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A CK2-DEPENDENT MECHANISM FOR ACTIVATION OF THE JAK-STAT SIGNALING PATHWAY: IMPLICATIONS FOR CANCER BIOLOGY

by

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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2010

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A CK2-DEPENDENT MECHANISM FOR ACTIVATION OF THE JAK-STAT SIGNALING PATHWAY: IMPLICATIONS FOR CANCER BIOLOGY

YING ZHENG

CELL BIOLOGY

ABSTRACT

Janus Kinase (JAK)-Signal Transducer and Activator of Transcription (STAT) signaling is involved in regulation of cell survival, proliferation and differentiation. JAK tyrosine kinases can be transiently activated by cytokines or growth factors in normal cells, while they become constitutively activated as a result of mutations that affect their function in tumors. Specifically, the JAK2V617F mutation is present in the majority of patients with myeloproliferative disorders (MPDs) and is implicated in the pathogenesis of these diseases. In this dissertation, we report the identification of the serine/threonine kinase CK2 as a novel interactor and important regulator of activation of the JAK-STAT signaling pathway in normal cells as well as in tumor cells. Briefly, we demonstrate that OSM-induced activation of JAKs and STATs, and expression of SOCS-3, a downstream gene, are inhibited by CK2 siRNAs or pharmacological inhibitors. IFN-γ and growth hormone (GH)-induced JAK-STAT signaling are also suppressed by inhibition of CK2 activity. Endogenous CK2α and β are associated with JAKs, and this association is not dependent on JAK kinase activity. In addition, CK2 can phosphorylate JAK2 *in vitro*, which suggests that CK2 may facilitate JAK activation by interaction with and/or phosphorylating JAKs. To extend these findings, we demonstrate that CK2 also interacts with JAK2V617F, and CK2 inhibitors suppress JAK2V617F autophosphorylation and downstream signaling in homozygous JAK2V617F-expressing human erythroleukemia

HEL92.1.7 cells (HEL). CK2 inhibitors also potently induce apoptotic cell death of HEL cells.

These data provide evidence for novel crosstalk between CK2 and JAK-STAT signaling, with implications for cancer therapy. In addition to MPDs, constitutive activation of JAKs and STATs also mediate neoplastic transformation and promote cell proliferation in many other human malignancies, such as glioblastoma (GBM). Expression of STAT-3 downstream genes such as IL-6, Mcl-1 and Pim-1 in GBM cells, which may contribute to the uncontrolled proliferation and anti-apoptotic features of GBM, can be suppressed by CK2 inhibitors. Future studies should be aimed at further clarifying the mechanisms by which CK2 regulates JAK activation by determining the interaction domains and phosphorylation sites of JAKs by CK2.

Keywords: CK2, JAK, JAK2V617F, MPDs, STAT, OSM

DEDICATION

This dissertation is dedicated to my parents and my husband Jun. Without their love and support, I could not have achieved my PhD.

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I thank every person in Dr. Benveniste's lab. Everyone taught me something. They care about me and gave me support during my pregnancy. I cherish the chance to work with such a group of talented and nice people.

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Finally, I want to thank my entire family, who were always behind me. Their love motivates me to overcome the difficulties in science and in my life. I love them.

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INTRODUCTION

The JAK-STAT Pathway

The Janus Kinase (JAK)-Signal Transducer And Activator (STAT) pathway is crucial in transmitting signals from many extracelluar cytokines, interferons and growth factors into the nucleus, regulating a large number of genes. It is crucial for many cellular functions including cell survival, proliferation and differentiation (1). There are four members of the JAK family including JAK1, JAK2, JAK3 and TYK2, and seven members of the STAT family including STAT-1, STAT-2, STAT-3, STAT4, STAT-6, and the closely-related STAT-5a and STAT-5b. The JAK-STAT signaling pathway has been extensively studied. The current model of JAK-STAT signaling is as follows: engagement of cytokines or growth factors to their receptors activates the receptor-associated JAKs, which in turn phosphorylate the receptor cytoplasmic region, allowing recruitment of STATs, which in turn are phosphorylated by JAKs, dimerize, translocate to the nucleus, bind to specific regulatory elements of genes, and activate gene transcription (**Figure 1**) (2). Knock-out of several key components of this signaling pathway such as JAK2 and STAT-3 is embroynic lethal (**Table 1**). Different cytokines and growth factors bind to different receptors, and signal through different combination of JAKs and STATs (**Table 2**).

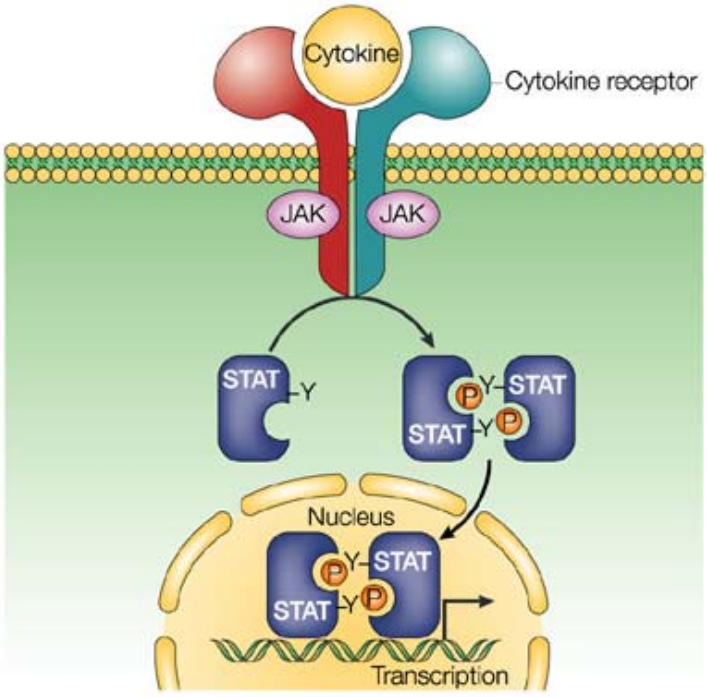

Figure 1. Schematic Representation of the JAK-STAT Pathway. The activation of JAKs after cytokine stimulation results in the phosphorylation of STATs, which then dimerize and translocate to the nucleus to activate gene transcription.

Note: From "Regulation of JAK-STAT Signaling in the Immune System" by Ke Shuai and Bin Liu, 2003, Nature Reviews Immunology, 3, p. 900-911. Copyright 2003 by the Nature Publishing Group. Adapted with permission.

Table 1. Phenotype of Knock-out of JAK-STAT Signaling Components

Gene	Phenotype
Jak1	Viable but perinatal lethality due to neurologic deficits, SCID.
Jak2	Embryonically lethal due to failure in erythropoiesis.
Jak3	Viable and fertile, SCID.
Tyk2	Viable and fertile, defective IL-12 signaling especially in NK cells, increased susceptibility to selected viral infections.
Stat1	Viable and fertile, defective IFNα/β and IFN-γ functions, increased tumorigenicity.
Stat2	Viable and fertile, defective IFN-α/β functions, reduced Stat1 expression in some tissues.
Stat3	Embryonic lethal, conditional knockouts define tissue specific functions (see text).
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.
Stat5 A/B	Viable, female infertility, defective mammary gland development, reduced body mass in males and females, defective T cell proliferation.
Stat6	Viable and fertile, defective IL-4-driven Th2 differentiation, increased susceptibility to helminthic infestation.
Stat4/6	Viable and fertile, defective Th-2 differentiation (Th1 skewed).
SOCS1	Viable but perinatal lethality severe IFN-γ-dependent inflammatory disease.
SOCS2	Viable and fertile, Gigantism due to increased growth hormone and IGF-1.
SOCS3	Embryonically lethal due to placental defects and erythrocytosis.
CIS	Viable and fertile — no phenotype reported.

Note: From "Cytokine Signaling in 2002: New Surprises in the Jak/Stat Pathway" by John J. O'Shea, Massimo Gadina and Robert D. Schreiber, 2002, Cell, 109, p. S121-S131. Copyright 2002 by Elsevier. Reprinted with permission.

Table 2. Utilization of JAKs and STATs by IL-6 Family Members, Interferons and Growth Hormone (GH)

Ligand	Receptors	JAKs	STATs
IL-6	IL-6R + gp130	JAK1, JAK2, TYK2	STAT-1, STAT-3
CNTF CT-1 LIF OSM	CNTFR + gp130 CT-1R + gp130 LIFR + gp130 OSMR + gp130	JAK1, JAK2, TYK2	Predominant: STAT-3 Secondary: STAT-1, STAT-5
IFN-α/β	IFNAR1 + IFNAR2	JAK1, TYK2	Predominant: STAT-1, STAT-2 Secondary: STAT-3, STAT-4, STAT-5
IFN-γ	IFN-γR1 + IFN-γR2	JAK1, JAK2	STAT-1, STAT-3
GH	GHR	JAK2	STAT3, STAT5 (mainly STAT5b)

OSM-JAK-STAT Signaling

The Interleukin-6 (IL-6) family has nine members: IL-6, Oncostatin M (OSM), interleukin-11 (IL-11), leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), cilary neurotrophic factor (CNTF), neuropoietin and interleukin-27 (IL-27). Many members in this family are involved in inflammatory regulation, especially IL-6 (3). Expression of many family members is elevated in diseases such as coronary heart disease (4), inflammatory bowel disease, rheumatoid arthritis (5) or psoriasis (6). All the IL-6 family members signal through receptor complexes containing the gp130 (glycoprotein 130) signal transduction subunit (**Table 2**).

OSM is mainly secreted by activated T lymphocytes, macrophages, and neutrophils (7, 8) and has predominantly proinflammatory properties (9, 10). Increased levels of OSM have been detected in synovial fluid of rheumatoid arthritis patients (11) and in dermal lesions of psoriasis patients (6). OSMR knock-out mice are viable and fertile, but show defects in hematopoiesis and liver regeneration after injury (12, 13). Besides the MAPK and PI3K pathways, OSM is a potent activator of the JAK-STAT signaling pathway (14, 15). Human OSM signals through gp130-OSMRβ as well as gp130-LIF receptor (LIFR) heterodimers, while mouse OSM uses only gp130-OSMRβ (**Figure 2**) (16). Upon OSM binding, receptor-associated JAKs, including JAK1, JAK2 and TYK2, become activated and lead to the phosphorylation of gp130 tyrosine residues. The phosphorylated residues direct the SH2-dependent recruitment of STAT proteins, including STAT-1, STAT-3

and STAT-5, which in turn become JAK substrates. Activated tyrosine-phosphorylated STATs are released from the receptors as they reorient into an antiparallel dimer, where the SH2 domain of one STAT binds the phospho-tyrosine residue of another STAT. Activated STAT dimers (homo- and hetero-dimers) translocate to the nucleus, and bind to specific consensus sequences in the promoters of OSM-responsive genes, inducing transcription (**Figure 2B**).

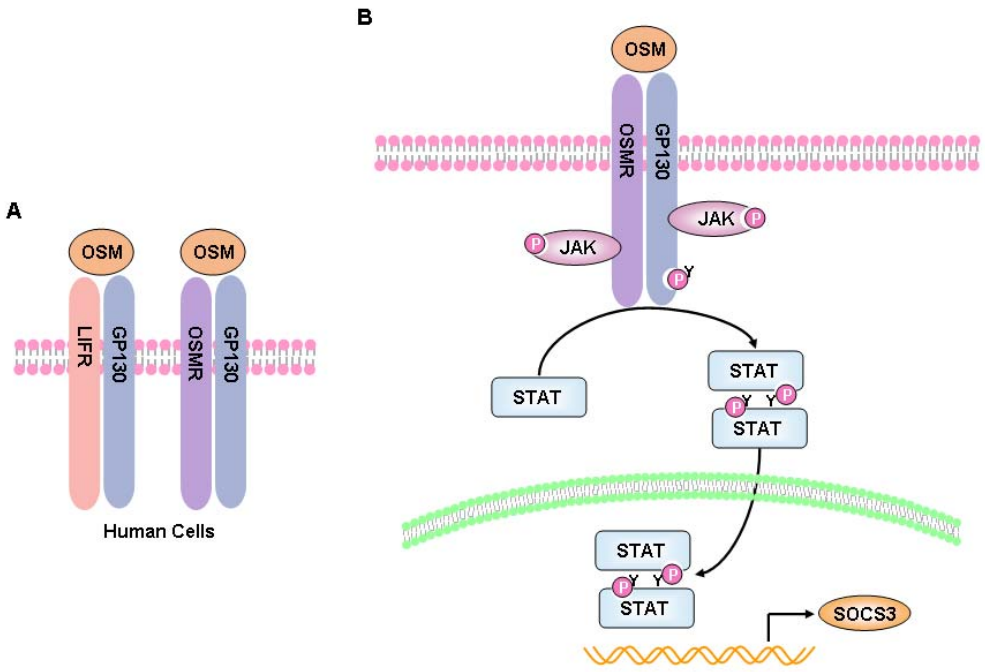

Figure 2. OSM-JAK-STAT Signaling Pathway. A. OSM receptor complexes in human cells. **B.** OSM activates JAK-STAT signaling. OSM binding to the gp130/OSMRβ complex leads to activation of STATs and expression of downstream genes, such as SOCS3.

JAKs and Associated Proteins

JAKs are a family of cytoplasmic non-receptor tyrosine kinases. Except for the restrictive expression of JAK3 in hematopoietic cells, they are widely expressed in a variety of tissues. JAK proteins range in size from 120 to 140 kDa, and they have seven homology domains (JH1-JH7) based on amino acid sequence similarity. JAKs contain four putative domains: FERM (four point one, ezrin, radixin, and moesin) containing JH7-5 and half of JH4, Src homology-2 (SH2) domain containing JH3 and half of JH4, pseudokinase (JH2), and kinase domains (JH1) (**Figure 3A**). The FERM domain is found in several proteins, and is the protein-interacting domain required for targeting proteins to various membrane proteins. The FERM domain is responsible for binding to the cytoplasmic tail of cytokine receptors (17, 18) and regulates the catalytic activity of the kinase domain (19). The SH2 domain is important for cytokine receptor binding and surface expression of OSMR (20). The pseudokinase domain regulates the kinase domain and interactions with cytokine receptors (21, 22).

JAK proteins are endogenously bound to many cytokine receptors. For example, the receptors for OSM, gp130 and OSMRβ, stably bind to JAK1 and JAK2 and TYK2 (23). In addition to cytokine receptors, there are many other molecules that can bind JAKs. For example, SH3- and ITAM-containing molecules (STAM1) associate with JAK2 and JAK3, and can be phosphorylated by them (35). The SH2 containing proteins SH2Bβ and APS (adaptor molecule containing Pleckstrin homology (PH) and SH2 domains) also bind and regulate the catalytic activity of some JAKs (36). Compared to thirty-six cytokine receptor combinations that respond to thirty-eight

cytokines, there are only four mammalian JAKs which are conserved from human to chicken. Thus, JAKs are very critical to cytokine signaling and their activity must be tightly regulated.

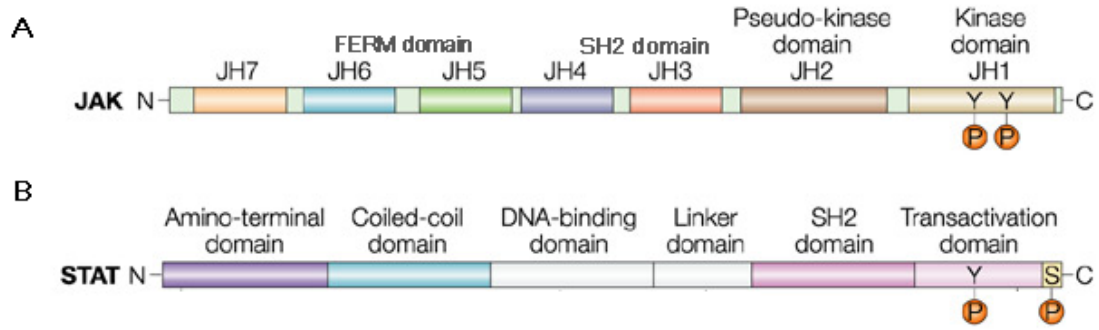

Figure 3. Domain Structure of JAKs and STATs.

