Regular Article

LYMPHOID NEOPLASIA

Pervasive mutations of JAK-STAT pathway genes in classical Hodgkin lymphoma

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KEY POINTS

- **Identification of genes frequently mutated in cHL, fostering tumor growth in a manner amenable to pharmacological targeting.**

- **Mutated genes include the almost ubiquitous targeting of JAK-STAT pathway members, as well as GNA13, XPO1, and ITPKB.**

Dissecting the pathogenesis of classical Hodgkin lymphoma (cHL), a common cancer in young adults, remains challenging because of the rarity of tumor cells in involved tissues (usually <5%). Here, we analyzed the coding genome of cHL by microdissecting tumor and normal cells from 34 patient biopsies for a total of ∼50 000 singly isolated lymphoma cells. We uncovered several recurrently mutated genes, namely, *STAT6* (32% of cases), *GNA13* (24%), *XPO1* (18%), and *ITPKB* (16%), and document the functional role of mutant STAT6 in sustaining tumor cell viability. Mutations of *STAT6* genetically and functionally cooperated with disruption of *SOCS1*, a JAK-STAT pathway inhibitor, to promote cHL growth. Overall, 87% of cases showed dysregulation of the JAK-STAT pathway by genetic alterations in multiple genes (also including *STAT3*, *STAT5B*, *JAK1*, *JAK2*, and *PTPN1*), attesting to the pivotal role of this pathway in cHL pathogenesis and highlighting its potential as a new therapeutic target in this disease. (*Blood*. 2018;131(22):2454-2465)

Introduction

Classical Hodgkin lymphoma (cHL), a common cancer in young adults, can be cured by chemotherapy and/or radiotherapy in the majority of cases.[1] However, about 20% of patients are refractory to, or relapse early after, treatment, with consequent suboptimal outcome, thus representing an unmet medical need. Furthermore, the burden of late-onset therapy-induced toxicities (eg, lung and heart dysfunction, secondary cancers, infertility) is of increasing concern in all patients with cHL receiving chemoradiotherapy at a young age.[1]

A deep understanding of cHL biology is therefore highly desirable. However, the pathogenesis of cHL in general, and its underlying genetic lesions in particular, have proven difficult to elucidate because of the rarity of Hodgkin and Reed-Sternberg (HRS) tumor cells in the involved lymph nodes. HRS cells usually account for <5% of the total cellularity, and are embedded in an immune suppressive inflammatory background that is thought to be recruited by the HRS cells themselves for fostering their growth and for evading the host antitumor response.[2,3]

Progress in understanding the biology of cHL has therefore required the laborious purification of HRS cells from tissues (by microdissection or fluorescence-activated cell sorter sorting),

which led to clarifying the genetic origin of HRS cells from normal germinal center B cells, despite the dramatic loss of the typical B-cell phenotype.[2,3] Various genetic lesions have then been found to be recurrent in fractions of cHL cases, which result in constitutive activation of the anti-apoptotic and pro-inflammatory NF-κB and JAK-STAT signaling pathways (eg, *TNFAIP3* and *SOCS1* disruption, respectively), as well as in immune evasion (eg, *PDL1/PDL2* copy number gain; *B2M* and *CIITA* disruption).[2,3]

However, a comprehensive characterization of cHL by whole-exome sequencing (WES), beyond that of a few cell lines[4,5] and primary cases,[6] is currently lacking. Here, we fill this gap and identify several genes that are mutated at notable frequencies and may represent new therapeutic targets in this disease.

Materials and methods

Laser microdissection and WES

Full details are provided in the supplemental Data, available on the *Blood* website, including a novel bioinformatics pipeline for calling somatic mutations and the methodological approaches (targeted sequencing and digital polymerase chain reaction) used to validate it.

Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) for *JAK2*, *TNFAIP3*, and *B2M* was performed according to standard protocols described in the supplemental Data.

Functional experiments in cHL cell lines

L1236, HDLM2, L540, and L428 cells were subjected to lentiviral transduction of anti-*STAT6* short-hairpin RNAs (shRNA) or the *SOCS1* coding sequence, followed by monitoring of cell death, as described in the supplemental Data. These data are shown in the main text as raw percentages of viable cells (and in supplementary figures as percentage of viable cells relative to the corresponding infected negative control set at 100%) because cHL cell lines are notoriously difficult to infect and their viability often decreases after infection, which may potentially influence the sensitivity of each cell line to different treatments.

