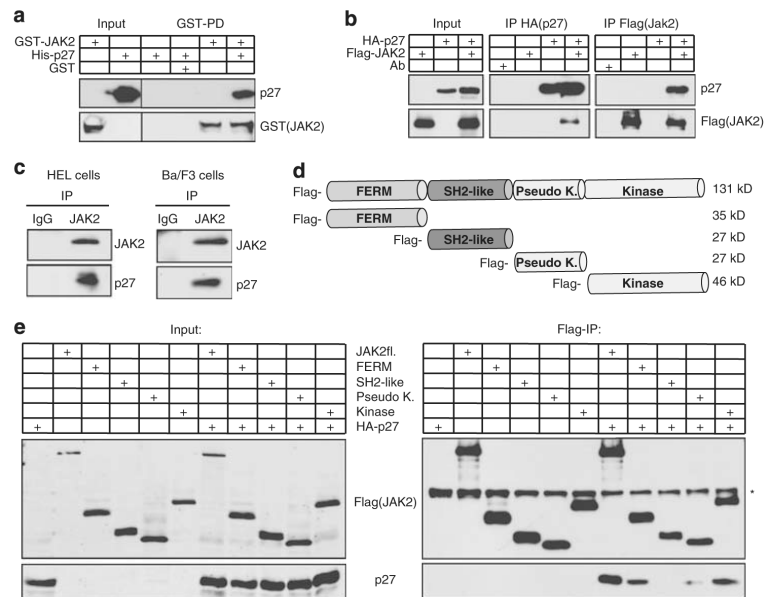
Figure 1.
IL-3 induces phosphorylation of p27^{Kip1} on tyrosine 88. (**a**) Ba/F3 cells were starved (6 h) for IL-3 and stimulated with 5 ng/ml rIL-3 as indicated. Level of p27^{Kip1}-phospho-Y88 immunoprecipitates, JAK2-phospho-Y1007/1008 immunoprecipitates, STAT5A/B-phospho-Y694/699, Src family kinase-phospho-Y416, p27^{Kip1} and α-tubulin were determined by immunoblot analysis. Representative blots of three independent experiments are shown. (**b**) Tyrosine-88-phosphorylation of p27^{Kip1} correlates with JAK2 activation. Ba/F3 cells were starved (6 h) for IL-3 and stimulated with 5 ng/ml recombinant IL-3 for 15 min in the absence and presence of 3 μ$_M$ JAK inhibitor-I. Immunoblot analysis of p27^{Kip1}-phospho-Y88 immunoprecipitates, JAK2-phospho-Y1007/1008 immunoprecipitates, STAT5A/B-phospho-Y694/699, JAK2, p27^{Kip1} and α-tubulin as a loading control. Signals decreased to 0.89% for p27^{Kip1}-phospho-Y88, to 1,22% for JAK2-phospho-Y1007/1008 and to 0.28% for STAT5A/B-phospho-Y694/699 after JAK inhibitor treatment of IL-3 stimulated cells (vehicle control=100%; Gel Pro Analyser software, Media Cybernetics, Bethesda, MD, USA). Mouse IgG was used in control IPs (CTRL IP), coupled p27^{Kip1}-phospho-Y88-specific monoclonal antibodies were loaded to exclude unspecific signals of the antibody (CTRL AB). IP, immunoprecipitation; WCE, whole cell extracts.