Note: From "Regulation of JAK-STAT Signaling in the Immune System" by Ke Shuai and Bin Liu, 2003, Nature Reviews Immunology, 3, p. 900-911. Copyright 2003 by the Nature Publishing Group. Adapted with permission.

STATs

The seven mammalian STAT genes are located in three chromosome clusters, suggesting that they have evolved by gene duplication. STAT proteins range in size from 750 to 900 amino acids, and contain several conserved domains including amino-terminal domain, coiled-coil domain, DNA-binding domain, linker domain, SH2 domain and transactivation domain (**Figure 3B**). STAT proteins have dual roles, that is they can transduce signals in the cytoplasm and also function as transcription factors in the nucleus. The SH2 domain is responsible for binding of STATs to

tyrosine-phosphorylated receptors, and also for the homo- or hetero-dimerization with other tyrosine-phosphorylated STATs in a reciprocal manner.

Cytokine receptors usually do not have intrinsic tyrosine kinase activity, and they activate STATs by phosphorylated JAKs. Tyrosine phosphorylation of STATs (Tyr 705 in STAT-3, Tyr701 in STAT-1, and Tyr 694 in STAT-5) by JAKs is the indicator of STAT activation, and serine phosphorylation (Ser 727 in both STAT-3 and STAT-1) by other kinases after tyrosine phosphorylation is required for the maximal transcriptional activity of STATs (24). STAT-3 can also be acetylated by the histone acetyltransferase p300 at lysine 685, which resides in the C-terminal transcriptional activation domain, and regulate its dimerization, DNA binding and transactivating activities (25, 26).

STAT activation is inducible by many cytokines including the IL-6 family of cytokines and interferons. Activated STATs bind to specific elements in the promoters of genes, and facilitate gene transcription. For example, IFN-γ-induced STAT-1 dimers bind GAS (γ activated sequence) elements ($TTCN_{2-4}AA$), while type I interferons induce formation of complex of STAT-1, STAT-2 and IRF9, which then bind to ISREs (IFN-α/β-stimulated response elements), $AGTTTN_3TTTC$ (27). In addition to cytokines, STATs are also activated by many growth factor receptors with intrinsic tyrosine-kinase activity, such as the epidermal growth factor receptor (EGFR), insulin-like growth receptor (IGFR) and vascular endothelial growth factor (VEGFR) (28). Many genes encoding cytokines and growth factors are in turn the target of the same STATs.

Negative Regulation of the JAK-STAT Pathway

The JAK-STAT signaling pathway is negatively regulated at different stages (**Figure 4**). Tyrosine phosphatases can de-phosphorylate and inactivate cytokine receptors, JAKs and STATs (29). Protein Inhibitors of Activated STATs (PIAS) proteins inhibit STAT transcription through different mechanisms including sequestering activated STATs in the nucleus, blocking the DNA-binding activity of STATs in the nucleus, and recruiting transcriptional co-repressors (30).

The Suppressor Of Cytokine Signaling (SOCS) proteins are key physiological regulators of the immune system (31). SOCS is a family of proteins including CIS and SOCS-1 through SOCS-7. SOCS proteins are induced upon cytokine binding, bind to cytokine receptors or JAKs, inhibit JAK activity and prevent STATs from becoming activated (32). SOCS proteins contain a central SH2 domain, a C-terminal SOCS box, and an N-terminal kinase inhibitory region (KIR), which resembles the activation loop of JAKs. The SH2 domain is responsible for binding to tyrosine phosphorylated JAKs or receptors, or competing for STAT binding. SOCS-3 inhibits many cytokine signaling pathways such as IL-6 and OSM signaling (33, 34).

Previous studies in our laboratory identified the tumor suppressor PML as a negative regulator of the JAK-STAT pathway (35). PML binds to STAT-1 and inhibits its transcriptional activity upon IFN-γ stimulation (35). PML can also bind to STAT-3, inhibits DNA-binding activity and abrogates gp130-mediated growth (36).

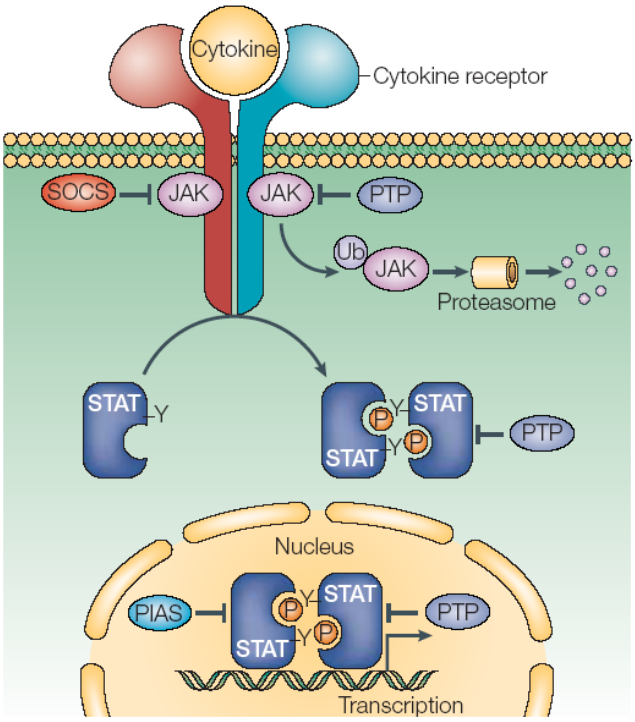

Figure 4. Negative Regulation of the JAK-STAT Signaling Pathway. The JAK-STAT signaling is negatively regulated at many levels. Protein Tyrosine phosphatases (PTPs) such as SHP-1, CD45 and PTP1b de-phosphorylate and inactivate phosphorylated JAKs and cytokine receptors, but the phosphatase in the nucleus needs to be determined. SOCS proteins such as CIS, SOCS-1 and SOCS-3 inhibit signaling by different mechanisms including binding to and inhibiting JAK activity by their KIR domain, binding receptors and blocking STAT recruitment, and promoting degradation of JAK/receptor complexes by the proteosome via its ubiquitin E3 ligase activity. PIAS proteins inhibit activated STAT dimers in the nucleus, presumably by its SUMO E3 ligase activity.

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Aberrant JAK-STAT Activation in Human Malignancies

Kinases and Hematopoietic Malignancies

The human genome contains 518 putative protein kinase genes, 244 of which map to disease loci or cancer amplicons (37). There are approximately 100 tyrosine kinases in mammalian cells, which are classified into two subfamilies, receptor and non-receptor kinases. In normal cells, activation of kinases is tightly regulated by the absence or presence of ligand binding. However, mutations or altered expression may lead to constitutive activation of kinases, therefore constitutively activating downstream signaling cascades and promoting unregulated cell growth, or other characteristics of tumor cells. Notably, aberrant activation of kinases plays a critical role in many human malignancies such as myeloid malignant diseases (38). The Philadelphia chromosome is caused by t(9;22)(q34;q11) reciprocal chromosomal translocation, and the result is Bcr-Abl, a fusion gene on Chromosome 22. Abl is a tyrosine kinase gene, and the Bcr-Abl protein is constitutively active and strongly associated with chronic myeloid leukemia (39). Constitutive activation of FLT3 (FMS-like tyrosine kinase, CD135) is associated with acute myeloid leukemia (40). Upon ligand binding, the tyrosine kinase c-Kit activates JAK-STAT, PI3K and MAP kinase pathways and promotes cell growth and differentiation, while mutations of c-Kit are strongly linked to the development of mast cell malignancy (40). Platelet-derived growth factor receptor β (PDGFRβ), a receptor tyrosine kinase, is disrupted by translocations and becomes constitutively active in atypical chronic myelomonocytic leukemia (40). Other examples include the PDGFRα fusion gene in

idiopathic hypereosinophilic syndrome (41), and FGFR1 gene rearrangements in stem-cell leukemia/lymphoma syndrome (42). The identification of these activating mutations provides an opportunity to develop molecularly targeted therapies for patients with these disorders. Inhibition of these activated kinases and their signaling pathways by small-molecule inhibitors is becoming an intriguing strategy to suppress neoplastic growth and survival. A good example of this strategy was demonstrated by therapy with imatinib mesylate (formerly known as STI571; drug name: Gleevec; Novartis, Basel, Switzerland) in chronic myelogenous leukemia (CML). Imatinib mesylate binds to the ATP-binding site in the catalytic domain of the Bcr-Abl oncoprotein, thereby inhibiting its kinase activity. Therapy with imatinib mesylate successfully results in durable and complete cytogenetic response in the early stages of CML (43-45).

Aberrant JAK activation is often associated with human malignancies. JAK2 mutations occur in several myeloid as well as lymphoid malignancies. Three gain-of-function mutations of JAK3, A572V, V722I and P132T, were identified in acute megakaryoblastic leukemia patients and cell lines (46). Amplification of JAK2 has been detected in Hodgkin's lymphoma (47), and an activating mutation of JAK2 was found in B-cell precursor acute lymphoblastic leukemia (48). Degradation of JAK2 is impaired and JAK2 phosphorylation is sustained in mediastinal B-cell lymphoma, which may be due to SOCS-1 mutations that disrupt the SOCS box, or by complete deletion of the SOCS-1 gene (49, 50). The SOCS-1 promoter is hypermethylated in multiple myeloma, resulting in inactivation of SOCS-1, which

may contribute to enhanced responsiveness to cytokines and tumor cell survival and expansion (51).

Myeloproliferative Disorders (MPDs)

MPDs are a group of clonal hematopoietic disorders characterized by excessive production of blood cells by hematopoietic precursors. Polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF), which is also known as myeloid metaplasia with myelofibrosis (MMM), or idiopathic myelofibrosis, were first described in the late 1890s and early 1900s. They were three clinicopathologically distinct diseases, but believed to be closely related (52). In 1951, PV, PMF, and ET were classified as pathogenetically related MPDs by William Dameshek (52). PV is characterized by increased production of blood cells, especially red blood cells, while ET is characterized by elevation of platelets. In PMF, fibrous tissues form in the bone marrow as a result of abnormal production of blood cells including platelets, white blood cells, and red blood cells. The progenitor cells of MPDs are hypersensitive to growth factors. For example, progenitor cells of PV patients are hypersensitive to erythropoietin, insulin-like growth factor-1, thrombopoietin, interleukin-3, and granulocyte/monocyte-colony stimulating factor. The main complication in PV and ET is thrombosis, although haemorrhage can also occur. PV and ET may develop into myelofibrosis or acute myeloid leukemia (AML) in the long term. The late stage of PMF is characterized by bone marrow fibrosis, cytopenia, and splenomegaly, and can also transform to AML.

In a broader classification, chronic myeloid leukemia (CML) is also a type of MPDs, and characterized by the increased and unregulated growth of predominantly myeloid cells in the bone marrow and the accumulation of these cells in the peripheral blood. CML now is defined by its cause, the Bcr-Abl gene, and can be effectively treated with imatinib mesylate. Thus far, there are no effective cures for PV, ET and PMF, the three Bcr-Abl negative MPDs.

The genetic basis of PV, ET and PMF was not clear until 2005. Four independent groups of scientists identified that the majority of these diseases are caused by mutations that constitutively activate JAK2 (53-56). The William Vainchenker group first found that JAK2 inhibitors and siRNAs suppress endogenous erythroid colonies (EEC) formation, which is a hallmark of abnormal *in vitro* growth of hematopoietic progenitors. They then sequenced the JAK2 gene and found the JAK2V617F mutation in 40 out of 45 PV patients, but not in controls. They demonstrated that the mutation is acquired because it does not involve all cell types. Mitotic recombination (genetic recombination occurs in mitosis) may be the cause of homozygous mutations in some patients. The JAK2V617F mutation is located in the pseudokinase domain, which negatively regulates JAK2 catalytic activity, probably through an interaction with the kinase domain (21). In contrast to wildtype JAK2, expression of JAK2V617F in JAK2-deficient γ2A cells activates STAT-5 transcription in the absence of erythropoietin, and induces growth factor-independent cell growth in JAK2V617F-expressing BaF/3, BaF/3-EpoR, and FDCP-EpoR factor-dependent cell lines (55). Robert Kralovics, Radek Skoda and their colleagues used microsatellite

mapping to identify loss of heterzygosity (LOH) on the short arm of chromosome 9 (9pLOH) in MPDs, and found JAK2 as a candidate gene. They then sequenced JAK2 in MPD patients, and found the JAK2V617F mutation in 83 of 128 patients with PV, 21 of 93 patients with ET, and 13 of 23 patients with PMF (54). Compared to patients with wildtype JAK2, patients with the JAK2V617F mutation have a higher rate of complications including fibrosis, hemorrhage and thrombosis (54). Expression of JAK2V617F in interleukin-3-dependent BaF3 cells enhances cell survival and proliferation in the absence of cytokines (54). Anthony Green and colleagues sequenced the JAK2 gene followed by allele-specific PCR, and identified JAK2V617F in peripheral-blood granulocytes of 53 of 73 patients with PV, 6 of 51 with ET, and 7 of 16 with PMF (56). Levine and colleagues performed high-throughput DNA resequencing and systematic survey of the tyrosine kinome in MPDs, and identified JAK2V617F in granulocytes of 121 of 164 PV patients, 37 of 115 ET and 16 of 46 PMF patients (53). Taken together, these independent studies identified a guanine-to-thymidine substitution, causing a valine to phenylalanine mutation of JAK2. JAK2V617F is a somatic point mutation occurring in most PV patients, more than 50% of ET and PMF patients, but not in controls. It has constitutive kinase activity, which activates STAT-5 and promotes cytokine-independent cell survival and growth. In addition, expression of JAK2V617F in mice recapitulates many of the pathologic characteristics observed in human PV, ET and PMF patients (57-59). For example, transgenic expression of JAK2V617F results in erythroid, megakaryocytic, and granulocytic hyperplasia in the

bone marrow, splenomegaly, and reduced levels of plasma erythropoietin and thrombopoietin (57). The transgenic mice also had increased numbers of hematopoietic progenitor cells in peripheral blood, spleen and bone marrow, where they can form autonomous colonies in the absence of growth factors and cytokines (57). Interestingly, although JAK2V617F mutations are common in MPDs, they occur less commonly in other myeloid diseases, while they do not occur in lymphoid malignancies or in solid tumors.

In vitro studies suggest that JAK2V617F activates multiple downstream signaling pathways, including the STAT family, predominantly STAT-5 and STAT-3, the mitogen activated protein kinase (MAPK) and the phosphotidylinositol 3-kinase (PI3K) pathways. These signaling pathways are involved in cell survival and proliferation, and are often activated in many human malignancies. The role of these pathways in JAK2V617F-mediated MPDs is not fully understood; however, several lines of evidence have shown that STAT-5 and STAT-3 are important in JAK2V617F-mediated transformation. Expression of constitutively activated STAT-5 or its downstream gene, Bcl-xL, in erythroid progenitor cells can induce EEC formation, the hallmark of PV (60). Constitutive STAT-3 activation and expression of Bcl-xL was detected in PV cells (61, 62).

The human erythroleukemia cell line, HEL92.1.7 cells (HEL), has been widely used in MPD studies as an *in vitro* cell model (53, 63). Sequence analysis identified a homozygous JAK2V617F allele in HEL cells, but not in K562, MOLT16, or RPMI-8402 cell lines (53). In HEL cells, JAK2V617F, STAT-5 and ERK are

constitutively phosphorylated (53).