The same 4 cHL cell lines, as well as 2 additional ones (ie, SUPHD1 and UHO1), were also treated with the JAK2 inhibitor fedratinib and/or the XPO1 inhibitor selinexor, and then monitored for apoptosis and/or viability, as detailed in the supplemental Data. The experiments with fedratinib, which were aimed at confirming pharmacologically the apoptosis induction observed on genetic silencing of the JAK-STAT pathway with *STAT6* sh-RNAs, were performed with fedratinib concentrations in the low micromolar range (1.5 and 3 μM), based on the drug concentration (1.5 μM) previously established to cause 50% of maximal growth inhibition (IC50) in the STAT6 wild-type cHL cell line L428.[7] The experiments with selinexor aimed at providing an initial assessment of the potential dependency of HRS cells on XPO1 and were performed at the dose of 100 nM, based on the median IC50 value of 123 nM that was previously established in 23 hematological and solid tumor cell lines (including the B-cell lymphoma line Ramos, where selinexor IC50 was also 123 nM).[8]

Western blotting was performed to verify STAT6 downregulation and exogenous SOCS1 expression after lentiviral transduction, as well as to analyze the phosphorylation status of STAT transcription factors basally and after JAK2 inhibition, using the procedures and reagents described in the supplemental Data.

All experiments were independently performed at least twice, giving reproducible results.

Results

The cHL coding genome

To define the genetic basis of cHL, we laser-microdissected HRS cells[9] (n = 1200-1800 per case), along with a similar number of adjacent nonneoplastic cells, from hematoxylin/eosin-stained frozen lymph node sections of 34 patients with cHL (supplemental Table 1; supplemental Figure 1). DNA from each tumor and matched normal sample was subjected in duplicate to whole-genome amplification (WGA) and independent WES of the duplicates to control the bias introduced by the WGA reaction through a novel bioinformatics pipeline ad hoc designed (supplemental Data). Unamplified germline DNA from peripheral blood cells was also included as control in 26/34 patients.

The median coverage depth in WGA-tumor, WGA-normal, and unamplified normal samples was 99, 114, and 142, respectively

(supplemental Table 2; supplemental Figure 2). We identified a median of 47 nonsilent somatic mutations per tumor that were present at ≥20% variant allele frequency, and hence, presumably in the major tumor clone (median: 43 single-nucleotide variants and 3 short indels per tumor; supplemental Figure 3; supplemental Table 3). Deeper sequencing analysis of 150 candidate tumor-specific changes identified across 26 samples previously subjected to WES confirmed the presence of 139 mutations (93%), including 130/139 (94%) single-nucleotide variants and 9/11 (82%) short indels, validating the high specificity of the approach (supplemental Table 4). Importantly, allele frequency estimates of somatic mutations in the deep targeted sequencing experiment were highly similar to those obtained in the WES experiment (correlation, 0.88; *P* value < 2.2e-16; supplemental Figure 4). Somatic mutations of selected genes were also validated by Sanger sequencing on tumor vs normal WGA-DNA (supplemental Table 5), and somatic variants of the most recurrently targeted gene (*STAT6*) were also confirmed by digital polymerase chain reaction to be present in unamplified DNA from whole-tissue sections at frequencies (supplemental Table 6) consistent with the low percentage of HRS cells typically present in lymph node biopsies.

In addition to previously known targets of genetic lesions in cHL (*B2M*, *TP53*; *SOCS1*, *JAK2*, and *PTPN1*; and the NF-κB pathway inhibitors *TNFAIP3* and *NFKBIE*),[2,3,10] this analysis revealed several recurrently mutated genes that had not been previously implicated in the genetics of cHL or had not been functionally assessed, the most common of which were *STAT6* (32% of cases), *GNA13* (24%), *XPO1* (18%), and *ITPKB* (16%) (Figure 1; supplemental Table 3).

