

## Brief report

Somatic hypermutation of *SOCS1* in lymphocyte-predominant Hodgkin lymphoma is accompanied by high *JAK2* expression and activation of *STAT6*Anja Mottok,<sup>1</sup> Christoph Renné,<sup>1</sup> Klaus Willenbrock,<sup>1</sup> Martin-Leo Hansmann,<sup>1</sup> and Andreas Bräuninger<sup>1</sup><sup>1</sup>Senckenberg Institute of Pathology, University of Frankfurt, Frankfurt, Germany

**Aberrant activities of JAK/STAT signaling pathways have been observed in several hematologic malignancies. Here, we show high expression of JAK2 in the tumor cells of lymphocyte-predominant Hodgkin lymphoma in 85% of cases and activation of JAK2 in 39% of cases. STAT6, which is a target of JAK2, was activated in 50% of the cases. SOCS1 controls JAK2 activity**

**and degradation. Mutations in SOCS1 of either somatic or germ-line origin were observed in micromanipulated tumor cells of 50% of cases. Most mutations truncated SOCS1 or caused replacement of amino acids in functional important regions. Activating mutations in exon 12 of JAK2, which are frequent in myeloproliferative diseases, were not observed. In**

**lymphocyte-predominant Hodgkin lymphoma SOCS1 function may thus be frequently impaired by mutations, and this may contribute to high JAK2 expression and activation of the JAK2/STAT6 pathway. (Blood. 2007;110:3387-3390)**

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## Introduction

Hodgkin lymphoma (HL) is distinguished into the classical (c) and nodular lymphocyte-predominant (lp) forms. While the derivation of the lymphocytic and histiocytic (L&H) tumor cells of lpHL from germinal center (GC) B cells is well established,<sup>1</sup> knowledge about their pathogenesis is limited. Besides *BCL6-IGH* translocations in fractions of cases,<sup>2</sup> aberrant somatic hypermutation (SHM) of *PIM1*, *PAX5*, *RHOH*, and *c-MYC* has been observed.<sup>3</sup>

JAK/STAT signaling pathways are constitutively activated in several hematologic malignancies.<sup>4</sup> In a survey of *JAK2* expression in lymphomas, we observed high *JAK2* expression in lpHL and analyzed the causes and consequences of the high *JAK2* expression in L&H cells.

microwave oven in citrate buffer (10 mM, pH 6) and incubated at room temperature overnight with 1:1000 dilutions of the antibodies. All p-STAT and p-JAK2 IHCs were developed with the CSAII system (Dako, Hamburg, Germany). For specificity tests of antibodies, see Data Supplements (available on the *Blood* website; see the Supplemental Materials link at the top of the online article). For picture acquisition, an Olympus BX40 (Olympus, Hamburg, Germany) microscope equipped with UPLANapo objective lenses (10×/0.4, 20×/0.7, 40×/0.65) and a Nikon Coolpix 4500 (Nikon, Düsseldorf, Germany) digital camera were used. All pictures were processed with Photoshop 7.0d (Adobe, San Jose, CA).

## Western blot analysis

Cells were lysed in Lämmli buffer (8 minutes, 100°C). Lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto PDVF membranes (BioRad, Munich, Germany), and incubated overnight with 1:1000 dilutions of primary antibodies.

## Micromanipulation, PCR, and sequence analysis

Single CD20<sup>+</sup> L&H cells were micromanipulated as previously described.<sup>1</sup> For every 10 cells, 4 aliquots of buffer covering the sections were used as negative controls. The complete coding region of *SOCS1* (a single exon of 633 bp) was amplified in 3 overlapping fragments and exon 12 of *JAK2* as a single PCR product from genomic DNA of single cells in seminested 2-round PCRs. For each case and *SOCS1* fragment, usually 5 to 6 PCR products from different cells and for *JAK2* amplicates from at least 4 different cells were analyzed. Primer sequences and PCR details are given in Table S1. All PCR products were directly sequenced.

## Materials and methods

Approval was obtained from the University of Frankfurt School of Medicine Institutional Review Board for these studies. Informed consent was obtained in accordance with the Declaration of Helsinki.

