

SOCS3 tyrosine phosphorylation as a potential bio-marker for myeloproliferative neoplasms associated with mutant JAK2 kinases

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ABSTRACT

JAK2 V617F, identified in the majority of patients with myeloproliferative neoplasms, tyrosine phosphorylates SOCS3 and escapes its inhibition. Here, we demonstrate that the JAK2 exon 12 mutants described in a subset of V617F-negative MPN cases, also stabilize tyrosine phosphorylated SOCS3. SOCS3 tyrosine phosphorylation was also observed in peripheral blood mononuclear cells and granulocytes isolated from patients with JAK2 H538QK539L or JAK2 F537-K539delinsL mutations. JAK kinase inhibitors, which effectively inhibited the proliferation of cells expressing V617F or K539L, also caused a dose-dependent reduction in both mutant JAK2 and SOCS3 tyrosine phosphorylation. We propose, therefore, that SOCS3 tyrosine phosphorylation may be a novel bio-marker of myeloproliferative neoplasms resulting from a

JAK2 mutation and a potential reporter of effective JAK2 inhibitor therapy currently in clinical development.

Key words: myeloproliferative neoplasms, JAK2, V617F, exon 12, SOCS3.

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Introduction

Myeloproliferative neoplasms (MPNs) like polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF), are clonal stem cell disorders attributed to a somatic mutation in Janus kinase (JAK) 2, resulting in a valine-to-phenylalanine substitution at position 617 (V617F).¹⁻⁵ Recently, other mutations within JAK2 have been identified in patients with V617F-negative PV.^{6,8} Like JAK2 V617F, these exon 12 mutations render JAK2 constitutively active and confer cytokine-independent growth *in vitro*.⁶ Suppressor of cytokine signaling (SOCS) 3 is a negative regulator of the erythropoietin receptor (EPOR) and the receptor-associated JAK2 kinase. SOCS3 contains a SOCS-box region at the C-terminus allowing it to form a multi-subunit E3 ligase complex consisting of ElonginBC, Cullin5 and Rbx1.^{9,10} In doing so, SOCS3 can target associated proteins for ubiquitination and degradation.¹¹⁻¹⁴ We have reported that, in contrast to wild-type JAK2, JAK2 V617F cannot be regulated by

SOCS3. Furthermore, turnover of SOCS3 is inhibited by JAK2 V617F and this correlates with marked tyrosine phosphorylation of SOCS3 protein.¹⁵ The aim of this study was to establish if SOCS3 was stabilized and phosphorylated by the other MPN-associated mutant JAK2 kinases. Our observations suggest that SOCS3 tyrosine phosphorylation may be a novel MPN bio-marker and a potential mechanism enabling the mutant JAK2 kinases to overcome SOCS3 inhibition.

Design and Methods

Constructs, reagents and transfections

293T cells were transfected using calcium phosphate.¹⁶ FLAG-tagged SOCS3 and JAK2 were expressed in pME18S and pRK respectively. JAK2 mutations were created in pRK-JAK2 and pMXI-JAK2-IRES-GFP using the QuikChange® site-directed mutagenesis kit (Stratagene, CA, USA). BaF3 cells were retrovirally infected with supernatants containing

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EPOR and JAK2, V617F or K539L expressed in pMXI-IRES-CD2 and pMXI-IRES-GFP, respectively.¹⁷ Prof. Terry Lappin (CCRCB, QUB) provided erythropoietin (EPO). AG490 and JAK inhibitor 1 were obtained from Calbiochem.

Immunoprecipitations and Western Blotting

293T cells were maintained and lysed as described.¹⁶ Lysates were immunoprecipitated with anti-JAK2 (Upstate Biotechnology, NY, USA) or anti-SOCS3 (clone 008) (Fusion Antibodies, Belfast). Western blots were probed with anti-phosphotyrosine (clone 4G10) (Upstate Biotechnology), anti-Flag (M2) or anti- γ -tubulin (Sigma Aldrich, UK), anti-JAK2 or anti-SOCS3.