Pervasive mutational targeting of JAK-STAT pathway genes in cHL

The most prominent genetic hallmark of cHL was represented by mutations in the JAK/STAT signaling pathway (supplemental Figure 5), with *STAT6* and *SOCS1* being the 2 most commonly affected targets (32% and 59% of cases, respectively). Missense mutations (n = 18) of the STAT6 DNA binding domain were found in 11/34 (32%) cases (Figures 1 and 2), including a highly recurrent hotspot at the N417 residue (9/18 mutations) and 2 additional hotspots at D419 and N421 within the same binding loop region (3/18 mutations each). These variants largely overlap with those reported in primary mediastinal B-cell lymphoma, relapsed-refractory germinal center B-cell type diffuse large B-cell lymphoma, and follicular lymphoma, where their role as gain-of-function or loss-of-function events with respect to STAT6 transcriptional activity is controversial.[11-14] Mutations were heterozygous in all cases, except 1 (no. 8) showing an 83% variant allele frequency, possibly indicating loss of the wild-type allele or, as already shown to occur in cHL, amplification of the mutant allele.[12,15] In 6 cases, the same allele was targeted by multiple mutations affecting up to 3 distinct codons (Figures 2A; supplemental Figure 6).

Because gains/amplifications of *JAK2* and mutations of the JAK-STAT pathway inhibitor *SOCS1* are frequent in cHL,[2,16-18] we examined the distribution of these lesions relative to STAT6 mutations across the 34 cases. JAK2 copy number increase, detected in 11/33 evaluable cases, was not preferentially associated with *STAT6* mutations (4/10 STAT6 mutated cases [40%] vs 7/23 wild-type cases [30%]; *P* value = .69, Fisher's exact

test). Conversely, *SOCS1* mutations, composed predominantly of frame-shifting and non-sense events, were significantly enriched in *STAT6*-mutated cases (n = 9/10 vs 7/17 *STAT6*-unmutated lymphomas; *P* = .018) (Figure 1). This preferential association was significant even when considering only SOCS1 disruptive variants (n = 8/10 vs 6/17; *P* = .046) and suggests a genetic interaction between *STAT6* mutations and *SOCS1*-inactivating events in cHL.

Besides *STAT6* and *SOCS1* mutations, multiple genetic lesions predicted to activate transducers of JAK-STAT signaling (JAK1, JAK2, STAT3, STAT5B) or to inactivate its inhibitor PTPN1 were found in 8 additional *STAT6* wild-type cases lacking (or not evaluable for) *SOCS1* (Figure 1; supplemental Tables 3 and 5). Thus, 87% (26/30) of the evaluable cHL cases carried genetic lesions in members of the JAK/STAT cascade (Figure 1). Together, these findings point to a key pathogenetic role for this signaling pathway in cHL through pervasive genetic targeting of its members (supplemental Figure 5).

Genetic and functional cooperation between STATs mutation and inactivation of the JAK-STAT inhibitor SOCS1

STAT transcription factors become activated on phosphorylation by JAK tyrosine kinases that transduce cytokine receptor signals, leading to STATs dimerization and translocation to the nucleus[19] (supplemental Figure 5). In cHL, phosphoSTAT6 expression by primary HRS cells is observed in the vast majority of cases (~80%),[20-22] suggesting constitutive activation of this transcription factor downstream of JAK signaling. To assess the relevance of *STAT6* and *SOCS1* mutations in the growth and survival of the cHL clone, we independently tested the consequences of *STAT6* silencing or *SOCS1* reconstitution on cell viability, using 3 phosphoSTAT6[+] cHL cell lines harboring *STAT6* and *SOCS1* alleles in different configurations (ie, L428 and HDLM2, both carrying inactivating *SOCS1* mutations and wild-type *STAT6*, and L1236, carrying an inactivating *SOCS1* mutation and expressing an amplified N417Y-STAT6 mutant allele but not wild-type STAT6[12]), whereas the L540 cHL cell line, which lacks mutations in both genes and shows no STAT6 phosphorylation, was used as control.[4-7,12,20]

ShRNA-induced knockdown of total (and phospho) STAT6 with either of 2 independent shRNAs consistently caused marked apoptosis in the STAT6-mutated L1236 cells, whereas it had no or a less pronounced effect in the 3 STAT6 wild-type cell lines, independent of whether they showed STAT6 phosphorylation (Figure 3; supplemental Figure 7). Thus, *STAT6* mutations may contribute to sustaining the growth of cHL cells through a mechanism that is distinct from (and beyond that) of STAT6 phosphorylation.