## Tissue samples

Forty-seven formalin-fixed paraffin-embedded and 12 frozen lpHL biopsies were used for immunohistochemistry (IHC) and micromanipulation, respectively.

## Immunohistochemistry

IHC for *JAK2* was performed as previously described and verified for a fraction of cases with an additional *JAK2*-specific antibody (24B11; Cell Signaling, Beverly, MA).<sup>5</sup> P-STAT3 and p-STAT5 IHC was performed as recommended by the supplier (no. 9131 and no. 9359; Cell Signaling). For p-STAT6 (no. 611566; B&D Biosciences, San Diego, CA) and p-JAK2 (no. 3771; Cell Signaling) IHC sections were cooked for 15 minutes in a

## Results and discussion

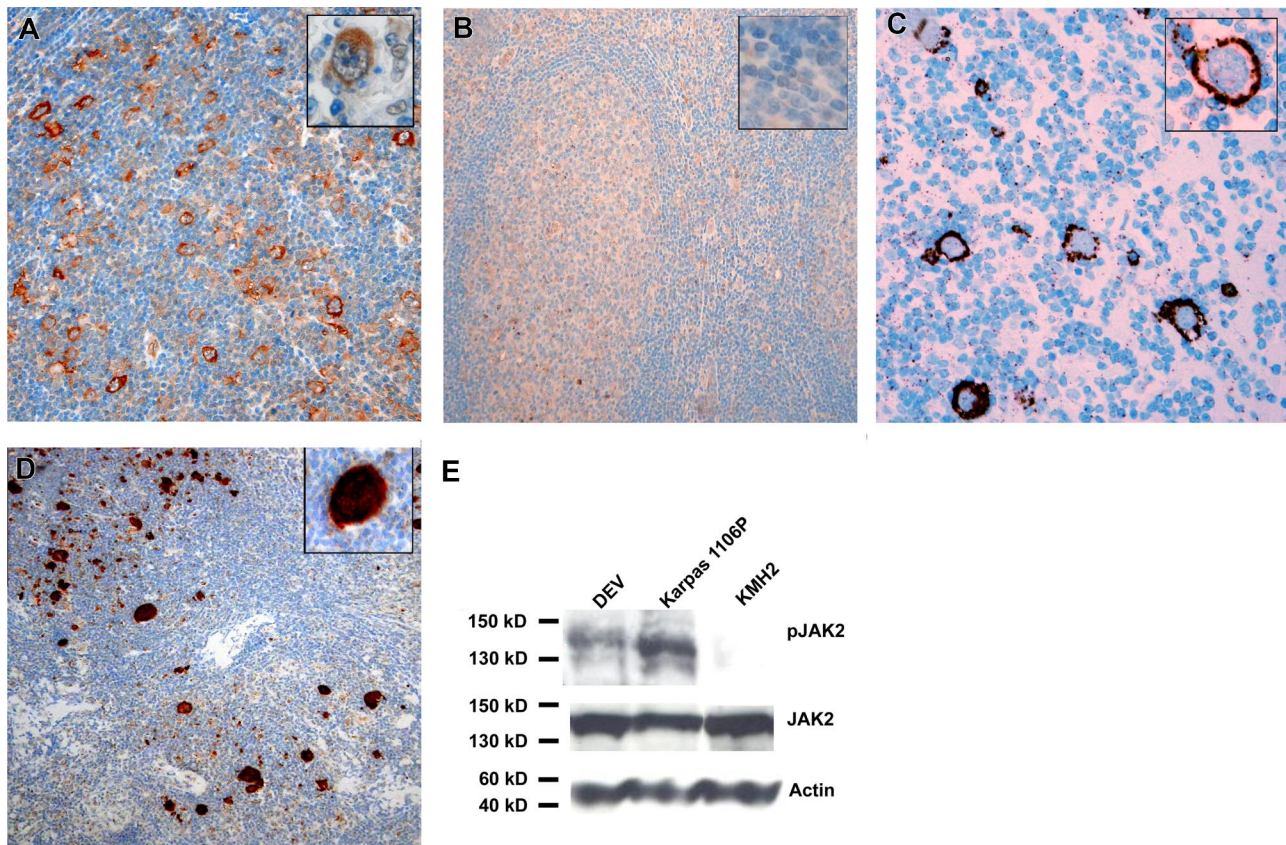
IHC revealed high *JAK2* expression in the majority of L&H cells in most lpHL cases (40/47 cases, 85%) (Figure 1). In GC B cells, from