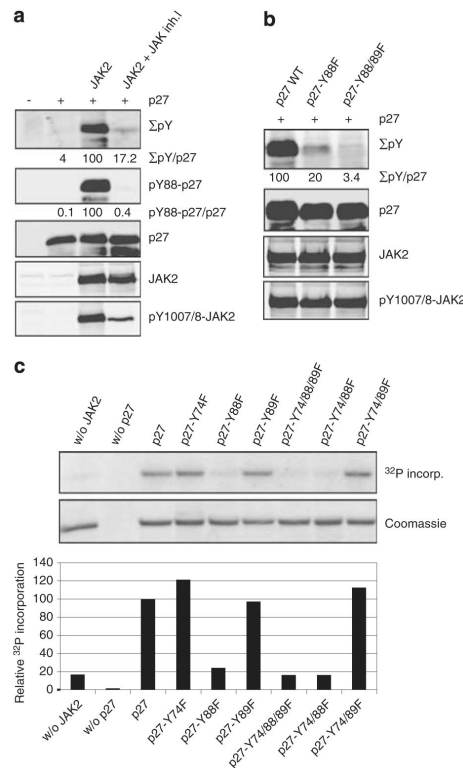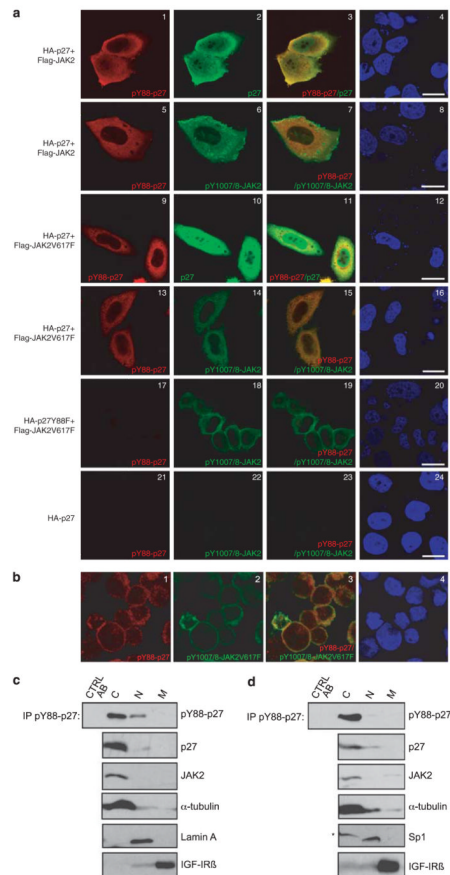
Figure 2.
JAK2 phosphorylates p27^{Kip1} on tyrosine residue 88 *in vitro* and *in vivo*. (**a**) Incubation of JAK2-transfected 293T cells with 3 μ$_M$ JAK inhibitor-I for 3 h impairs p27^{Kip1} tyrosine-88-phosphorylation. HA-p27^{Kip1} was co-transfected with JAK2 in 293T cells. Extracts were boiled (65 °C; 10 min) to precipitate the majority of the proteins. Levels of the heat stable p27^{Kip1} and its phosphorylation on tyrosines were simultaneously determined by the Odyssey infrared imaging system. Overall phospho-tyrosine was detected using the 4G10 antibody, and p27^{Kip1}-Y88 phosphorylation was detected by a specific p27^{Kip1}-pY88 monoclonal antibody. Quantitative analyses of these signals (adjusted to 100% for wild-type p27^{Kip1} when co-expressed with JAK2, and corrected for p27^{Kip1} expression levels) are listed below as indicated. JAK2 and its tyrosine phosphorylation on residues 1007/1008 were analyzed in whole cell extracts by the Odyssey infrared imaging system. A representative of three independent experiments is shown. (**b**) Tyrosine 88 is the main JAK2 phosphorylation site. JAK2 was transfected with p27^{Kip1} and p27^{Kip1} tyrosine to phenylalanine mutants as indicated. The analysis was performed as described above. (**c**) JAK2 phosphorylates Y88 of p27^{Kip1} *in vitro*. *In vitro* kinase assays were performed with recombinant purified His-p27^{Kip1} and His-p27^{Kip1} mutant proteins as indicated. Proteins were incubated with the kinase domain of JAK2 and γ-^{32}P-ATP. Incorporation of radioactive phosphate into p27^{Kip1} was detected after SDS–PAGE by autoradiography (upper panel). p27^{Kip1} loading was confirmed by Coomassie brilliant blue staining (middle panel). Incorporation of ^{32}P was quantified with a Typhoon 9410 scanner and ImageQuant software. Phosphorylation of wt p27^{Kip1} was set to 100% (lower panel).