We then transduced wild-type SOCS1 alleles in the same 4 cell lines. As expected, exogenous SOCS1 expression significantly suppressed STAT6 phosphorylation in the 3 pSTAT6^{+} cells (Figure 4, top). However, this was accompanied by significant apoptosis only in the 2 cell lines harboring concurrent mutations of *SOCS1* and *STAT* family members; that is, L1236 and HDLM2 (Figure 4, bottom), with the latter carrying an activating heterozygous D661Y hot-spot mutation of the STAT3 SH2 domain (COSMICv76 database; Hudnall et al[5]). The D661Y mutation is recurrent in T/NK-cell neoplasms and was previously shown to be associated with both STAT3 constitutive phosphorylation and

increased transcriptional activity.[23] Indeed, western blot analysis confirmed basal STAT3 phosphorylation in this cell line, which was abrogated on enforced expression of exogenous SOCS1 (supplemental Figure 8).

Consistent with these findings, treatment with the JAK2 selective inhibitor fedratinib was toxic to L1236 and HDLM2, but not to L428, despite efficient dephosphorylation of STAT6 and STAT3 (Figure 5).

Collectively, these data suggest a cooperative activity between mutations of *STAT* family members and genetic inactivation of *SOCS1* in sustaining cHL growth. In support of this model, 2/8 cHL tumors with *SOCS1* disruptive mutations and wild-type *STAT6* alleles carried activating mutations in other STAT family members, including a T628S substitution in the SH2 domain of STAT5B (also described in T-cell prolymphocytic leukemia)[24] and a D661Y substitution of STAT3 (Figure 1; supplemental Tables 3 and 5).

Recurrent GNA13 mutations in cHL

A second target of recurrent mutations in cHL was *GNA13*, encoding the Gα13 subunit of heterotrimeric G-proteins (n = 8/34 cases [24%]; Figures 1 and 6A; supplemental Table 5). By transmitting signals from the G-protein-coupled receptors S1PR2 and P2RY8 that result in inhibition of AKT phosphorylation, Gα13 ensures the proper confinement of proliferating germinal center B cells within secondary lymphoid follicles and at the same time constrains their expansion by facilitating apoptosis in this potentially dangerous niche.[25-27]

GNA13 variants in cHL were mostly heterozygous and included non-sense, frame-shifting, and missense mutations. This pattern overlaps with that observed in Burkitt lymphoma and diffuse large B-cell lymphoma of the germinal center B-cell type, and is compatible with loss of Gα13 signaling activity.[25,27-32] Mutations of *GNA13* in cHL were strongly associated with *STAT6* mutations, being detected in 7/11 STAT6-mutated samples (64%) compared with 1/25 *STAT6*-unmutated patients (4%; *P* value = .0003, Fisher's exact test; Figure 1).

Frequent inactivating mutations of ITPKB in cHL

Four (16%) of 25 evaluable case harbored heterozygous somatic mutations in *ITPKB* (Figure 6B; supplemental Table 5). *ITPKB* encodes for a kinase converting the second messenger inositol trisphosphate (IP3) to IP4, a soluble antagonist of the AKT-activating PI3K-product IP3.[33] All 4 *ITPKB*-mutated lymphomas showed disruptive frame-shifting variants predicted to generate truncated proteins lacking the kinase domain and, therefore, to boost PI3K-AKT signaling, a characteristic and growth-promoting feature of HRS cells that is amenable to pharmacologic inhibition.[34,35] In addition, 1 of these 4 cases harbored 3 missense mutations in the gene exon 2, the significance of which remains to be established.

Recurrent hot spot mutations of XPO1 correlating with cHL cell vulnerability to the clinical XPO1 inhibitor selinexor