Clinical Impact of JAK2V617F

The identification of JAK2V617F has had a broad impact on the classification, diagnosis, prognosis and treatment of MPDs (64). Current therapy of MPD is not curative and does not prevent evolution, and the therapeutic goal is to prevent and/or treat thrombohaemorragic events (either blood clot or bleeding), and to palliate symptoms (64). The findings of JAK2V617F in MPDs, and the remarkable success of clinical use of the tyrosine kinase inhibitor imatinib for the treatment of Bcr-Abl positive CML, has spurred developmenet of selective JAK2V617F inhibitors for the treatment of MPDs (65). These small molecular inhibitors suppress JAK2V617F-mediated cell growth at nanomolar concentrations, and *in vivo* therapeutic efficacy has been demonstrated in mouse models. Some of these compounds are currently in clinical trials (65). Many questions remain regarding the future of JAK2 inhibitors, such as how to limit off-target effects on JAK3 and monitor treatment responses. In addition, patients may develop resistance to JAK2 inhibitors, as in imatinib-resistant CML (66, 67).

Aberrant JAK-STAT Activation in Other Hematopoietic Malignancies

In addition to the JAK2V617F mutation in MPD patients, several other activating mutations occur in the JAK-STAT signaling components, which are associated with hematopoietic malignancies (**Figure 5**).

JAK2 Exon 12 and MPLW515L Mutations

Although significant portions of MPD patients are JAKV617F positive, a subset are JAK2V617F negative. Based on the hypothesis that there may be other mutations that activate JAK-STAT signaling in JAK2V617F negative MPDs, at least eight mutations in exon 12 of JAK2 (68-72), and one mutation in the thrombopoietin receptor (MPL), have been identified in those patients (73). JAK2 exon 12 mutations are found in PV patients, but not in ET or PMF patients, while MPLW515L occurs in approximately 8.5% of JAK2V617F negative ET patients (74) and 10% of JAK2V617F negative PMF patients (73, 75, 76). They are transforming *in vitro*, and confer cytokine-independent growth of hematopoietic cells as JAK2V617F does, demonstrating that aberrantly activated JAK2 signaling is seen in both JAK2V617F-positive and JAK2V617F-negative disease, either through mutations in JAK2 itself or in cytokine receptors.

TEL-JAK2

The TEL-JAK2 fusion protein is found in hematopoietic malignancies as a consequence of the t (9,12)(p24;p13) chromosome translocation. It contains the oligomerization domain of TEL, a member of the ETS transcription factor family, and the kinase domain (JH1) of JAK2. Oligomerization of TEL-JAK2 results in constitutive kinase activity and confers cytokine-independent growth in the IL-3-dependent Ba/F3 hematopoietic cell line (77). TEL-JAK2 lacks JH2-JH7, the region containing the FERM domain, which is required for interaction with cytokine

receptors; therefore, constitutive activation of TEL-JAK2 does not require association with cytokine receptors. Unlike TEL-JAK2, JAK2V617F requires coexpression of growth factor receptors such as the erythropoietin receptor, thrombopoietin receptor, or the granulocyte-colony stimulating factor receptor, for constitutive activation of JAK2V617F and STAT-5, and JAK2V617F-mediated transformation of Ba/F3 cells (78). This suggests that the autonomous activation of JAK2V617F requires a cytokine receptor scaffold (intracellular component of cytokine receptors that engages JAKs and enables the activation of signal transduction).

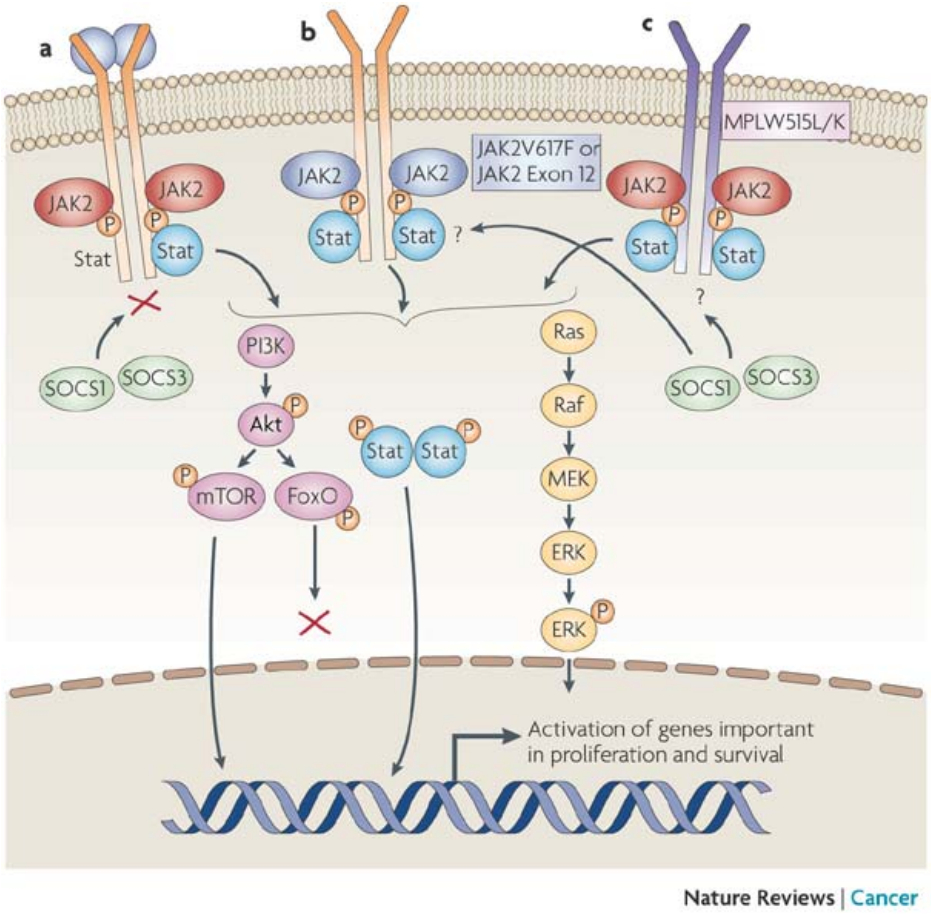

Figure 5. Cytokine-dependent or Independent Activation of the JAK2 Signaling Pathway. a. In normal cells, cytokines bind to their receptor, and receptor-associated JAK2 becomes activated as a result of auto- or trans-phosphorylation, and then activates downstream signaling including STAT, PI3K-AKT and Ras-MAPK signaling pathways, thereby increasing transcription of genes that are important in proliferation and survival. **b**. JAK2V617F and JAK2 exon 12 bind to cytokine receptors, and become phosphorylated and activated in the absence of cytokines. **c**. The thrombopoietin receptor mutation MPLK515W/L is also able to phosphorylate and activate wildtype JAK2 in the absence of thrombopoietin, resulting in activation of downstream signaling pathways. SOCS-1 and SOCS-3 are important negative regulators of JAK2 signaling. Escape from negative regulation by SOCS proteins could be one mechanism that contributes to aberrant JAK2V617F autonomous phosphorylation (79).

Note: From "Role of JAK2 in the pathogenesis and therapy of myeloproliferative disorders" by Ross L. Levine, Animesh Pardanani, Ayalew Tefferi and D. Gary Gilliland, 2007, Nature Reviews Cancer, 7, p. 673-683. Copyright 2007 by the Nature Publishing Group. Reprinted with permission.

Aberrant Activation of STATs in Tumors

In normal cells, activation of STATs is rapid and transient, because it is tightly regulated by the absence or presence of cytokines or growth factors. Negative regulation by phosphatases, SOCS and PIAS proteins is also important to suppress activation. However, STAT proteins, particularly STAT-3 and STAT-5, are frequently overactivated in a variety of human solid tumors and blood malignancies (**Table 3**). The constitutive activation of STATs may be due to multiple defects in regulation of this pathway. In tumor cells, the tyrosine kinase activity of JAKs or growth-factor receptors can become constitutively activated due to mutations, over-expression, or continuous engagement of ligands that come from autocrine or paracrine sources (28). STATs can also be activated by non-receptor, cytoplasmic kinases such as Bcr-Abl (80) and SRC-related kinases (81) without receptor engagement. In addition, unrestrained STAT activation can also arise from loss of function of negative regulators. However, the exact molecular mechanism of how STATs become activated in tumors is still not clear. In normal cells, STATs regulate many genes controlling fundamental biological functions. Many proteins that are crucial for cell proliferation such as c-Myc, cyclin D1 and cyclin D2, cell survival such as Survivin, anti-apoptotic responses such as Bcl-xL and Mcl-1, metastasis such as MMP-2 and MMP-9, and angiogenesis such as VEGF and HIF-1α, have subsequently been found to be regulated by STAT-3 (82). As a result of constitutive STAT activation in tumors, the expression pattern of STAT-regulated genes will be changed accordingly, therefore promoting tumor cell survival and proliferation.

Table 3. Activation of STATs in Human Cancers

Tumour type	Activated STAT
Blood tumours	
Multiple myeloma	STAT1, STAT3
Leukaemias:	
HTLV-I-dependent	STAT3, STAT5
Erythroleukaemia	STAT1, STAT5
Acute myelogenous leukaemia (AML)	STAT1, STAT3, STAT5
Chronic myelogenous leukaemia (CML)	STAT5
Large granular lymphocyte leukaemia (LGL)	STAT3
Lymphomas:	
EBV-related/Burkitt's	STAT3
Mycosis fungoides	STAT3
Cutaneous T-cell lymphoma	STAT3
Non-Hodgkins lymphoma (NHL)	STAT3
Anaplastic large-cell lymphoma (ALCL)	STAT3
Solid tumours	
Breast cancer	STAT1, STAT3, STAT5
Head and neck cancer	STAT1, STAT3, STAT5
Melanoma	STAT3
Ovarian cancer	STAT3
Lung cancer	STAT3
Pancreatic cancer	STAT3
Prostate cancer	STAT3

Note: From "The STATs of Cancer-New Molecular Targets Come of Age" by Hua Yu and Richard Jove, 2004, Nature Reviews Cancer, 4, p. 97-105. Copyright 2004 by the Nature Publishing Group. Adapted with permission.

CK2

General Structural and Enzymic Features of CK2

Protein kinase CK2 derives from the acronym of casein kinase 2 or II, which was shown to be a misnomer because casein is very unlikely to be its physiological substrate (83). CK2 was originally identified in a mixture with another different kinase CK1 in 1954, and later purified in the 1970s (84). Different isoenzymatic

forms of CK2 catalytic subunits have been identified in different organisms including human (85), mouse (86), bovine (87) and yeast (88). In humans, CK2 often exists as a tetramer, which is composed of two catalytic subunits, α and α' of 42-44 and 38 kD respectively, and two regulatory β subunits of 28 kD. CK2α and α' share a common catalytic domain but differ in their C-terminal region, and several proteins such as HSP90, Pin1, PP2A and protein CK2 interacting protein-1 (CKIP-1) specifically interact with CK2α, but not α' (89-92). A third catalytic subunit, CK2α'', has recently been identified and shown to play an important role in the endocytic pathway (93). The quaternary complex of CK2 may contain identical (αα or α'α') or non-identical catalytic subunits (αα') (94). The crystal structure of the human CK2 tetramer shows the shape of a butterfly, in which the two β subunits form a stable dimer linking the two catalytic subunits (**Figure 6**) (95). Each of the two β subunits touches both the catalytic subunits, which do not make direct contact with one another (95). Unlike most other kinases, CK2 has a "dual-cosubstrate specificity", which means it can efficiently utilize either adenosine triphosphate (ATP) or guanine triphosphate (GTP) as the donor of phosphate (96). CK2 phosphorylates serine or theronine residues, and it has been shown that CK2 can also phosphorylate tyrosine residues *in vitro*, in yeast and mammalian cells (97-101). The yeast protein Fpr3 is tyrosine phosphorylated by CK2 following serine phosphorylation (98). CK2β undergoes autophosphorylation (102-104), which may be mediated by CK2α (105). No other physiological tyrosine phosphorylated substrates has been identified thus far.

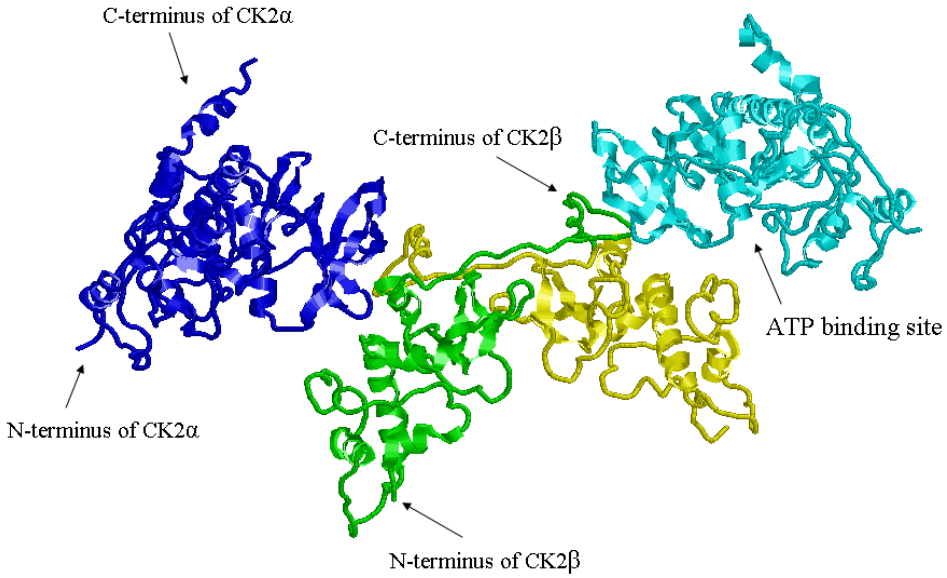

Figure 6. Diagram Illustrating the Structure of the CK2 Tetramer. The coordinates (PDB database identification number 1JWH) of two C-terminally truncated catalytic and two regulatory subunits of CK2 at 3.1 Å resolution (95) were displayed with RASMOL 2.7.2.1.1 (106) in cartoon mode. The two CK2α are in light and dark blue, while the two CK2β are in green and yellow, respectively.

CK2β plays a regulatory role in the following aspects: first, in the tetrameric complex, the catalytic subunits do not interact with each other; instead, each of them interacts with both CK2β subunits (95). Second, the formation of a CK2β dimer is required for the formation of the CK2 tetramer, because disruption of a zinc finger region of CK2β, which is responsible for CK2β dimer formation, will also impair its association with the catalytic subunits (107-109). Third, the CK2β subunit regulates the stability, activity and specificity of the catalytic subunits (110-113), and serves as a docking site for interacting proteins (114-118).

CK2 Regulation

Unlike many other kinases, CK2 is considered to be constitutively active, because

CK2 activity is detected in cells or tissue extracts even in the absence of any stimulation or addition of cofactors, or when expressed in bacteria. However, several mechanisms including regulation of expression and assembly, localization, protein-protein interactions and phosphorylation may contribute to the regulation of CK2 activity (119). For example, CK2α is phosphorylated and interacts with prolyl isomerase Pin1 in mitotic cells (120). CKIP-1 interacts with CK2, and recruits it to the plasma membrane (120). Thus far, there is no unifying mechanism to explain the regulation of CK2 that has been achieved.

CK2 Substrates and Function

An increasing number of CK2 substrates have been identified since the 1980s, and thus far, more than 300 CK2 targets have been reported (121). These substrates include signaling proteins such as β-catenin, calmodulin, caveolin and PTEN, transcription factors such as C-Myb, c-Myc, CREB, p53 and Tal-1, proteins affecting DNA/RNA functions and protein synthesis such as DNA topoisomerase I, eIF2, RNA polymerase I and DNA ligase, viral proteins such as HIV-1 Rev, HPV E7, SV40 large T Ag and influenza virus PA, cytoskeleton and structural proteins such as tubulin and connexin, and metabolic enzymes such as glycogen synthase (121). These targets are located throughout the cell, including the nucleus, cytoplasm and cellular membrane. CK2 phosphorylation sites usually contain minimum consensus sites, in which there are several acidic amino acids downstream from Ser/Thr at the n+3 (the most important one), n+1, and n+2 position, and the absence of positively charged residues

nearby (121).