Patient samples

Peripheral blood mononuclear cells (PBMCs) and granulocytes were isolated^{1,15} and analyzed as described¹⁵ from healthy donors or MPN patients who gave informed consent in accordance with the Declaration of Helsinki. Approval was obtained from the Research Ethics Committees, Northern Ireland and Addenbrooke's National Health Service Trust Research Ethics Committee.

Proliferation assays

BaF3-EPOR cell populations were seeded in triplicate at 3×10^4 per mL in the presence or absence of 5 u/mL EPO. Viable cells were counted in quadruplicate by trypan exclusion at 24h, 48h and 72h. Statistical analysis was carried out using a Student's *t* test.

Results and Discussion

JAK2 V617F is resistant to the inhibitory effects of SOCS3.¹⁵ In the presence of EPOR, wild-type JAK2 induced a loss of SOCS3 protein while JAK2 V617F tyrosine phosphorylated and stabilized both endogenous and exogenous SOCS3 (Figure 1A). Recently, a number of mutations within JAK2 (including JAK2 K539L, JAK2 N542-E543del, JAK2 H538QK539L and JAK2 F537-K539delinsL)^{6,8} have been identified in a subset of V617F-negative MPN patients. We wished to determine whether these mutations also induced SOCS3 tyrosine phosphorylation. Co-expression studies of SOCS3 and wild-type or mutant JAK2 were performed in 293T cells, in the presence of the EPOR.¹⁸ Compared to wild-type JAK2, expression of K539L or N542-E543del stabilized SOCS3 protein, as did JAK2 V617F (Figure 1B). Additionally, endogenous and exogenous SOCS3 was tyrosine phosphorylated in the presence of these MPN-associated mutant JAK2 kinases (Figure 1A and 1B). Similar results were obtained when JAK2 H538QK539L and JAK2 F537-K539delinsL were tested (Figure 1C). We next investigated if SOCS3 tyrosine phosphorylation was observed in JAK2 V617F-negative MPN patients. PBMCs and granulocytes were isolated from a healthy individual and 2 patients, with either H538QK539L or F537-K539delinsL mutations. Protein concentration was estimated and equalized for each sample. Compared with

controls, both MPN patients showed greater levels of tyrosine phosphorylated SOCS3 in both PBMCs and granulocytes, while SOCS3 protein levels were similar (Figure 1D). We next investigated whether SOCS3 tyrosine phosphorylation is specific to the MPN-associated V617F or exon 12 mutations in JAK2. To investigate this we determined whether other activating mutations in JAK2 resulted in stabilization of tyrosine phosphorylated SOCS3. To do this we created the JAK2 T875N mutation identified in an acute megakaryoblastic leukemia cell line and expressed this mutation in the presence of SOCS3. As before, V617F caused stabilization of tyrosine phosphorylated SOCS3, while both wild-type JAK2 and JAK2 T875N failed to do so in the absence (Figure 1E, left hand panels) or presence (Figure 1E, right hand panels) of EPO stimulation. These data demonstrated that SOCS3 tyrosine phosphorylation may be a useful bio-marker specific to the MPN-associated V617F or exon 12 mutations.

We next established if SOCS3 could regulate the phosphorylation of the exon 12 JAK2 mutants. Following EPO stimulation, SOCS3 potently inhibited wild-type JAK2 phosphorylation, with a concomitant reduction in SOCS3 protein levels, while SOCS3 was unable to significantly down-modulate mutant JAK2 phosphorylation and in all cases SOCS3 protein was stabilized (Figure 2). This may indicate that tyrosine phosphorylation of SOCS3 abrogated its ability to negatively regulate JAK2 K539L, H538QK539L, or F537-K539delinsL.