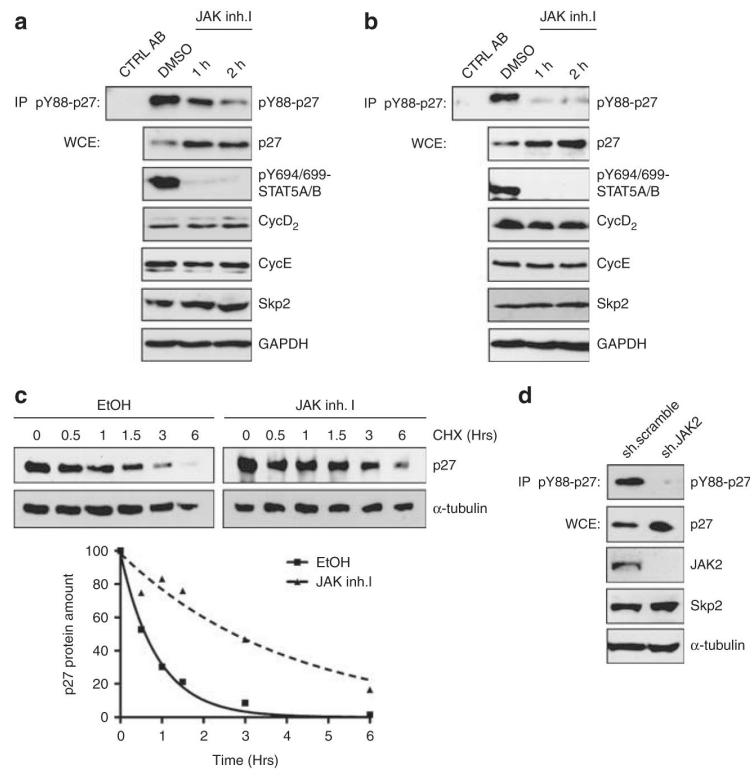
Figure 3.
JAK2 directly interacts with p27^{Kip1} through its FERM and kinase domain. (**a**) GST and GST-JAK2 fusion proteins isolated from *E.coli* were bound to glutathione sepharose and incubated with recombinant purified His-p27^{Kip1}. After extensive washing, GST-JAK2-associated p27^{Kip1} was detected in immunoblots. (**b**) p27^{Kip1} interacts with JAK2 in 293T cells. HA-p27^{Kip1} and Flag-JAK2 were overexpressed and expression was verified by immunoblotting. For the co-preciptiation analysis, HA-p27^{Kip1} or Flag-JAK2 were immunoprecipitated using tag-specific antibodies. Immunoprecipitated proteins were detected in immunoblots. (**c**) Endogenous p27^{Kip1} co-immunoprecipitates with endogenous JAK2 and JAK2V617F in hematopoietic cells. JAK2V617F was immunoprecipitated from HEL cells and JAK2 from IL-3-starved (16 h) Ba/F3 cells and analyzed for co-immunoprecipitated p27^{Kip1} in immunoblots. (**d**) Schematic representation of JAK2 domains that are used in (**e**). p27^{Kip1} interacts with JAK2, its isolated FERM- and kinase domains. Flag-tagged JAK2 and Flag-tagged JAK2 domains were expressed with HA-p27^{Kip1} in 293T cells. Their expression was confirmed in immunoblots ((**e**), left panel, 'Input'). Flag-tagged proteins were immunoprecipitated with Flag-antibodies and precipitates were analyzed in immunoblots ((**e**), right panel, 'Flag-IP'). p27^{Kip1} co-immunoprecipitates with JAK2, its FERM domain and its kinase domain. A weak interaction could be observed with the pseudokinase domain. The asterisk (*) indicates heavy chain.

Figure 4.