CK2 is essential for cell viability, as disruption of either CK2α or β is embryonic lethal (122, 123). Knockout of CK2α by homologous recombination leads to death of embryos in mid-gestation, with abnormalities including open neural tubes, reduction in the branchial arches, and defects in heart formation (122). CK2α' knockout mice are viable but are defective in spermatogenesis (124).

CK2 and Cancer

Elevated CK2 expression and activity has been reported in blood tumors including multiple myeloma (125) and leukemia (126), and solid tumors including kidney (127), mammary gland (128), lung (129), prostate (130), and head and neck cancers (131). Targeted overexpression of CK2 in transgenic mouse models is capable of transforming lymphocytes (132) and mammary epithelium (128). CK2 cooperatively promotes oncogenesis and tumor progression with overexpression of other oncogenes such as c-myc (133) or Tal-1 (134) in T-cells , or with loss of tumor suppressors such as p53 in $p53^{-/-}$ mice (135).

The role of CK2 in tumorigenesis may be due to its regulation of a number of tumor suppressor proteins, pro-apoptotic proteins, as well as oncogenes (136). Two examples of tumor suppressors as CK2 targets are PML (137), expression of which is lost in a large number of tumors, and PTEN (138-140), which negatively regulates the PI3K pathway that promotes cell survival. In the case of PML, CK2 phosphorylates PML at Ser517 and promotes its degradation through the proteosome. Inhibition of

CK2 enhances the tumor suppressor activity of PML in a lung cancer mouse model (137). Phosphorylation of PTEN by CK2 leads to stabilization in its inactive form, therefore inhibiting its activity and perpetuating PI3K signaling (138-140). A number of signaling components of pro-survival pathways, such as IκB and NF-κB in the NF-κB pathway, β-catenin, Dishevelled-2, Dishevelled-3 and APC in the Wnt pathway, and PTEN and Akt in the PI3K pathway, have been shown to be targets and/or interactors of CK2 (136). Increasing reports suggest that CK2 has an anti-apoptotic role through directly phosphorylating components of the apoptotic machinery, or protection of pro-apoptotic proteins from caspase cleavage, which may be due to the high similarity between the CK2 consensus motif (pT/pS-X-X-D/E)) and caspase cleavage sites (136). As an example, phosphorylation of PTEN by CK2 can protect it from caspase cleavage (141).

Significance

The JAK-STAT signaling pathway is pivotal for cellular functions, and has been intensively studied. However, many questions remain regarding regulation of activation of this signaling pathway. Both CK2 and JAK-STAT signaling play crucial roles in cell survival, proliferation and anti-apoptotic mechanisms, and elevated CK2 activity and constitutive JAK-STAT activation occur in many human malignancies. However, little is known of potential crosstalk between CK2 and the JAK-STAT pathway. In this dissertation, we provide primary data on a novel interaction between

CK2 and JAK proteins, which promotes a positive regulatory node in the JAK-STAT signaling pathway. For the first time, the regulatory role of CK2 in the JAK-STAT signaling pathway was identified. Importantly, we demonstrate that JAK2V617F, the constitutively active JAK2 mutant which is present in the majority of patients with MPDs, also interacts with CK2. CK2 pharmacological inhibitors suppress constitutively activated JAK-STAT signaling and induce apoptosis in JAK2V617-expressing cells. Therefore, there are disease-related implications of these findings, in that constitutive activation of STATs, particularly STAT-3 and STAT-5, and heightened CK2 activity are frequently detected in a variety of blood tumors and solid tumors. CK2 upregulation may be one novel mechanism contributing to aberrant JAK and STAT activation through association with JAKs.

A CK2-DEPENDENT MECHANISM FOR ACTIVATION OF THE JAK-STAT SIGNALING PATHWAY: IMPLICATIONS IN MYELOPROLIFERATIVE DISORDERS

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Abstract

Janus Kinase (JAK)-Signal Transducer and Activator of Transcription (STAT) signaling is involved in regulation of cell survival, proliferation and differentiation. JAK tyrosine kinases can be transiently activated by cytokines or growth factors in normal cells, while they become constitutively activated as a result of mutations that affect their function in tumors. Specifically, the JAK2V617F mutation is present in the majority of patients with myeloproliferative disorders (MPDs) and is implicated in the pathogenesis of these diseases. Here we report that the serine/threonine kinase CK2 is a novel interaction partner of JAKs, and is essential for JAK-STAT activation. We demonstrate that cytokine-induced activation of JAKs and STATs, and expression of SOCS-3, a downstream gene, are inhibited by CK2 siRNAs or pharmacological inhibitors. Endogenous CK2α and β are associated with JAKs, and this association is not dependent on JAK kinase activity. To extend these findings, we demonstrate that CK2 also interacts with JAK2V617F, and CK2 inhibitors suppress JAK2V617F autophosphorylation and downstream signaling in HEL92.1.7 cells (HEL). Furthermore, CK2 inhibitors potently induce apoptosis of HEL cells. Taken together, these data provide evidence for novel crosstalk between CK2 and JAK-STAT signaling, with implications for therapeutic intervention in JAK2V617F positive MPDs.

Introduction

The Janus Kinase (JAK) - Signal Transducer and Activator of Transcription (STAT) pathway is crucial in transmitting signals from many cytokines and growth factors into the nucleus, regulating gene expression. Cytokines of the IL-6 family, type I and II interferons, and growth factors such as growth hormone (GH) activate the JAK-STAT signaling pathway. Oncostatin M (OSM), a cytokine belonging to the IL-6 family, is a potent activator of the JAK-STAT signaling pathway.[1] Binding of OSM induces heterodimerization of its receptors gp130 and OSMRβ, and receptor-associated JAKs, JAK1 and JAK2, become activated, leading to phosphorylation of gp130 tyrosine residues. The phosphorylated residues direct the recruitment of STAT proteins, including STAT-3, STAT-1 and STAT-5, which in turn become JAK substrates. Activated tyrosine phosphorylated STATs form homodimers or heterodimers, translocate to the nucleus, and bind to consensus sequences in the promoters of OSM-responsive genes, inducing transcription.[2]

The JAK tyrosine kinase family comprises four mammalian members: JAK1, JAK2, JAK3 and TYK2. JAK2 is essential in erythropoiesis,[3] and its dysfunction has been implicated in myeloproliferative disorders (MPDs) and leukemias.[4] MPDs are a group of clonal hematopoietic disorders including polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF).[4] Recent studies have identified

that JAK2V617F, a somatic, activating, point mutation, occurs in most PV patients and more than 50% of ET and PMF patients, and is involved in the pathogenesis of MPDs.[5-8] Over-expression of JAK2V617F in murine Ba/F3 cells leads to cytokine-independent growth,[5,9] and expression of JAK2V617F in mice recapitulates many pathologic characteristics observed in human PV, ET and PMF patients.[10-12] Among the signaling pathways activated by JAK2V617F are the STATs, predominantly STAT-5.[13] Therefore, JAK2V617F represents an ideal target for therapeutic intervention, especially in JAK2V617F positive MPDs.

Protein kinase CK2 (formerly known as casein kinase 2 or II) is a ubiquitous, highly conserved serine/threonine kinase, while recent studies have also shown that CK2 can phosphorylate tyrosine residues.[14] CK2 phosphorylates more than 300 substrates which are involved in DNA replication, gene transcription, signal transduction, cell growth and apoptosis.[15,16] CK2 presents as a tetramer composed of two catalytic subunits (α or α') and two β regulatory subunits. CK2 is essential for cell viability as disruption of either CK2α or CK2β is embryonic lethal.[17,18]

CK2 expression and activity is upregulated in blood tumors including multiple myeloma[19] and leukemia[20], and solid tumors including kidney, mammary gland, lung, prostate, head and neck cancers.[21,22] Overexpression of CK2 is capable of transforming lymphocytes and mammary epithelium.[23,24] In mouse models, CK2 cooperatively

promotes oncogenesis and tumor progression with overexpression of other oncogenes such as c-myc,[25] or with loss of tumor suppressors such as p53.[26] Pro-survival genes such as β-catenin, oncogenes such as c-Myc, c-Myb and c-Jun, and tumor suppressors such as PML, PTEN and p53 have been shown to be targets and/or interactors of CK2.[21] Several signaling pathways such as the NF-κB, Wnt and PI3K pathways are regulated by CK2 in a manner that promotes cell survival signaling.[21]

Both CK2 and JAK-STAT signaling play pivotal roles in cell survival, proliferation and anti-apoptotic mechanisms, and their dysregulation is associated with human malignancies. However, little is known of potential crosstalk between CK2 and the JAK-STAT pathway. To test this, we first utilized small interfering RNA (siRNA) against CK2α and/or β as well as the pharmacological inhibitors 4,5,6,7-tetrabromobenzotriazole (TBB) or Emodin to knockdown CK2 expression or inhibit its activity, respectively, and then examined effects on activation of the JAK-STAT pathway. Our results indicate that OSM, IFN-γ and GH-induced JAK-STAT activation is dependent on the presence or activity of CK2. In addition, CK2 is constitutively associated with JAK2 and JAK1. Identifying CK2 as a novel interactor of JAKs and positive regulator of JAK and STAT activation, we extended these studies to examine JAK2V617F activation in MPD cells. Using JAK2V617F-expressing erythroid leukemia cells HEL as a model, we demonstrate that the CK2 inhibitor TBB suppresses autonomous JAK2V617F activation and downstream signaling, and also potently induces

apoptosis of HEL cells. These results suggest that CK2 inhibitors are of potential value for the treatment of JAK2V617F positive MPDs.

Materials and methods

Recombinant proteins and reagents. Recombinant human and murine Oncostatin M (OSM), and murine IFN-γ were obtained from R&D Systems (Minneapolis, MN). Antibodies to p-Y-STAT3, total STAT3, p-Y-STAT1, p-Y-STAT5, total STAT5 and caspase 8 were from Cell Signaling Technology (Danvers, MA). Antibodies to gp130, OSMRβ, p-Y1007/1008-JAK2, CK2α and JAK1 were from Santa Cruz Biotechnology (Santa Cruz, CA). STAT1, JAK2 and anti-phosphotyrosine antibodies were from Millipore (Billerica, MA). Antibodies to caspase 3 were from Abcam (Cambridge, MA). TBB (#218697) and antibody to CK2β were from EMD Biosciences (Madison, WI). Emodin and antibodies to actin and HA were from Sigma-Aldrich (St. Louis, MO). Monoclonal antibodies specific for Bcl-xL were a generous gift of Dr. Tong Zhou (University of Alabama at Birmingham). Murine CK2α and CK2β siRNA and transfection reagents were from Dharmacon (Lafayette, CO).

Cells. Primary or immortalized MEFs were kindly provided by Dr. P-P. Pandolfi (Harvard University, Boston, MA). The CH235-MG human astroglioma cell line was cultured as previously described.[27] γ2A-JAK2 is a JAK2-deficient human fibrosarcoma cell line (γ2A) that was stably transfected with JAK2, and γ2A-JAK2-GHR (Clone 14) is γ2A cells that were stably transfected with JAK2 and growth hormone receptor (GHR) and maintained as described previously.[28] γ2A-GHR-JAK2$_{KD}$ cells were maintained as

previously described.[29] MDA-MB-231 cells were maintained in DMEM media. HEL 92.1.7 (HEL) cells were obtained from ATCC,[30] and maintained in RPMI media.

Plasmid constructs. HA-CK2α and inhibitor-resistant HA-CK2α mutant (HA-CK2α-IR), which contains V66A and I174A double mutations, were constructed as previously described.[31]

RNA interference. MEFs were seeded at 0.5 x 10^5/well in 6-well plates overnight, and transfected with 100 nM CK2α or β siRNA or 50 nM CK2α plus β siRNA for 48 h using Dharmacon transfection reagent 4 and the provided protocol.

Immunoprecipitation and immunoblotting. For coimmunoprecipitation assays, confluent cells in 150 mm dishes were harvested in cell lysis buffer. One to two mg of total cell lysate was incubated with 1-2 μg of antibody overnight at 4°C, and then with 35 μl of protein A/G beads for 1 h. The beads were washed, boiled in 2X loading buffer, separated by SDS-PAGE gel and immunoblotted with antibody. For immunoblotting, ~20-30 μg of total protein was used to detect STATs or CK2 on 8 or 10% gels, respectively, and ~50-80 μg of total protein used to detect JAK2, gp130 or OSMRβ on 6% gels.

ELISA. Whole cell lysates were prepared from MEFs and HEL cells, and 65 μg of total protein were assayed using JAK2 [pYpY1007⁄ 1008] ELISA kit (Invitrogen, Carlsbad, CA) according to the provided protocol.

RNA isolation, riboprobes, RNase protection assay (RPA) and RT-PCR. Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA). Riboprobes for murine SOCS-3 and GAPDH were prepared, and RPA performed as previously described.[32] For RT-PCR, 1 μg of total RNA was used and the primer pairs 5'-aggagagcggattctactgga-3' and 5'-tggccgttgacagtcttccgaca-3' used to amplify murine SOCS-3.

Apoptosis assay. HEL cells were treated with TBB for 24 h, and cells were stained with Annexin V and propidium iodide using Clontech (Mountain View, CA) ApoAlert Annexin V-FITC Apoptosis Kit, and examined by flow cytometry. The percentage of Annexin V-positive cells was determined by FlowJo 7.5.5 software.

Cell cycle analysis. HEL cells were treated with TBB for 24 h, fixed with 70% ethanol overnight, stained with propidium iodide, digested with RNase for 45 min, and examined by flow cytometry. The percentage of cells in different cell-cycle stages was determined by FlowJo 7.5.5 software.

Densitometric and statistical analysis. Densitometric quantitation of immunoblotting, RPA or RT-PCR images in the linear range was performed using an image analysis program, ImageJ 1.41o (National Institute of Health, Bethesda, MD). Levels of significance for comparison between samples were determined by the Student's *t*-test distribution. *P* values of ≤ 0.05 were considered to be statistically significant.

Results

CK2 is required for cytokine-induced STAT activation and downstream gene expression. Previous studies in our laboratory had identified the tumor suppressor PML as a regulator of the JAK-STAT pathway.[33] Furthermore, PML was shown to be a substrate of CK2, and targeted for degradation by the proteosome.[34] Thus, we were interested to determine if the JAK-STAT pathway may be regulated by CK2, in a PML-dependent or independent manner. To test whether there is a crosstalk between CK2 and the JAK-STAT pathway, we initially examined whether CK2 is involved in regulation of STAT-3 activation. We utilized siRNA to specifically inhibit expression of CK2α and/or CK2β, and then examined the influence on OSM-induced signaling cascades. Mouse embryonic fibroblasts (MEFs) were transfected with 100 nM of CK2α or CK2β siRNAs, or 50 nM CK2α and CK2β siRNAs for 48 h, stimulated with OSM and assayed for phospho-tyrosine STAT-3 and total STAT-3 levels. Silencing of CK2α or CK2β expression led to reduced OSM-induced STAT-3 activation (**Fig. 1A**). The inhibitory effect was most pronounced when CK2β or both CK2α and CK2β were diminished in expression. We observed that CK2α siRNA inhibited CK2β levels as well (**Fig. 1A, lanes 3-4**). This has been previously observed *in vivo* with CK2α knockout mice[17] and *in vitro* in MCF7 cells[35]. The CK2 pharmacological inhibitor TBB is one of most selective inhibitors of CK2 available. We confirmed that TBB suppresses CK2 catalytic activity (data not shown). MEFs pretreated with TBB for 30 min exhibited

reduced OSM-induced STAT-3 tyrosine phosphorylation in a dose-dependent manner (**Fig. 1B**). Longer pre-incubation times with TBB (2 and 4 h) completely inhibited OSM-induced STAT-3 activation (**Fig. 1C, lanes 3** and **5**), without any observable toxic effect on cell survival of MEFs. Comparable results were obtained using $PML^{-/-}$ MEFs (data not shown), indicating that the CK2-mediated effects on STAT activation occurred in a PML-independent manner. In order to confirm that the inhibitory effect of TBB on STAT-3 activation is dependent on inhibition of CK2, we overexpressed either wildtype CK2α or CK2α-IR, which contains V66A and I174A double mutations, thus rendering cells resistant to three specific CK2 inhibitors including TBB.[31] As shown in **Figure 1D**, TBB inhibited OSM-induced STAT-3 activation in HA-CK2α transfected cells (compare **lanes 3** and **2**), while the inhibitory effect of TBB on STAT-3 activation was abolished by introducing HA-CK2α-IR (compare **lanes 6** and **5**). We conclude that CK2 expression and activity is required for OSM-induced STAT-3 tyrosine phosphorylation.