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**Figure 1. Immunohistochemistry for JAK2 and p-STAT6.** IHC for JAK2 with an IphL case (A) and a tonsil (B) is shown. Inserts show higher magnifications of an L&H cell in panel A and the mantle zone/GC border in panel B. While JAK2 amounts in normal B cells were below the detection limit of IHC, 40 of 47 cases of IphL showed positivity in L&H cells. (C) IHC with an IphL case and a p-JAK2 specific antibody is shown. Fractions of L&H cells were positive in 13 of 33 analyzed cases. (D) IHC for p-STAT6 with an IphL case is shown. Twenty-one of 43 analyzed IphL cases showed positivity for p-STAT6. All cases were from the files of the Department of Pathology of the University of Frankfurt. Bound antibodies were detected using the Envision system (Dako, Hamburg, Germany) for anti-JAK2 and the CSA11 signal amplification system for anti-p-JAK2 and anti-p-STAT6 with horseradish peroxidase and 3,3'-diaminobenzidine as substrate. Specificity of antibodies was verified by comparison of IHC with Western blot analysis (JAK2 and p-STAT6; Figures S1,S2) and/or by the use of 2 different antibodies directed against the same antigen (JAK2 and p-JAK2). Specificity of anti-p-JAK2 and anti-p-STAT6 antibody for phosphorylated JAK2 and STAT6 was tested by pretreatment of several cases with T-cell phosphatase,<sup>5</sup> which abolished staining completely (Figure S3). (E) Western blot analysis of JAK2 expression and activation in the IphL-derived cell line DEV is shown. Whereas JAK2 was expressed in the MLBCL-derived cell line Karpas1106P, the cHL-derived cell line KM12, and DEV, activation of JAK2 was observed only in Karpas1106P and DEV, in line with the presence of SOCS1 mutation only in Karpas1106P and DEV.<sup>6</sup>

which L&H cells are derived, and other normal B cells JAK2 protein amounts were below the detection limit of IHC. Besides the previously reported high JAK2 expression in cHL and mediastinal large B-cell lymphoma (MLBCL),<sup>5</sup> JAK2 expression was only occasionally observed in other B-cell lymphomas (3/20 diffuse large B-cell, 0/10 mantle cell, and 2/16 follicular lymphomas and 1/9 chronic lymphocytic leukemias). With a p-JAK2-specific antibody, JAK2 activation was observed in 39% (13/33) of cases (Figure 1). Western blot analysis with the IphL-derived cell line DEV revealed that JAK2 was expressed and also activated (Figure 1).