To investigate if SOCS3 could be a novel bio-marker of MPN, we analyzed the effect of JAK inhibitors on SOCS3 tyrosine phosphorylation. Firstly, we created stable BaF3 cells lines expressing EPOR along with wild-type JAK2, V617F or K539L. Trypan exclusion counts confirmed that although BaF3 cells expressing wild-type JAK2 were unable to proliferate in the absence of EPO, cells expressing V617F or K539L continued to proliferate (Figure 3A). Next we investigated the effects of AG490 or JAK inhibitor 1 on the proliferation of BaF3 cells expressing V617F and K539L in the absence of EPO stimulation. Both AG490 and JAK inhibitor 1 severely impaired the proliferation of BaF3 cells expressing V617F or K539L in a dose-dependent manner (Figure 3B). Since AG490 gave the most pronounced growth inhibitory effects, we investigated the impact of AG490 on SOCS3 tyrosine phosphorylation (Figure 3C). 293T cells expressing EPOR and SOCS3 along with V617F (Figure 3C, left panels) or K539L (Figure 3C, right panels) were treated with AG490 for 24h. AG490 caused a dose-dependent reduction in SOCS3 tyrosine phosphorylation correlating with a reduction in mutant JAK2 phosphorylation. These findings suggest that SOCS3 tyrosine phosphorylation mirrors JAK2 activity and may highlight SOCS3 tyrosine phosphorylation as a marker of MPN and a potential therapeutic read-out of JAK2 inhibitor therapy currently in clinical trials.

SOCS3 tyrosine phosphorylation may also hint at a mechanism by which these mutant JAK2 kinases cause transformation. The inhibitory function of SOCS3 is attributed to the C-terminal SOCS-box which enables