We then tested whether activation of other STATs, including STAT-1 and STAT-5, is also dependent on CK2. As shown in **Fig. 1E**, OSM-induced activation of STAT-1 and STAT-5 tyrosine phosphorylation was inhibited by TBB or Emodin, another CK2 inhibitor. We next examined the effect of inhibition of CK2 on SOCS-3, a downstream STAT-3 target gene. OSM induced expression of SOCS-3 mRNA (**Fig. 1F**, **lane 2; Fig. 1G**, **lanes 3** and **5**), which was inhibited by CK2 siRNAs (**Fig. 1F**, **lanes 4**, **6** and **8**) or

TBB (**Fig. 1G, lanes 4** and **6**). These observations indicate that OSM-induced SOCS-3 gene expression is dependent on CK2.

We also observed the inhibitory effects of CK2 inhibitors on OSM-induced STAT-3 tyrosine phosphorylation in human cancer cell lines including CH235-MG human astroglioma cells, γ2A-JAK2 human fibrosarcoma cells and MDA-MB-231 breast tumor cells (**Fig. 2**), indicating this effect is not restricted to MEFs.

We next examined whether signaling by other cytokines was affected by the CK2 inhibitors. We utilized IFN-γ, an activator of STAT-1 and STAT-3, and GH, an activator of STAT-5. As shown in **Fig. 3**, the CK2 inhibitors TBB and Emodin suppressed IFN-γ-induced tyrosine phosphorylation of both STAT-1 and STAT-3, and GH-induced tyrosine phosphorylation of STAT-5. Taken together, these results indicate that CK2 is required for cytokine (OSM, IFN-γ) and GH-induced activation of the JAK-STAT signaling pathway.

Given that OSM-induced activation of three STATs, STAT-3, STAT-1 and STAT-5, is suppressed by inhibiting CK2 expression or activity, this suggests that the inhibitory effect may involve a common mediator(s) capable of activating the different STAT proteins, which could be JAKs or the cytokine receptors.

OSM-induced activation of JAK2 is dependent on CK2. OSM binds to the gp130 receptor subunit, and recruits the OSMRβ subunit.[1] Receptor-associated JAK tyrosine kinases, JAK1 and JAK2, are tyrosine phosphorylated and activated, and then phosphorylate gp130. To determine the effect of CK2 siRNAs or inhibitors on JAK2 activation, we assayed JAK2 tyrosine phosphorylation by ELISA (**Figs. 4A-4B**). OSM induced JAK2 tyrosine phosphorylation (**column 2** of **Figs. 4A-4B**), which was inhibited by CK2 siRNAs (**Fig. 4A**, **columns 3-5**) or by pre-incubation with TBB or Emodin (**Fig. 4B**, **columns 3-4**). OSM stimulation induced gp130 tyrosine phosphorylation (**Fig. 4C**, **lane 2**), which was inhibited by TBB (**lane 3)**. Total levels of gp130 were also slightly inhibited (**Fig. 4C**, **lane 3**). OSM treatment did not affect the total levels of OSMRβ (**Fig. 4D**, **lane 2**), which were also largely unaffected by treatment with TBB or Emodin (**lanes 3-4**). These findings suggest that CK2 may function to regulate JAK activation, therefore controlling activation of STATs and expression of downstream genes.

CK2 is associated with JAK2 and JAK1. Our results thus far suggest that JAK activation may be directly or indirectly dependent on the presence/activity of CK2. Thus, we tested the possible association of endogenous JAKs and CK2 by co-immunoprecipitation experiments. JAK2 forms a complex with endogenous CK2α and CK2β (**Figs. 5A-5B**), and this association was enhanced in response to OSM (**Fig. 5B**, **lane 3**). To test whether the association of JAK2 and CK2 is dependent on JAK2 kinase activity, we used a γ2A-JAK2$_{KD}$ stable cell line expressing kinase-dead JAK2.[29]

As shown in **Fig. 5C**, CK2α was capable of associating with $JAK2_{KD}$ (**lane 3**), which suggests that JAK2 kinase activity is not required for association with CK2α. In addition to JAK2, endogenous CK2α and CK2β associate with JAK1 (**Figs. 5D-5E**). Co-immunoprecipitation experiments did not indicate an association of endogenous STAT-3 and CK2α or CK2β (**Figs. 5F-5G**).

CK2 is required for JAK2V617F phosphorylation and JAK-STAT signaling in HEL cells. CK2 and JAKs are dysregulated in different types of blood tumors as well as solid tumors. JAK2V617F, an acquired point mutation causing constitutive activation of JAK2 and STAT-5, is implicated in the pathogenesis of MPDs.[5-8] Based on our findings that JAK2 activation is dependent on CK2, and CK2 is constitutively associated with JAK2, we hypothesized that JAK2V617F activation may also depend on CK2. We first examined whether JAK2V617F could associate with CK2. We used the JAK2V617F mutant-expressing erythroid leukemia cell line HEL, because it expresses homozygous JAK2V617F. As shown in **Figure 6A**, endogenous JAK2V617F is associated with CK2α. Furthermore, treatment with the CK2 inhibitor TBB for 4 h suppressed autonomous JAK2V617F tyrosine phosphorylation in a dose-dependent manner in HEL cells (**Fig. 6B**) without affecting the total levels of JAK2 (**Fig. 6C**). We also detected constitutive activation of STAT-5 and STAT-3, and expression of the downstream, anti-apoptotic gene Bcl-xL (**Fig. 6C, lane 1**), which were also inhibited by TBB in a dose-dependent manner (**Fig. 6C, lanes 2-4**).

Role of CK2 in apoptosis and cell cycle regulation of HEL cells. Given that JAK2V617F phosphorylation, STAT activation and downstream gene expression of Bcl-xL can be inhibited by TBB, we hypothesized that inhibition of CK2 may promote apoptosis of HEL cells. As shown in **Figure 7A**, TBB induced an increase in apoptosis of HEL cells in a dose-dependent manner within 24 h, as assayed by Annexin V staining. Cell-cycle analysis also showed that the number of HEL cells in sub-G1 phase, which is used as an index for the degree of apoptosis, increased with TBB treatment in a dose-dependent manner (**Fig. 7B**). The percentage of cells in each phase of the cell cycle was altered, and fewer cells were present in the G2/M phase upon treatment with TBB (**Fig. 7B**). To further confirm the incidence of apoptosis, we evaluated caspase activation by immunoblotting. Upon treatment with TBB for 24 h, HEL cells display caspase 8 and caspase 3 activation (**Fig. 7C**). These data indicate that TBB potently induces apoptosis in HEL cells.

Discussion

We provide evidence that activation of the JAK-STAT signaling pathway is dependent on the presence and/or activity of CK2 in normal and tumor cells. Silencing CK2 expression by CK2 siRNA or inhibiting CK2 activity by CK2 inhibitors abrogates OSM-induced activation of JAK2, STAT-3, STAT-1 and STAT-5, and expression of the SOCS-3 gene. In addition to OSM signaling, CK2 activity is required for IFN-γ and GH activation of the JAK-STAT pathway. Furthermore, constitutive activation of the mutant JAK2, JAK2V617F, and constitutive STAT-3 and STAT-5 signaling was also abrogated by inhibition of CK2. The suppressive effects of CK2 inhibition on activation of JAK-STAT signaling were observed in normal cells such as MEFs, solid tumor cell lines such as CH235-MG, γ2A-JAK2 and MDA-MB-231, as well as the blood tumor cell line HEL. This suggests CK2-dependent activation of JAKs is a general regulation mechanism of JAK-STAT signaling pathway in different type of cells. We also demonstrated that CK2α and CK2β are constitutively associated with JAK2 and JAK1, but not with STAT-3, suggesting that JAKs are the regulatory node linking CK2 and the JAK-STAT signaling pathway. To our knowledge, our studies are the first to demonstrate crosstalk between CK2 and the JAK-STAT pathway.

A number of cell signaling components/transcription factors such as IKKα, IκBα, NF-κB p65, and ALK-1, a member of the TGF-β superfamily of receptors, are direct

substrates or interactors of CK2.[36] In the case of ALK-1, CK2β was shown to be an enhancer of ALK-1 signaling[36], while CK2-mediated activation of IKKα leads to subsequent activation of the NF-κB pathway.[37] Our findings of the association of endogenous JAKs and CK2, and preliminary results from *in vitro* phosphorylation assays (data not shown) suggest that CK2 may directly phosphorylate JAKs. JAK2 undergoes multisite phosphorylation upon cytokine stimulation, including tyrosine and serine/threonine phosphorylation.[38] Using the Scansite prediction program (http://scansite.mit.edu), T308, T310 and T817 of murine JAK2 were predicted to be potential CK2 sites. These sites are in agreement with consensus CK2 sites, in that there are several negatively charged amino acids downstream from Ser/Thr especially at the n+3 position, and the absence of positively charged residues nearby.[16] In addition, recent data have suggested that CK2 can phosphorylate tyrosine residues.[14] It will be important to demonstrate that phosphorylation occurs *in vivo*, identify the JAK2 site(s) phosphorylated by CK2, and examine how these site(s) affect JAK2 function in cells. In addition to JAKs, there may be other CK2 targets regulating the JAK-STAT pathway. PIAS1, a negative regulator of activated STAT-1, is also a CK2 substrate, and its regulation of transcription factor function is negatively affected by CK2 phosphorylation.[39]

Persistent STAT-3 and STAT-5 phosphorylation is prevalent in many types of human solid tumors as well as hematopoietic tumors, contributing to survival, growth,

anti-apoptotic mechanisms, angiogenesis and immune evasion of tumor cells.[40] A recent study demonstrated that JAKs, especially JAK2, are essential for constitutive STAT-3 signaling in solid tumor cells.[41] By using CK2 siRNA and/or inhibitors, we found that cytokine-induced JAK2 phosphorylation and constitutive JAK2V617F phosphorylation are dependent on the presence or activity of CK2. Therefore, inhibition of CK2 could be used to control persistent STAT-3 phosphorylation in a number of tumors.

CK2 protects cells from apoptosis through the regulation of tumor suppressors and oncogenes; therefore, downregulation of CK2 using various strategies potently induces apoptosis and inhibits tumor growth in a number of cancers.[42] CK2 inhibitors such as TBB, TBCA and DMAT induce apoptosis through activation of caspases in a variety of cancer cells.[43-45] In a study using CK2 inhibitors to investigate the anti-tumorigenic function of PML, Emodin was shown to inhibit tumor growth in a mouse model of lung cancer.[34] Antisense CK2α oligodeoxynucleotides potently induced cell apoptosis and suppressed tumor growth in a xenograft mouse model of human prostate cancer.[46] Considering the potential problems of drug resistance, human cancer therapy is moving away from drug monotherapy to treatment with more than one drug. Panobiostat, a pan-histone deacetylase inhibitor which can suppress the chaperone function of heat shock protein 90, has recently been proposed for combination therapy with JAK2V617F inhibitors, as it can deplete mRNA levels and promote proteosomal degradation of JAK2V617F.[47] CK2α has been shown to interact with the Bcr/Abl oncogene, and promote proliferation of Bcr/Abl expressing cells.[48] Treatment of PLC1 Bcr/Abl

lymphoblast leukemia cells with the CK2 inhibitor DMAT in combination with the Bcr/Abl inhibitor Imatinib lead to a synergistic reduction of cell viability.[49] We have shown that inhibition of CK2 is particularly potent in inducing apoptosis in JAK2V617F-expressing cells, which is associated with suppression of constitutive activation of JAK2V617F, STAT-3 and STAT-5. Preclinical and clinical trials using several small molecule JAK2 inhibitors has been reported for treatment of MPDs.[50] CK2 could be an additional therapeutic target for JAK2V617F positive MPDs. In particular, CK2 inhibitors may be effective in tumors that display aberrant CK2, JAK and STAT activation.

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Authorship

Contribution: Y.Z. and E.N.B. designed the research plan, analyzed and interpreted data, and wrote the manuscript; Y.Z. performed the experiments; and Y.Z., H.Q., S.J.F., L.D., and D.W.L. contributed vital new reagents.

Conflict-of-interest disclosure: All the authors declare no conflicts of interest.

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Figure legends

Figure 1. CK2 is required for OSM-induced STAT activation and gene expression. (A) MEFs were transfected with non-target (NT) siRNA (100 nM), CK2α siRNA (100 nM), CK2β siRNA (100 nM) or CK2α (50 nM) plus CK2β siRNA (50 nM) for 48 h, then stimulated with 1 ng/ml of OSM for 10 min. Total cell lysates were analyzed by immunoblotting with antibodies against p-Y-STAT3, STAT3, CK2α, CK2β and Actin. The densitometric ratios of p-Y-STAT3 versus STAT3 were calculated. The values of **lanes 4**, **6** and **8** were compared with that of **lane 2** (control, no inhibition), and percentage of inhibition determined. (B**)** MEFs were pretreated with 25-100 μM of TBB for 30 min, and then stimulated with 1 ng/ml of OSM for 10 min. The densitometric ratios of p-Y-STAT3 versus STAT3 were calculated. The values of **lanes 3**, **4** and **5** were compared with that of **lane 2** (control, no inhibition), and percentage of inhibition determined. (C) MEFs were pretreated with TBB (50 μM) for 2 and 4 h, and then stimulated with 0.1 ng/ml of OSM for 30 min. Antibodies recognizing p-Y-STAT3, total STAT3 and Actin were used in immunoblotting. Representative of at least three experiments. (D) MEFs were transfected with HA-tagged CK2α or CK2α-inhibitor resistant (HA-CK2α-IR) constructs for 24 h, pretreated with TBB (50 μM) for 2 h, and then stimulated with 1 ng/ml of OSM for 30 min. Antibodies recognizing p-Y-STAT3, STAT3 and HA tag were used in immunoblotting. (E) MEFs were pretreated with CK2 inhibitors for 2 h, and then treated with OSM (10 ng/ml) for 30 min. Antibodies

recognizing phospho-tyrosine STAT1, total STAT1, P-Y-STAT5, total STAT5 and Actin were used in immunoblotting. (F) MEFs were transfected with non-target (NT) siRNA (100 nM), CK2α siRNA (100 nM), CK2β siRNA (100 nM) or CK2α (50 nM) plus CK2β siRNA (50 nM) for 48 h, then stimulated with 0.1 ng/ml of OSM for 30 min. RNA was prepared and analyzed by RT-PCR for expression of SOCS3 and GAPDH. The densitometric ratios of SOCS3 versus GAPDH were calculated. The values of **lanes 4**, **6** and **8** were compared with that of **lane 2** (control, no inhibition), and percentage of inhibition determined. (G**)** MEFs were pretreated with TBB (50 μM) for 2 h, and then stimulated with different concentrations of OSM (0.1-0.5 ng/ml). Total mRNA was extracted, and analyzed by RPA with probes specific to SOCS3 and GAPDH (loading control). The densitometric ratios of SOCS3 versus GAPDH were calculated. The value of **lane 4** was compared with that of **lane 3** (control, no inhibition), and the value of **lane 6** with that of **lane 5**, and percentage of inhibition determined.