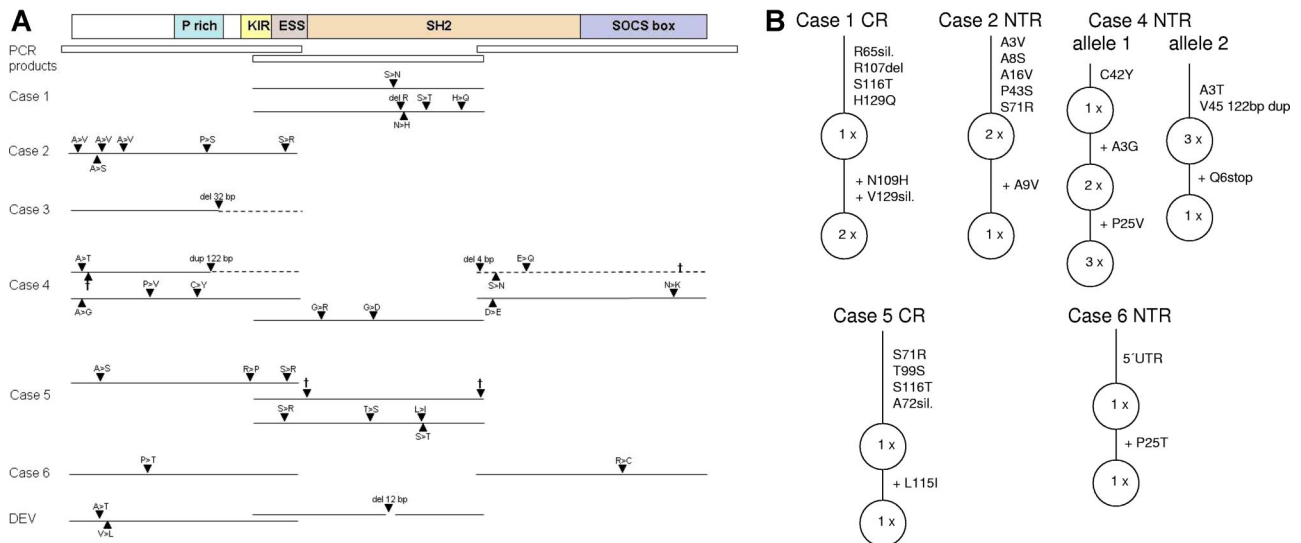
JAK2 phosphorylates STAT transcription factors. We thus investigated the activation status of STAT3, STAT5, and STAT6, which are constitutively phosphorylated in cHL and MLBCL,<sup>6-8</sup> in IphL. While no activation of STAT3 and STAT5 was observed, STAT6 was phosphorylated in 49% of cases (21/43). Twenty of the 21 p-STAT6-positive cases showed high JAK2 expression and 9 of 17 p-STAT6-positive cases were also p-JAK2 positive (Figure 1). Taking the technical problems performing p-Y-specific IHC into account,<sup>9</sup> it is likely that JAK2 and STAT6 are activated in most cases of IphL.

JAK2 activity and protein amount are regulated by a negative-feedback loop. P-STATs induce SOCS1 expression, which binds via its SH2 domain to p-JAK2, thereby inhibiting JAK2 kinase activity and initiating JAK2 degradation by recruitment of a E3-ubiquitin ligase.<sup>10</sup> In cHL and MLBCL, this negative-feedback

loop is disturbed by inactivating mutations in SOCS1 with subsequent accumulation of JAK2 protein.<sup>6,11</sup> We thus analyzed the SOCS1 gene in L&H cells for presence of mutations. Three overlapping fragments encompassing the complete coding region of SOCS1 were amplified from genomic DNA of single micromanipulated L&H cells and directly sequenced (Figure 2A; Table S2).

Mutations were found in 6 of 12 cases and the IphL cell line DEV. Comparisons with SOCS1 from corresponding nontumor cells revealed that a deletion in one case was of germ-line origin, while all other mutations were of somatic origin. In 3 cases, inactivating mutations were observed, and presence of several replacement mutations in functionally important regions in the 3 other cases suggest that SOCS1 function was also impaired in these cases.

In 3 cases (cases 1, 4, and 5) alleles with completely different mutation patterns and in 4 of 6 cases (cases 1, 2, 5, and 6) with mutated alleles, unmutated alleles were also repeatedly amplified (Figure 2A; Table S2). This could be due to the presence of different alleles in all L&H cells of a case or due to presence of different alleles only in subclones of L&H cells. In the former case, the different alleles should be occasionally coamplified from single cells. This was observed only for both mutated N-terminal fragments of case 4 and mutated and unmutated N-terminal, central, and C-terminal fragments of case 4 and the central fragment of case 5. For the other differing alleles, it remained thus unclear whether