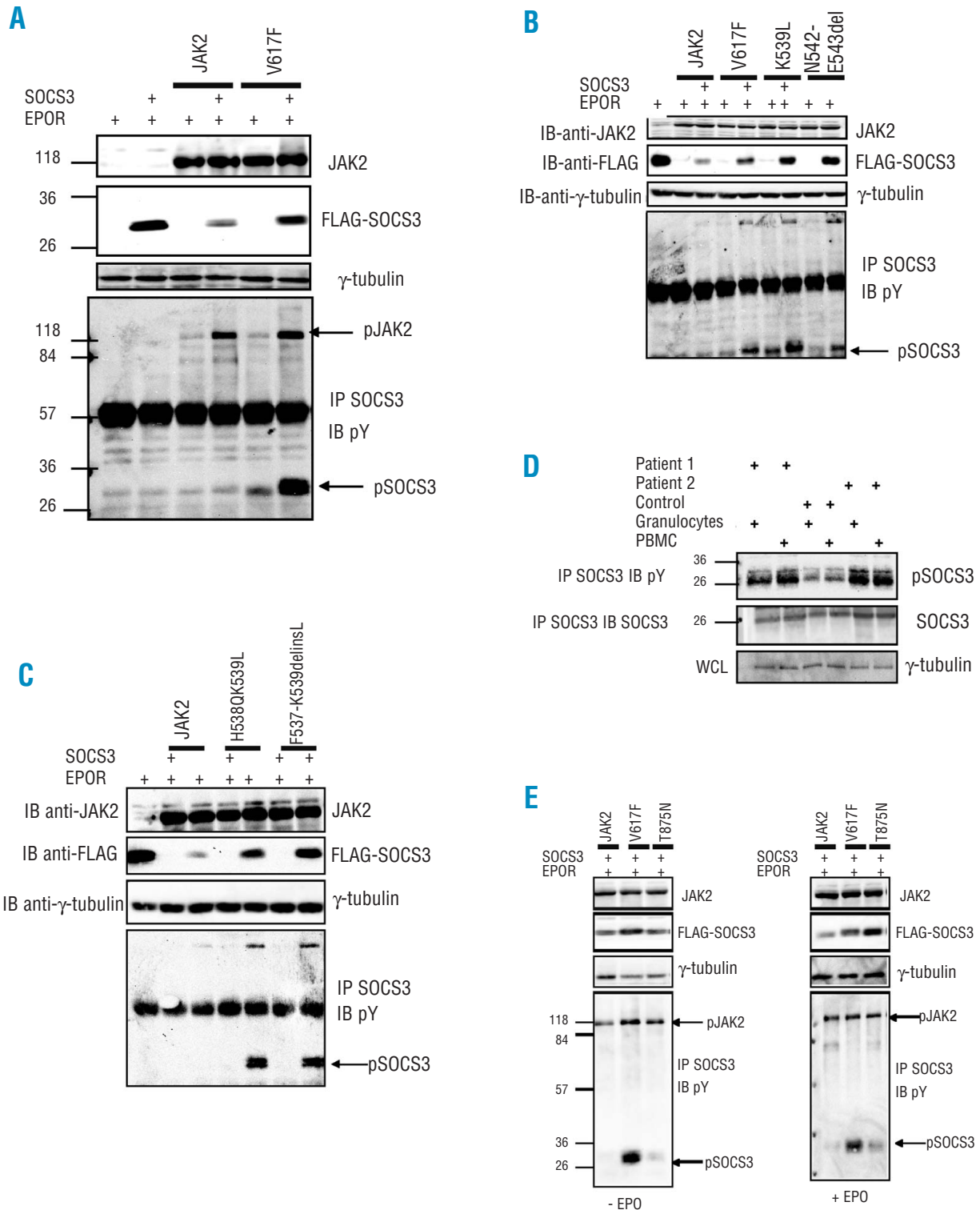


Figure 1. SOCS3 tyrosine phosphorylation by the MPN-associated mutant JAK2 kinases. (A) 293T cells were transfected with cDNA encoding EPOR with JAK2 or JAK2 V617F both in the presence and absence of FLAG-SOCS3. Whole cell lysates (WCLs) were immunoblotted with anti-JAK2 (top panel), anti-FLAG (panel 2) to detect SOCS3 or anti- γ -tubulin (panel 3). The remaining lysates were immunoprecipitated with anti-SOCS3 and immunoblotted with anti-phosphotyrosine (pY) (bottom panel). (B) 293T cells were transfected with EPOR and either JAK2, JAK2 V617F, JAK2 K539L or JAK2 N542-E543del in the presence and absence of FLAG-SOCS3. Lysates were analyzed as in (A). (C) 293T cells were transfected with cDNA encoding EPOR with JAK2 wild-type, H538Q/K539L or F537-K539delinsL in the absence and presence of SOCS3. Lysates were analyzed as in (A). (D) PBMCs and granulocytes were isolated from a control patient or MPN patients with a JAK2 H538Q/K539L mutation (patient 1) or a F537-K539delinsL mutation (patient 2). Lysates were immunoprecipitated with anti-SOCS3 and immunoblotted with anti-pY (top panel) and reprobed with anti-SOCS3 (middle panel). WCLs were also immunoblotted with anti- γ -tubulin (bottom panel). (E) 293T cells were transfected with cDNA encoding EPOR, SOCS3 and either JAK2 wild-type, JAK2 V617F or JAK2 T875N. Cells were left untreated (left hand panels) or treated with EPO (50 u/mL) for 15min prior to lysis (right hand panels). Lysates were analyzed as in (A).

assembly of a multi-subunit E3 ligase complex.^{9,10} We have previously reported that SOCS3 is tyrosine phosphorylated in response to many cytokines and that tyrosine phosphorylation of SOCS3 *in vitro* prevents the SOCS3-ElonginC binding.¹⁹⁻²¹ Constitutive SOCS3 tyrosine phosphorylation may prevent the SOCS3-ElonginC interaction thereby inhibiting SOCS3 E3 ligase activity. In other words, the MPN-associated mutant JAK2 kinases may constitutively phosphorylate SOCS3 and could indicate a mechanism by which the MPN-associated JAK2 mutations may induce transformation. Regulation of SOCS proteins by phosphorylation is not restricted to SOCS3, since v-ABL-mediated phosphorylation of SOCS1 disrupts the interaction with the ElonginBC complex, thus stabilizing SOCS1 and blocking JAK degradation.^{22,23} If the MPN-associated mutants bypass the suppressive function of SOCS3 by inhibiting E3 ligase recruitment through tyrosine phosphorylation, SOCS3 may not only be a suitable bio-marker of MPN but may be a potential therapeutic target of MPN arising from these JAK2 mutations.