Figure 2. Inhibition of OSM-induced STAT3 activation in human solid tumor cell lines by CK2 inhibitors. (A) CH235 human astroglioma cells were pretreated with TBB (50 μM) for 2 h, and then stimulated with different concentrations of human OSM (0.5 and 1 ng/ml) for 30 min. The densitometric ratios of p-Y-STAT3 versus STAT3 were calculated. The value of **lane 4** was compared with that of **lane 3** (control, no inhibition), and the value of **lane 6** with that of **lane 5**, and percentage of inhibition determined. γ2A-JAK2 human fibrosarcoma cells (B) and MDA-MB-231 human breast cancer cells

(C) were pretreated with TBB (50 μM) or Emodin (50 μM) for 2 h, and then stimulated with 0.5 ng/ml of human OSM for 30 min. The densitometric ratios of P-Y-STAT3 versus STAT3 were calculated. The values of **lanes 3** and **4** were compared with that of **lane 2** (control, no inhibition), and percentage of inhibition determined.

Figure 3. Inhibition of IFN-γ and GH signaling pathways by CK2 inhibitors. (A) MEFs were pretreated with TBB (50 μM) or Emodin (50 μM) for 2 h, and then stimulated with 5 ng/ml of IFN-γ for 30 min. The densitometric ratios of P-Y-STAT1 versus STAT1 were calculated. The values of **lanes 3** and **4** were compared with that of **lane 2** (control, no inhibition), and percentage of inhibition determined. (B**)** MEFs were pretreated with TBB (50 μM) or Emodin (50 μM) for 2 h, and then stimulated with 5 ng/ml of IFN-γ for 30 min. Antibodies recognizing p-Y-STAT3, total STAT3 and Actin were used in immunoblotting. (C**)** γ2A-JAK2-GHR cells were pretreated with TBB (50 μM) or Emodin (50 μM) for 2 h, and then stimulated with GH at 250 ng/ml for 10 min. Antibodies recognizing p-Y-STAT5, total STAT5 and Actin were used in immunoblotting. The densitometric ratios of P-Y-STAT5 versus ACTIN were calculated. The values of **lanes 3** and **4** were compared with that of **lane 2** (control, no inhibition), and percentage of inhibition determined.

Figure 4. **CK2 is required for OSM-induced JAK2 activation**. (A**)** MEFs were transfected with non-target (NT) siRNA (100 nM), CK2α siRNA (100 nM), CK2β

siRNA (100 nM) or CK2α (50 nM) plus CK2β siRNA (50 nM) for 48 h, and stimulated with 5 ng/ml of OSM for 10 min. (B) MEFs were pretreated with TBB (50 μM) or Emodin (50 μM) for 2 h, and then stimulated with 5 ng/ml OSM for 10 min. (A-B) The protein concentration of total cell lysates was measured in duplicate. Sixty-five μg of total protein was analyzed for JAK2 pYpY 1007/1008 expression by ELISA, and then normalized to JAK2 expression. The untreated sample was arbitrarily set as 1. Three independent experiments were performed, and error bars show ±S.D.. *, $p<0.05$. (C) MEFs were pretreated with TBB (50 μM) for 2 h, and then stimulated with 10 ng/ml of OSM for 30 min. Lysates were immunoprecipitated with anti-gp130 antibody, and analyzed by immunoblotting. The blot was detected with anti-phosphotyrosine antibody, and then reprobed with gp130 antibody after stripping. Representative of three experiments. (D) MEFs were pretreated with TBB (50 μM) or Emodin (50 μM) for 2 h, and then stimulated with 0.1 ng/ml of OSM for 30 min. Total cell lysates were analyzed by immunoblotting with antibodies against OSMRβ and Actin. Representative of three experiments.

Figure 5. **Co-immunoprecipitation of endogenous CK2 with JAK1 and JAK2.** Lysates of γ2A-JAK2 cells were immunoprecipitated with anti-CK2α and normal goat IgG (G-IgG, negative control) (A, D, G), or with anti-STAT3 or normal rabbit IgG (R-IgG, negative control) (F). The immunoprecipitates were analyzed by immunoblotting with anti-JAK2 and anti-CK2α (A), anti-JAK1 and anti-CK2α (D) or with anti-STAT3,

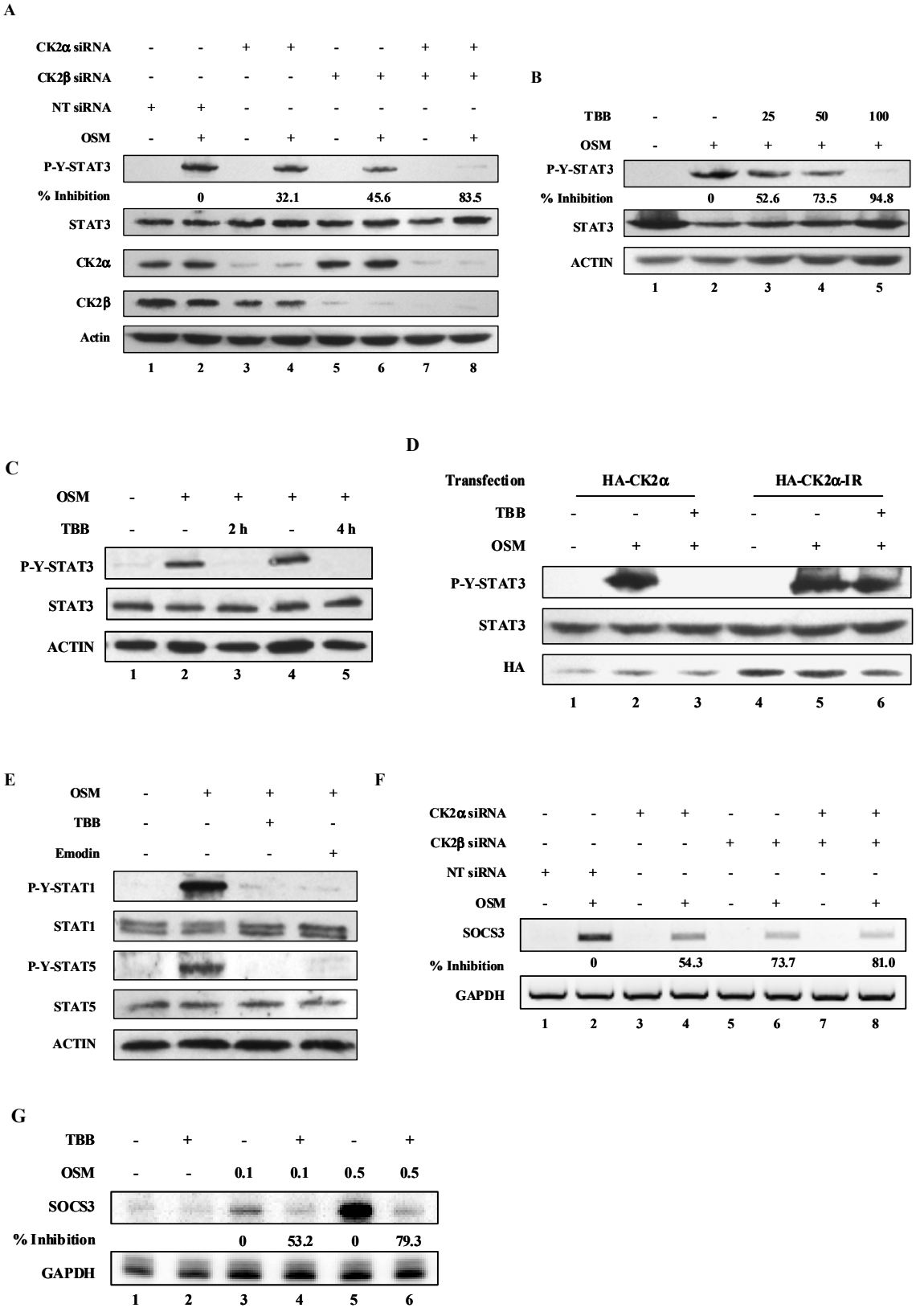
anti-CK2α and anti-CK2β antibodies (F-G). (B) γ2A-JAK2 cells were untreated or treated with 5 ng/ml of OSM for 15 min. Cell lysates were immunoprecipitated with anti-JAK2 and R-IgG. The immunoprecipitates were analyzed by immunoblotting with anti-CK2α, anti-CK2β and anti-JAK2 antibodies. (C) Lysates of γ2A-GHR-JAK2 cells (**lane 2**) and γ2A-GHR-JAK2$_{KD}$ cells (**lanes 1** and **3**) were immunoprecipitated with anti-JAK2 and R-IgG. The immunoprecipitates were analyzed by immunoblotting with anti-CK2α and anti-JAK2 antibodies. (E) Whole cell lysates were immunoprecipitated with anti-JAK1 or R-IgG. The immunoprecipitates were analyzed by immunoblotting with anti-JAK1 and anti-CK2α antibodies.

Figure 6. **Inhibition of autonomous JAK2V617F phosphorylation and JAK-STAT signaling in HEL cells by the CK2 inhibitor TBB**. (A) Lysates of HEL cells were immunoprecipitated with anti-JAK2 and R-IgG. The immunoprecipitates were analyzed by immunoblotting with anti-CK2α and anti-JAK2 antibodies. (B) HEL cells were treated with different concentrations of TBB (10-50 μM) for 4 h. The protein concentration of total cell lysates was measured in duplicate, 65 μg of total protein were analyzed for JAK2 pYpY 1007/1008 expression by ELISA, and then normalized to JAK2 expression. The untreated sample was arbitrarily set as 1. Three independent experiments were performed, and error bars show ±S.D.. *, $p<0.05$. (C) HEL cells were treated with different concentrations of TBB (10-50 μM) for 4 h. Antibodies recognizing p-Y-STAT5,

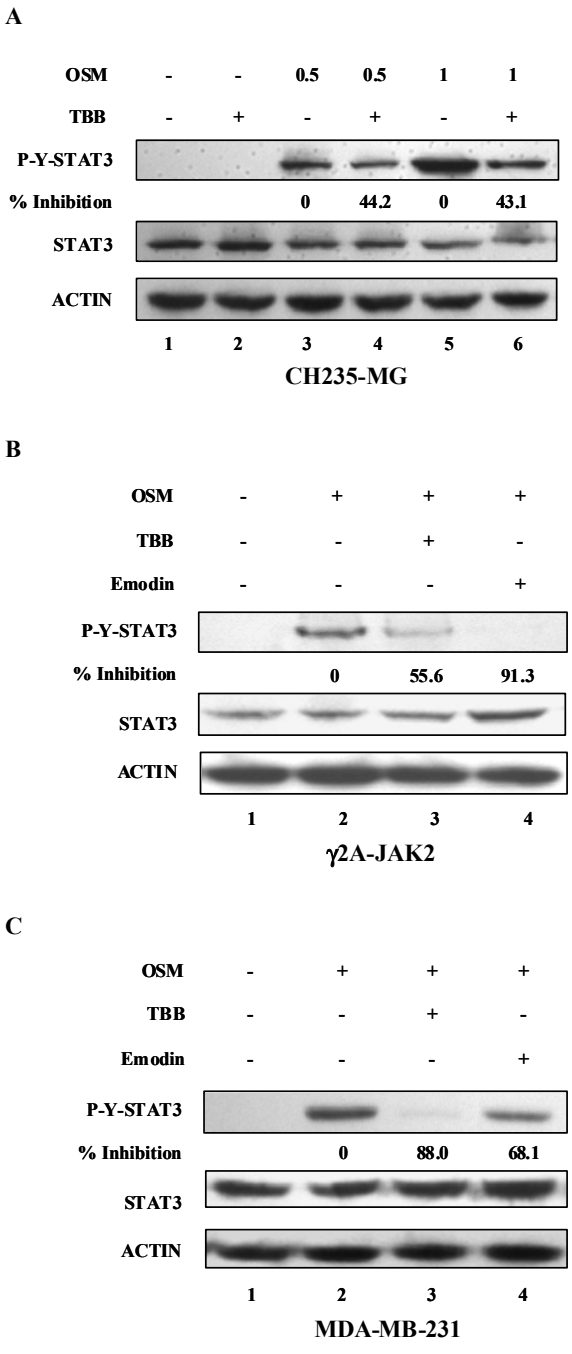
total STAT5, p-Y-STAT3, total STAT3, JAK2, Bcl-xL and Actin were used in immunoblotting.

Figure 7. **Induction of apoptosis in HEL cells by CK2 inhibitor TBB**. HEL cells were treated with different concentrations of TBB (5-25 μM) for 24 h. (A**)** Cells were stained with Annexin V and propidium iodide and examined by flow cytometry. Representative of three experiments. (B) HEL cells were fixed overnight, stained with propidium iodide and digested with RNase. The percentage of cells in the sub-G1, G1, S and G2/M phase were examined by flow cytometry. Representative of three experiments. (C) Whole cell lysates were collected and the levels of pro-caspase 3, cleaved caspase 3, pro-caspase 8, cleaved caspase 8 and Actin were analyzed using the antibodies specified.