Tyrosine 88-phosphorylated p27^{Kip1} is predominantly localized in the cytoplasm. (**a**) Immunofluorescence analyses of U2OS cells transfected with HA-p27^{Kip1}, Flag-JAK2 or Flag-JAK2V617F as indicated by confocal microscopy. pY88-p27^{Kip1} is shown in red and pY1007/1008-JAK2/pY1007/1008-JAK2V617F and p27^{Kip1} are shown in green. DNA of the corresponding cells is stained with Hoechst dye (blue). Panels 1–4 show the localization of p27^{Kip1} and pY88-p27^{Kip1}, panels 5–8 the localization of pY88-p27^{Kip1} and pY1007/1008-JAK2 after transfection of p27^{Kip1} and JAK2. Panels 9–12 show the localization of pY88-p27^{Kip1} and JAK2V617F and panels 13–16 the localization of pY88-p27^{Kip1} and pY1007/1008-JAK2 after transfection of p27^{Kip1} and JAK2V617F. As negative controls, cells transfected with HA-p27^{Kip1}–Y88F and JAK2V617F or cells transfected with HA-p27^{Kip1} only were stained with antibodies recognizing pY88-p27^{Kip1} and pY1007/1008-JAK2 (panels 17–24). Scale bar represents 20 μm; (**b**) Immunofluorescence analysis of endogenous pY88-p27^{Kip1} (panel 1) and pY1007/1008-JAK2V617F (panel 2) in HEL cells by confocal microscopy. The corresponding overlay is shown in panel 3 and Hoechst staining in panel 4. (**c**) Endogenous pY88-p27^{Kip1} is predominantly cytosolic in HEL cells. Whole cell extracts were separated in nuclear, cytosolic and membrane fractions and analyzed by immunoblots as indicated. Lamin A, α-tubulin and IGF-IRβ were used as markers for nuclear, cytoplasmic and membrane fractions. (**d**) Endogenous pY88-p27^{Kip1} is predominantly cytosolic in Ba/F3 cells. Analysis as in (**c**), transcription factor Sp1 was used as control for nuclear proteins. Representative blots of three independent experiments are shown. The asterisk (*) indicates a non-specific band.

Figure 5.
p27^{Kip1}-Y88 phosphorylation is decreased and p27^{Kip1} stability is increased upon JAK2V617F inhibition or small hairpin RNA-mediated JAK2V617F knockdown. (**a**) Inhibition of JAK2V617F reduces p27^{Kip1}-Y88 phosphorylation and increases p27^{Kip1} expression in HEL cells. Cells were incubated with solvent (dimethyl sulfoxide or ethanol, as indicated) or JAK inhibitor-I (JAK inh.I, 3 μM). p27^{Kip1}-pY88 immunoprecipitates, p27^{Kip1}, pY694/699-STAT5A/B, cyclin D$_2$, cyclin E, Skp2, GAPDH were detected by immunoblotting. IP, immunoprecipitation, WCE, whole cell extract. (**b**) SET-2 cells were analyzed as in (**a**). (**c**) p27^{Kip1} stability increases upon JAK2V617F inhibition. HEL cells were incubated with JAK inhibitor-I (3 μM) or the solvent (ethanol). After 4 h incubation, cycloheximide (CHX) was added to a final concentration of 100 μg/ml and cells were incubated for the indicated periods of time. p27^{Kip1} expression was determined in immunoblots. The α-tubulin was used as a loading control. Lower panel: immunoblot signals were quantified (Gel Pro Analyser software, Media Cybernetics) and intensities were adjusted to 100% at 0 h and plotted over time. The exponential decay curve, determined by regression analysis, is shown (GraphPad Prism5 software, La Jolla, CA, USA). (**d**) Knockdown of JAK2V617F in HEL cells reduces p27^{Kip1}-Y88 phosphorylation and increases p27^{Kip1} levels. HEL cells were electroporated with control ('scramble') or JAK2-specific small hairpin RNA (pLKO1.puro plasmids, (Neilson *et al.*, 2007) and incubated for 40 h. p27^{Kip1}-pY88 immunoprecipitates, p27^{Kip1}, JAK2, Skp2 and α-tubulin were detected in immunoblots.