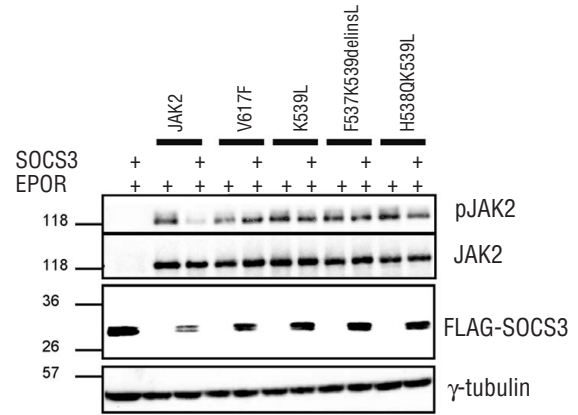


Figure 2. SOCS3 cannot down modulate phosphorylation of V617F or the exon 12 mutants. 293T cells were transfected with EPOR and either wild-type JAK2, V617F, K539L, H538Q/K539L or F537-K539delinsL mutation alone or in the presence of SOCS3. Cells were treated with EPO (50 u/mL) for 15 min prior to lysis. Whole cell lysates (WCLs) were immunoprecipitated with anti-JAK2 and immunoblotted with anti-phosphotyrosine (pY) (top panel) and reprobbed with anti-JAK2 (panel 2). WCLs were immunoblotted with anti-FLAG to detect SOCS3 (panel 3) or anti- γ -tubulin (bottom panel).