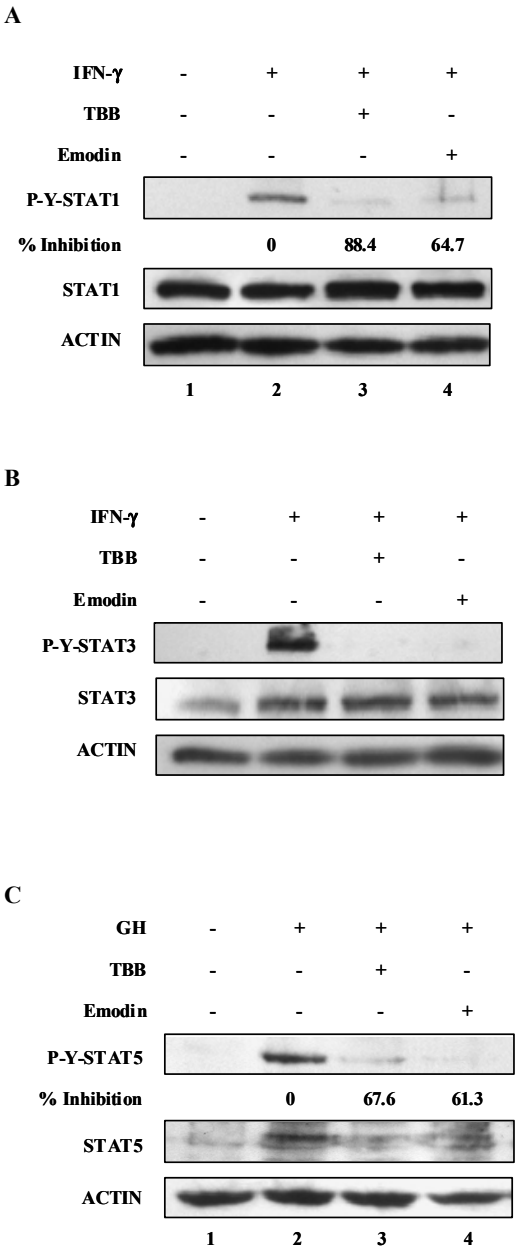
Zheng et al., Figure 1



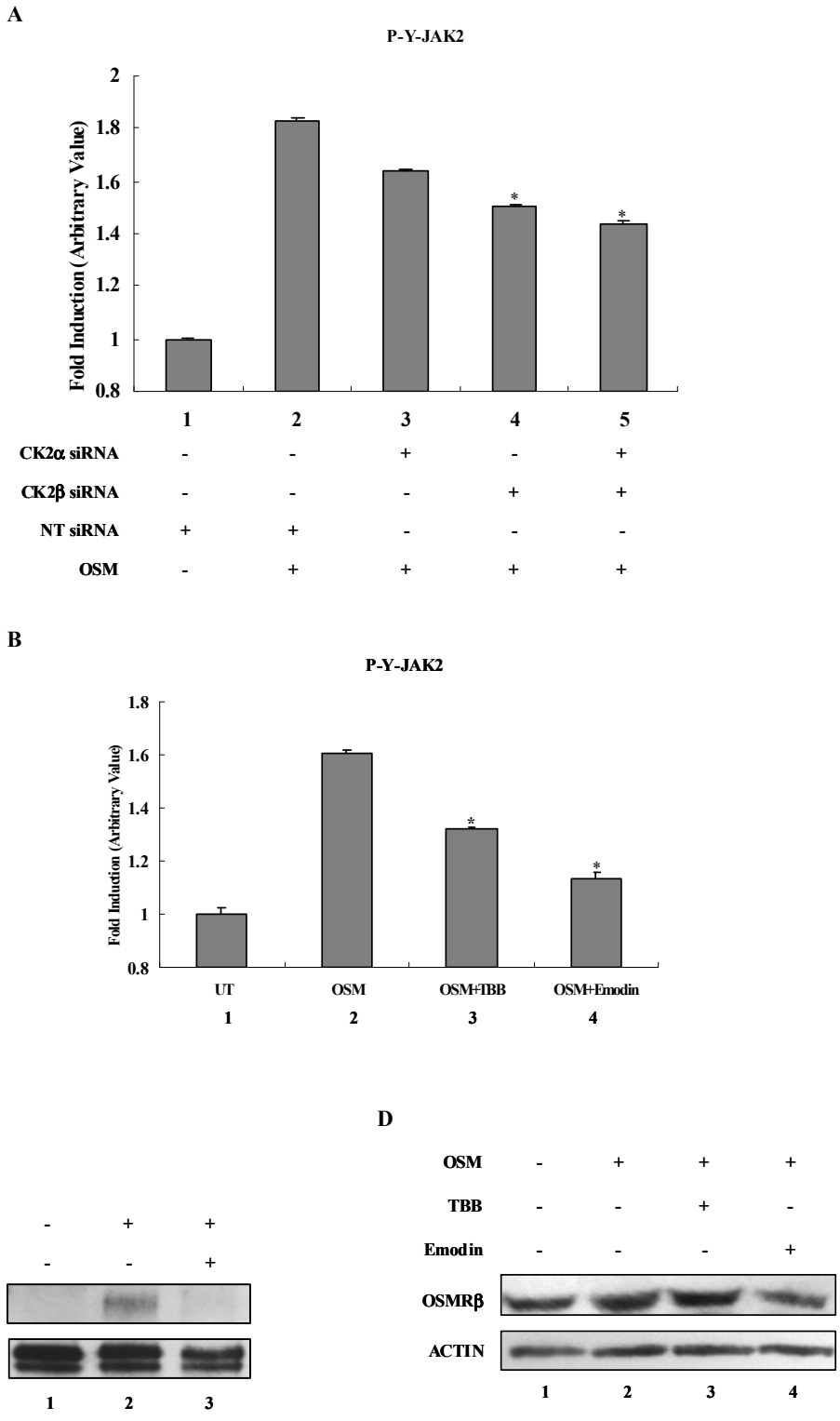
Zheng et al., Figure 2



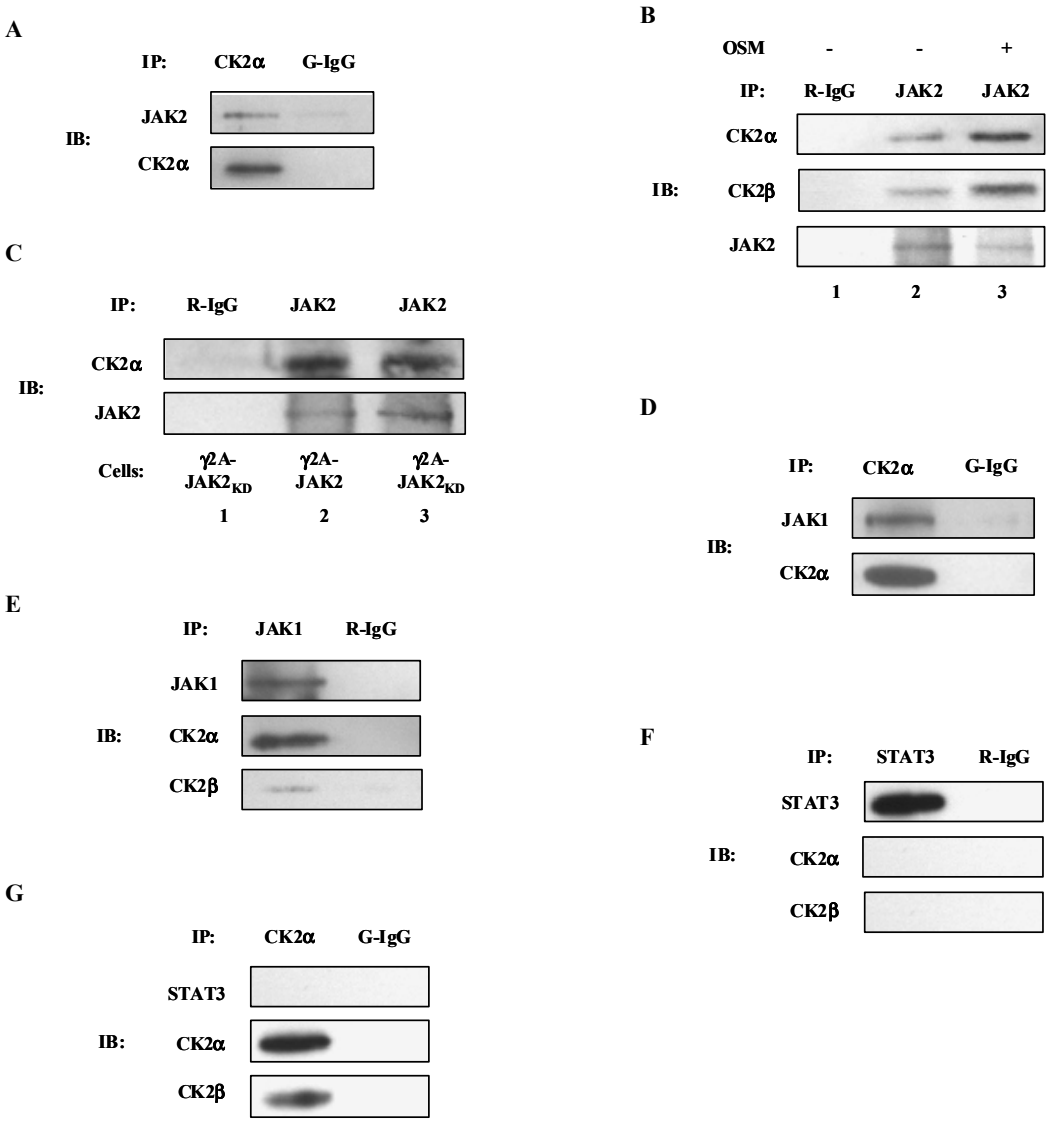
Zheng et al., Figure 3



Zheng et al., Figure 4



Zheng et al., Figure 5



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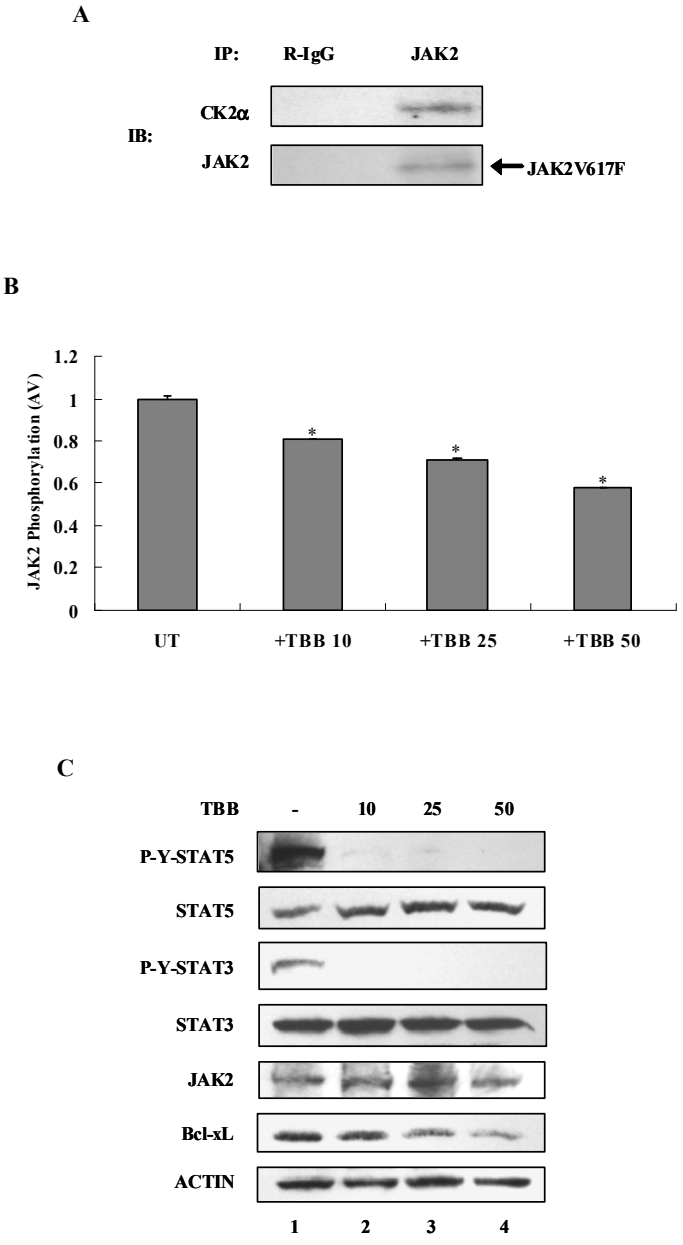

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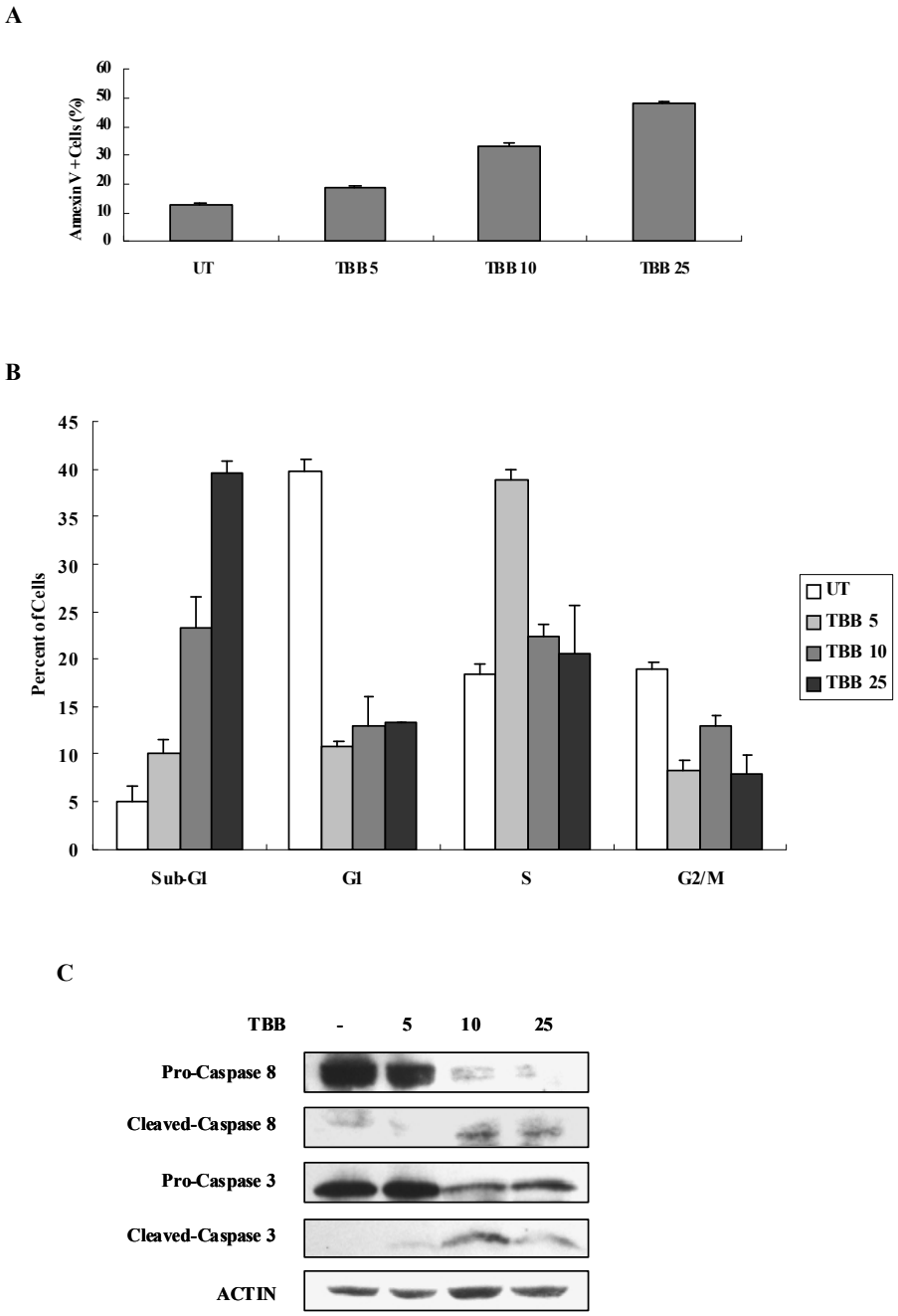

CONCLUSIONS

Model of CK2-dependent Activation of JAK-STAT Signaling

The JAK-STAT signaling pathway is regulated in normal cells by different mechanisms, and alterations in regulation can bypass the physiological mode of activation, giving rise to constitutive activation of this signaling pathway. As a result, aberrant activated JAK-STAT signaling is often associated with dysregulated cellular functions and human diseases. In this dissertation, we explored the regulatory role of CK2 in the JAK-STAT signaling pathway, first in normal cells, and then in tumor cells including several solid tumor cell lines and MPD cells. We found that CK2 is constitutively associated with JAKs, and essential for cytokine-induced or constitutive activation of JAKs, STATs and expression of downstream genes in normal and tumor cells. Based on the current model of JAK-STAT signaling and our findings, we propose a model for CK2-dependent activation of JAK-STAT signaling (**Figure 1**). As a novel interactor of JAKs and a positive regulator of JAK-STAT signaling, CK2 facilitates activation of JAKs in response to cytokines such as OSM and growth factors, while blockage of CK2 function will abolish this response.

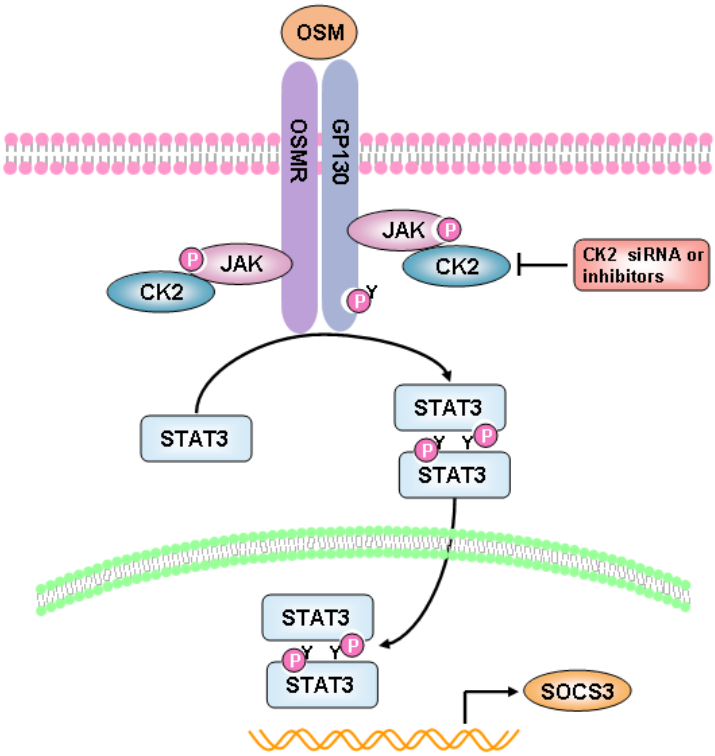

Figure 1. **Proposed Model of CK2-dependent JAK-STAT Activation**. CK2 constitutively binds JAKs, allowing activation of JAKs and STATs upon OSM stimulation. CK2 siRNAs or pharmacological inhibitors suppress activation of JAKs, STATs and expression of target genes such as SOCS3.