**Figure 2. Schematic presentation of *SOCS1* mutations in L&H cells.** The complete coding region of *SOCS1* was amplified and sequenced from genomic DNA of single micromanipulated L&H cells of 12 cases and the lpHL-derived cell line DEV. (A) A scheme of the *SOCS1* protein with functionally important regions and the 3 PCR fragments amplified are shown at top (P rich indicates proline rich; KIR, kinase inhibitory region; and ESS, extended SH2 domain). For each case with mutations in *SOCS1*, only regions and alleles that carried mutations are presented. Mutations are indicated by ▲. The deletion in case 3 is of germ-line origin. Protein sequences translated out of frame due to deletions or duplications are shown in — — and stop codons, by †. In addition to the mutated alleles, unmutated alleles were amplified for the central region (CR) of case 1 (7×), the N-terminal region (NTR) of case 2 (3×), the NTR of case 3 (1×), the CR of case 4 (1×), the NTR and CR of case 5 (1× and 2×), and the NTR and C-terminal region (CTR) of case 6 (2× and 3×). Amplification of only 1 unmutated allele for a fragment of a case (NTR of case 3, CR of case 4, and NTR of case 5) is most likely due to cellular contamination during micromanipulation. Apart from the replacement mutations shown, altogether 7 silent mutations were observed (not shown; see Table S2 for details). For alleles with ongoing somatic mutation, the variant carrying the most mutations is always presented. (B) Stepwise accumulation of mutations for alleles with intraclonal diversity is shown. Presence of truncating and replacement mutations in functional important regions of *SOCS1* suggest that *SOCS1* function is impaired in L&H cells.

they were present in all L&H cells of the respective cases or restricted to different subclones of tumor cells.

In all 5 cases with somatic mutations, intraclonal diversity of individual *SOCS1* alleles with stepwise accumulation of mutations was observed (Figure 2B). Hotspots of SHM (RGYW) were 4.5 times more frequently affected than expected (Table S2), and single base pair substitutions were much more frequent than deletions or insertions (39 versus 3, respectively). The somatic mutations are thus likely due to ongoing activity of SHM usually active in GC B cells.

In myeloproliferative disorders, *JAK2* is frequently activated by a replacement mutation in exon 12.<sup>12</sup> In micromanipulated L&H cells of 11 lpHLs, no such mutations were observed.

Taken together, these data suggest that in L&H cells *SOCS1* function might be impaired by mutations that are in most cases likely due to activity of SHM. This may cause increases in *JAK2* protein amount and activity and contribute to constitutive activation of *STAT6*, although *SOCS1* mutations are not necessarily accompanied by *JAK2* phosphorylation, and *JAK2* and *STAT6* phosphorylations did not correlate at the level of individual cases.<sup>13</sup> P-*STAT6* promotes expression of *AID* and *SOCS1*.<sup>14,15</sup> As SHM is dependent on transcription of target genes,<sup>16</sup> the constitutive p-*STAT6* activity in L&H cells may drive further acquisition of mutations in *SOCS1* and thus cause the high mutation frequency of *SOCS1* compared with other genes targeted by normal or aberrant SHM (average mutation frequency in mutated alleles: 1.2% for *SOCS1*; 0.7% for *BCL6*; 0.25% for *CD95* in GC B cells; and 0.1%-0.2% for aberrant SHM of *PAX5*, *PIMI1*, *RHOH*, and *c-MYC*).<sup>3,17,18</sup>

Although *SOCS1* mutations may in some cases be subclonal and thus a late event in tumor cell development, the selection of replacement mutations (observed ratio of replacement–silent mutations: 4.6 (32/7); expected 3)<sup>19</sup> indicates that potential impairment of *SOCS1* function is advantageous for L&H cells. In addition to the observed mutations, *SOCS1* may be affected by genomic deletions, as losses of the region encompassing *SOCS1* are

frequent in lpHL,<sup>20</sup> and inactivation by hypermethylation, which has been observed in several other malignancies.<sup>21,22</sup>

*STAT6* is also constitutively activated in cHL and MLBCL.<sup>7,8</sup> However, *STAT6* activation in the context of a GC B-cell expression program as in L&H cells may have different consequences. Thus, *BCL6* is expressed in L&H cells but not cHL and MLBCL, and *BCL6* may interfere with *STAT6* on several promoters.<sup>23</sup> Finally, potential impairment of *SOCS1* function could in addition to *JAK2* also influence the expression of other proteins such as *NFκB* and *VAV1* in lpHL.<sup>24,25</sup>

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## Authorship

Contribution: A.M. and C.R. designed and performed research and analyzed and interpreted data; K.W. performed research and analyzed data; M.-L.H. analyzed and interpreted data; A.B. designed research, analyzed and interpreted data, and drafted the paper. A.M. and C.R. contributed equally to this work.

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