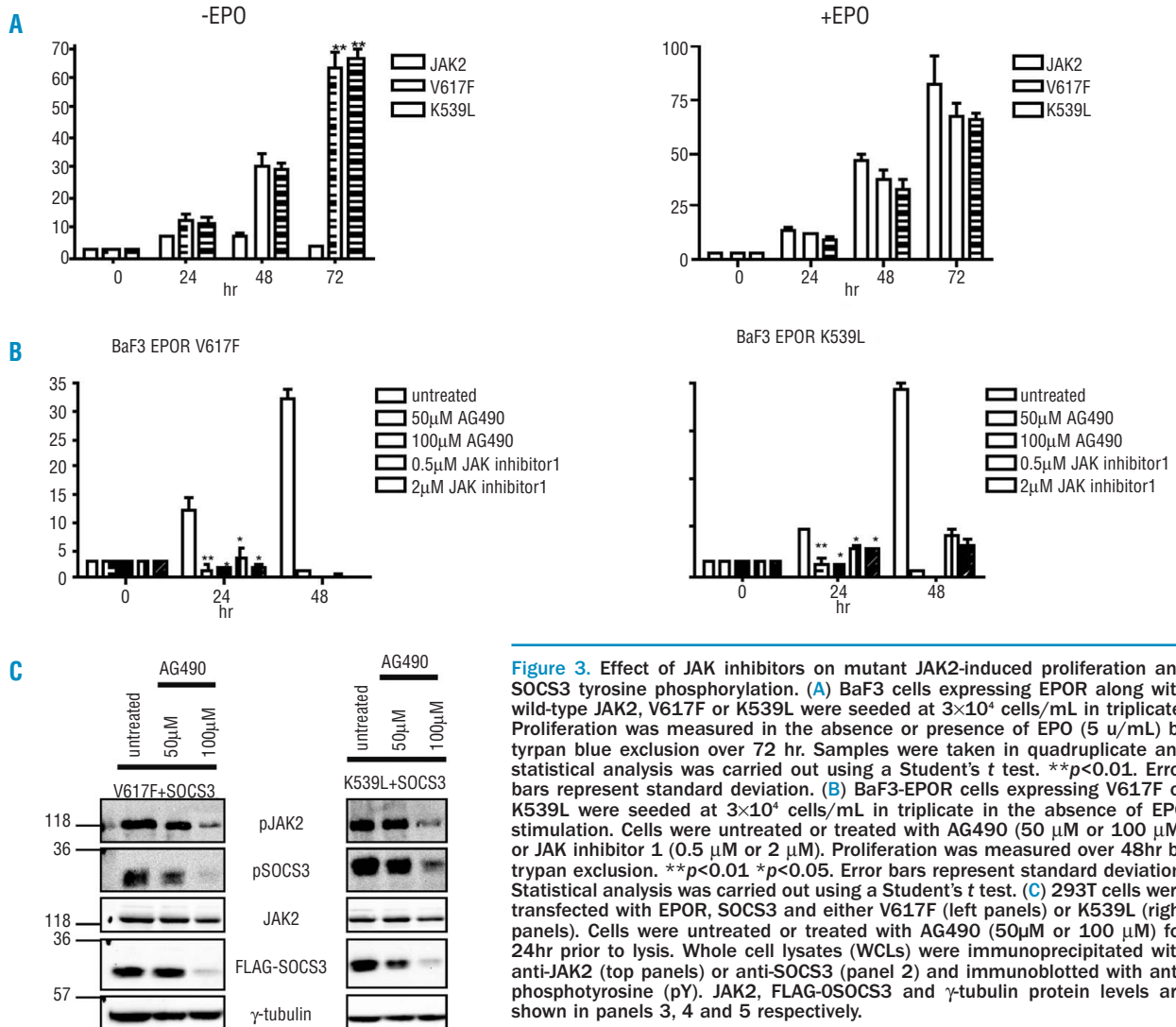


Figure 3. Effect of JAK inhibitors on mutant JAK2-induced proliferation and SOCS3 tyrosine phosphorylation. (A) BaF3 cells expressing EPOR along with wild-type JAK2, V617F or K539L were seeded at 3×10^4 cells/mL in triplicate. Proliferation was measured in the absence or presence of EPO (5 u/mL) by trypan blue exclusion over 72 hr. Samples were taken in quadruplicate and statistical analysis was carried out using a Student's *t* test. $**p < 0.01$. Error bars represent standard deviation. (B) BaF3-EPOR cells expressing V617F or K539L were seeded at 3×10^4 cells/mL in triplicate in the absence of EPO stimulation. Cells were untreated or treated with AG490 (50 μ M or 100 μ M) or JAK inhibitor 1 (0.5 μ M or 2 μ M). Proliferation was measured over 48hr by trypan exclusion. $**p < 0.01$ $*p < 0.05$. Error bars represent standard deviation. Statistical analysis was carried out using a Student's *t* test. (C) 293T cells were transfected with EPOR, SOCS3 and either V617F (left panels) or K539L (right panels). Cells were untreated or treated with AG490 (50 μ M or 100 μ M) for 24hr prior to lysis. Whole cell lysates (WCLs) were immunoprecipitated with anti-JAK2 (top panels) or anti-SOCS3 (panel 2) and immunoblotted with anti-phosphotyrosine (pY). JAK2, FLAG-SOCS3 and γ -tubulin protein levels are shown in panels 3, 4 and 5 respectively.

Authorship and Disclosures

JE designed, performed research and wrote the paper. YS, LMS and KN performed research, ARG and MFM

provided patient samples and advised on research. SC provided vital reagents and contributed to the manuscript preparation. JAJ advised on research and contributed to manuscript preparation.

The authors reported no potential conflicts of interest.

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