JAKs as New Members in the CK2 Interactome

We have shown that CK2 is constitutively associated with JAK proteins, and required for its activation by cytokines. It has been known that CK2 associates with numerous protein interactors. Studies in yeast and human cells demonstrate that CK2 interacts with a large number of cellular proteins (142). For example, CK2α (CKA1 in yeast) has an interaction network consisting of 154 proteins in yeast, and 43 in human that has been identified thus far (142). Among these interactors, the most abundant group is proteins involved in nucleic acid synthesis and processing, including a large number of transcription factors, transcription regulatory proteins, RNA or DNA binding proteins, deacetylases and cell cycle control proteins. The second most abundant group is proteins involved in cellular communication and signal transduction, including protein kinases and phosphatases, GTPases, GTPase activating proteins, adaptors, and adhesion and binding proteins. Studies of some of these interactors have been shown to be potential targets or regulators of CK2. These interactors highlight the critical role of CK2 in transcriptional regulation and signal transduction. The different subunits of CK2 interact with many cellular proteins, and there is considerable overlap between the interactomes of the individual CK2 subunits, providing evidence for the existence of tetrameric CK2 complexes, as well as independent existence of subunits outside of the tetramer (142). In our case, both CK2α and β are associated with JAK1 and JAK2, suggesting the JAK-CK2 complex contains the CK2 tetramer.

CK2 and JAK2V617F Activation

We have shown that CK2 is also associated with JAK2V617F, and required for its constitutive activation. Currently, the mechanism underling autonomous activation of JAK2V617F is not clear, as the structure of full-length JAK2 has not been resolved yet. V617F is located in the pseudokinase domain, which negatively regulates the kinase domain, because deletion or mutation in this domain leads to increased kinase activity of the protein (21, 48, 143). Only the crystal structures of the JH1 domain of JAK3 and JAK2 have been reported (144, 145), although homology modeling has been used to predict the overall structure for these kinases (144, 146). Three inhibitory regions of the pseudokinase domain, namely IR1 (residues 619-670), IR2 (725-757) and IR3 (758-807), have been identified and they can suppress JAK2 activity (147). V617F is just N-terminal to IR1, and it is believed to diminish the inhibitory effect that the pseudokinase domain imposes on the functional tyrosine kinase domain. However, the exact mechanism of how V617F affects the function of the pseudokinase domain as well as the kinase domain is not clear. We now know that JAK2V617F phosphorylation and activation is dependent on cytokine receptors (78, 148), which likely provide a scaffold upon which JAK2V617F interacts and transphosphorylates each other. In addition to cytokine receptors, our studies provide evidence that CK2 is also required for JAK2V617F activation, and it may function through interaction with JAK2. In addition, CK2 may be associated with cytokine receptors such as gp130 or OSMRβ, because JAKs are stably associated with these receptors (149). It is possible that CK2 is a component of that cytokine receptor

scaffold, required for JAK2V617F activation. In order to demonstrate how the association with CK2 regulates JAK activation, we need to locate the interaction domain(s) of JAKs with CK2. Thus far, we can not tell whether the association between CK2 and JAKs is direct or indirect. In either case, it will be informative to identify the interaction domain(s) of JAK proteins with CK2.

Phosphorylation of JAK2 by CK2

The fact that CK2 associates with JAKs suggests that JAKs may serve as biological substrates for CK2-mediated phosphorylation. We expressed JAK2 in *E. coli*, and tested this idea by *in vitro* phosphorylation assay. As shown in **Figure 2**, bacterially-expressed JAK2 is phosphorylated by CK2, and inclusion of the CK2 inhibitor TBB suppresses JAK2 phosphorylation by CK2. These findings suggest that JAK2 is a substrate for CK2 *in vitro*. Like many other tyrosine kinases, JAKs undergo autophosphorylation. Studies have demonstrated that phosphorylation of a conserved tyrosine residue in the activation loop of JAK2, Y1007, is required for JAK2 kinase activity (150). Y1007 phosphorylation is also required for association with SOCS-1, which mediates proteosomal degradation of activated JAK2 (151, 152). Many other tyrosine phosphorylation sites of JAK2 have been identified, and it is believed that they are the result of autophosphorylation (153). S523 was identified as a site for JAK2 serine phosphorylation, which represents another mechanism for JAK2 regulation (154, 155). In order to demonstrate whether JAK2 is a bona fide CK2 substrate and how CK2 exactly regulates JAKs, we need to further determine the

phosphorylation sites of JAK2 by CK2, and confirm this event *in vivo*. In addition to JAKs, it is possible that other components in this signaling pathway, such as the cytokine receptors, could also be substrates of CK2.

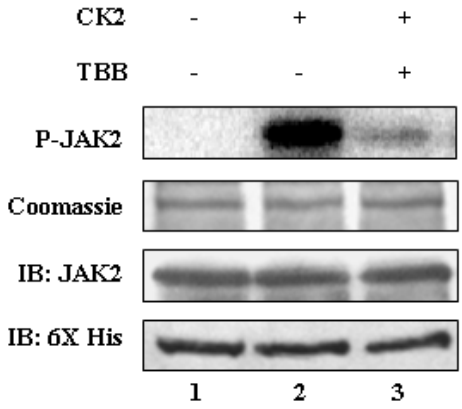

Figure 2. JAK2 is Phosphorylated by CK2 *In Vitro*. Bacterially produced His-JAK2 fusion protein bound to 25 µl of Ni-NTA agarose beads was incubated with 500 U of CK2 (α and β) in the absence or presence of 50 µM TBB. Phosphorylated proteins were resolved by 6% SDS-PAGE followed by autoradiography (top panel) and Coomassie Blue staining (second panel). The proteins bound to beads were also subjected to immunoblotting with JAK2 antibody (third panel) and 6X His antibody (bottom panel).

Possible Regulation of CK2 by JAKs

We demonstrate the essential role of CK2 in activation of JAK-STAT signaling. On the other hand, the association of CK2 with JAKs also suggests the possible regulation of CK2 by JAKs. Traditionally CK2 is regarded as an unregulated protein as it possesses constitutive activity. Recombinant CK2 is active when expressed in bacteria (156), and CK2 is also constitutively active in cells (136). In contrast, there

are many reports suggesting that CK2 is regulated by extracellular stimuli and/or intracellular factors (90, 157-161). These apparent discrepancies may be resolved by considering that discrete CK2 subpopulations in cells may be independently regulated by localization and/or association with distinct cellular components (161). Since protein–protein interactions are very important in the regulation of signal transduction events, CK2 function may be regulated by interacting proteins that target or localize CK2 to specific locations and/or serve as adaptors to modulate the phosphorylation of particular substrate proteins (161). JAKs may be such an example, recruiting CK2 to the cell membrane. Supportive evidence for this notion is that JAK overexpression promotes cytokine receptor(s) surface expression, which serves as a quality control mechanism, allowing only functional receptors to be expressed on the cell surface (162, 163). It will be interesting to examine whether the association with JAKs affects CK2 function. In addition, CK2 is known to undergo phosphorylation (157, 159, 164, 165). Therefore, it is possible that associated proteins such as JAKs may modulate its function by phosphorylation.

Regulation of JAK-STAT and NF-κB signaling in Glioblastoma (GBM)

Our findings that CK2 is required for JAK-STAT activation and the well-recognized roles of aberrantly upregulated CK2, JAK and STAT activities in human malignancies suggest that we may be able to control aberrant JAK-STAT activation in tumors through inhibition of CK2. In this dissertation, we demonstrate that by inhibition of CK2, we can suppress constitutive JAK2V617F activation and

downstream signaling, and induce apoptotic cell death of JAK2V617F-expressing cells, which suggests the potential therapeutic value of CK2 inhibitors in JAK2V617F positive MPDs. In addition to MPDs, constitutive activation of JAKs and STATs also mediates neoplastic transformation and promotes uncontrolled cell proliferation in many other human malignancies. Here, we focus on GBM. GBM, also known as glioblastoma, is WHO grade IV, the most aggressive malignant astrocytic glioma, because of its high degree of cellularity, vascular proliferation, and necrosis (166, 167). GBM is characterized by its propensity to infiltrate throughout the brain, which results in the inability of surgery to cure patients even when surgical resection is possible. The median life expectancy is less than 1 year after diagnosis. Radiotherapy and/or chemotherapy have been used to improve patient survival. Despite optimal treatment, nearly all GBMs eventually recur. Therefore, the need for effective new therapies is evident even in the few patients who survive long-term, as they suffer serious cognitive impairment from the effects of radiotherapy.

Previous studies in our laboratory had identified that STAT-3 protein activation is elevated in GBM tissues compared to control brain tissue (168). In this dissertation, we have provided data showing OSM-induced STAT-3 activation is suppressed by CK2 inhibitors in human CH235-MG astroglioma cells. In addition, OSM-induced expression of downstream target genes of STAT-3, including SOCS-3, IL-6, Mcl-1 and Pim-1 are also suppressed by CK2 inhibitors in the human glioblastoma cell line U251-MG (**Figure 3**). Human GBMs express elevated levels of IL-6 and OSM (14, 169, 170), which correlates with increasing tumor grade. In addition, in one study,

amplification of the IL-6 gene was detected in 41.7% of GBM patients, which was significantly associated with decreased survival (171). Expression of the anti-apoptotic genes Mcl-1 and Pim-1 may also contribute to GBM pathogenesis. Gliomas are defined by apoptotic resistance, uncontrolled cellular proliferation, angiogenesis, diffuse infiltration, and necrosis (166). In addition to these genes, it will be interesting to examine the effect of inhibition of CK2 on other STAT-3 target genes involved in cell growth and proliferation such as c-Myc, Cyclin D1, AKT, and Jun B, in apoptosis and drug resistance such as Bcl-2, Bcl-xL, Survivin, cIAP2, MDR1 and STAT-3, in cell migration such as MMP-2 and MMP-9, and in angiogenesis such as VEGF and HIF-1α.

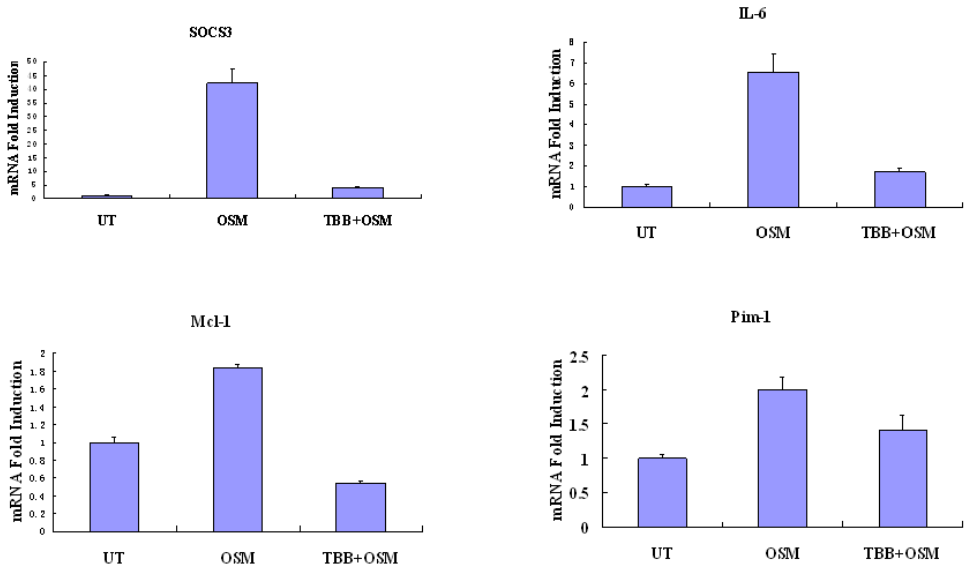

Figure 3. **CK2 Inhibitor Suppresses Expression of OSM-induced STAT-3 Target Genes**. U251-MG cells were pretreated with TBB (50 μM) for 2 h, and then stimulated with 5 ng/ml of OSM for 1 h. Total mRNA was extracted, and analyzed by real-time RT-PCR for expression of SOCS3, IL-6, Mcl-1 and Pim-1, which were then normalized to expression of 18S rRNA. Gene expression in the untreated sample was arbitrarily set to 1, and mRNA fold induction was calculated.

In addition to STAT-3, our lab also identified that the anti-apoptotic transcription factor NF-κB is elevated and activated in GBM tissue compared to non-tumor control tissue (172). NF-κB activates the expression of genes that mediate inflammatory responses, cell proliferation, invasion, migration, apoptotic resistance and angiogenesis. Interestingly, the NF-κB signaling is also positively regulated by CK2 at different levels. CK2 constitutively associates with and phosphorylates IκBα at multiple sites, which is required for IκBα degradation by the proteosome and activation of NF-κB (173-175). CK2 also phosphorylates NF-κB p65 at Serine 529 in response to TNF-α stimulation (176) and phosphorylation by CK2 enhances the transcriptional activity of p65 (177). Recent studies also suggest that CK2α can phosphorylate IKK2 *in vitro* (178), and inhibition of CK2 decreases expression of IKKε in breast cancer cells (179). Taken together, CK2 modulates this signaling pathway through positively regulating IKK and NF-κB, and negatively regulating IκB.

Thus far, there are only a few reports about the function of CK2 in GBM (180). Considering the well-recognized importance of aberrantly regulated JAK-STAT and NF-κB signaling in various types of tumors, it will be interesting to study the regulation of JAK-STAT signaling by CK2 in the context of GBM, and also examine how CK2 regulates the NF-κB signaling in GBM. This may provide novel clues for future drug targeting in GBM.

CK2 as a Cancer Therapeutic Target

Current trends in cancer therapy focus on “targeted therapy”, which refers to a new generation of cancer drugs designed to interfere with a specific molecule that is believed to play an important role in tumor growth or progression (181). Protein kinases are a promising class of cancer targets as some kinase inhibitors such as imatinib mesylate have shown clinical benefit in patients (182, 183). CK2 expression and activity are upregulated in almost every tumor that has been examined thus far. As a multifunctional kinase, the role of CK2 in normal and cancer cell growth and proliferation has long been recognized (119, 184-188). CK2 can also protect cells from apoptosis through the regulation of tumor suppressors and oncogenes (136). Survival of many blood tumors, such as T-cell leukemia, multiple myeloma and acute myeloid leukemia, and solid tumors, such as colon carcinoma, breast cancer and lung carcinoma, is dependent on CK2, as blockage of CK2 function can either induce apoptotic cell death or sensitize them to apoptosis (189). Therefore, CK2 has emerged as a potential cancer therapeutic target. The targeting of CK2 for cancer therapy has not been seriously considered until now, because of its ubiquitous and multifunctional nature, so its inhibition may have serious toxicity to non-tumor cells. Recent studies have suggested that tumor cells seem more “addicted” to high levels of CK2 compared to their non-tumor counterparts (189, 190), and promising preclinical results in xenograft models of prostate cancer and squamous cell carcinoma of head and neck indicate that CK2 could be a good molecular target for cancer therapy (190). Preliminary results from a clinical trial using a novel CK2 peptide inhibitor,

CIGB-300, on patients with cervical malignancies suggests this inhibitor is safe, well-tolerated, and may have possible clinical benefit (191). Further studies along this line may yield an important new approach to cancer therapy.

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