Heterozygous missense mutations of *XPO1* at the hot-spot residue E571 were found in 6/34 patients (18%; Figures 1 and 7A; supplemental Table 5); this amino acid is known to contact a prototypic nuclear export signal (NES)[36,37] and is also recurrently targeted by somatic mutations in primary mediastinal B-cell lymphoma and, at a lower frequency, in chronic lymphocytic
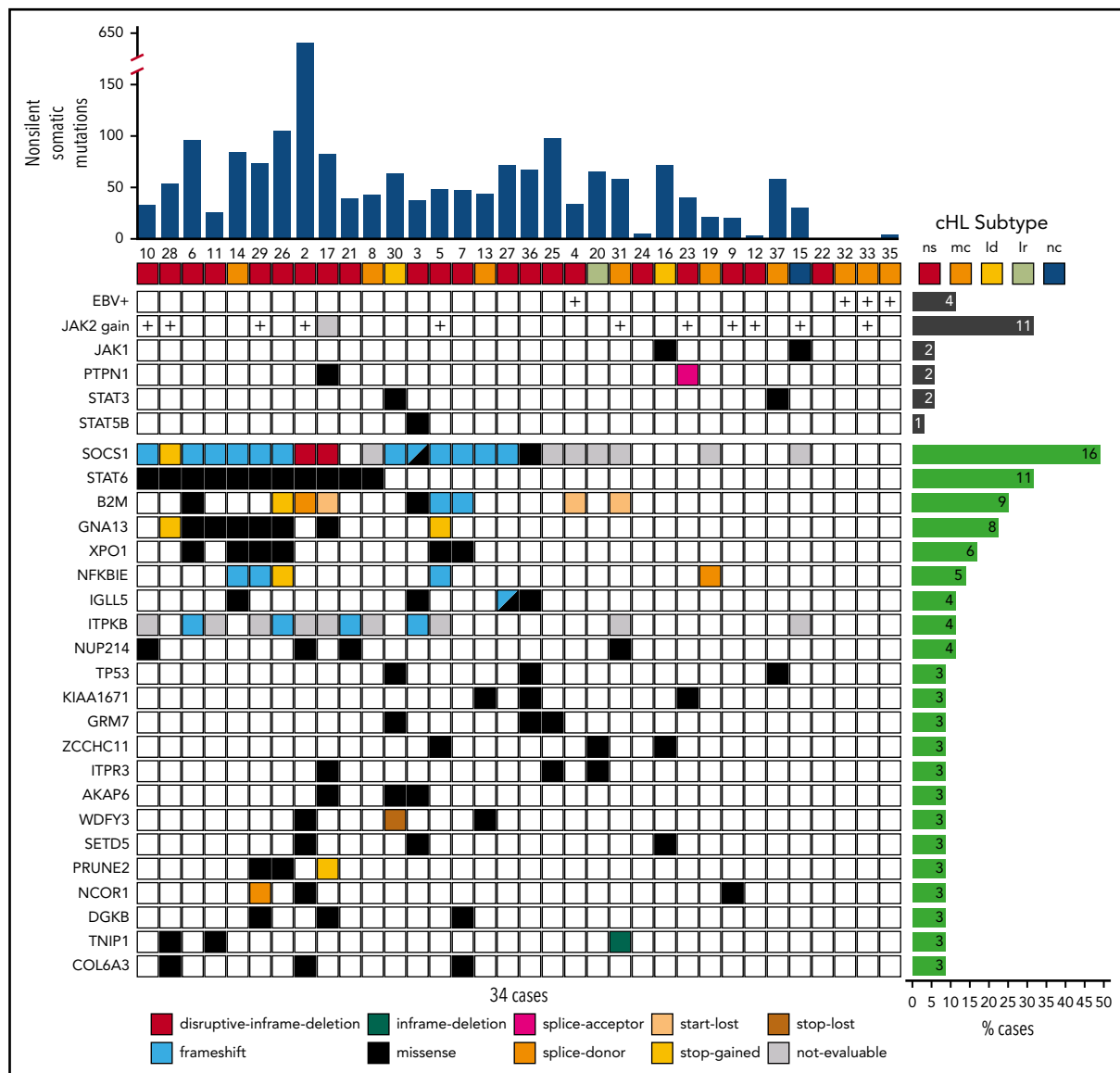
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Figure 1. Recurrently mutated genes in the tumor cells of cHL. (Top) Total number of nonsilent somatic mutations present in each of the 34 cHL cases, identified by their identification number and annotated based on histological subtype (ld, lymphocyte depletion; lr, lymphocyte-rich; mc, mixed cellularity; nc, not classifiable; ns, nodular sclerosis). EBV infection status, presence/absence of *JAK2* copy number gains, and the status of 3 JAK-STAT pathway genes (*PTPN1*, *STAT3*, and *STAT5B*) that were found mutated in <3 cases are provided for individual cases (columns) in the heat map below, along with the mutation pattern of genes found mutated in ≥ 3 cases (rows). Color codes at the bottom denote the type of mutation. The bar plots on the right give the percentage and absolute number of cases showing the feature displayed in the corresponding row across all samples.

leukemia.[14,38] E571-XPO1 mutations have been also detected in whole biopsies of cHL cases, although formal proof of their actual presence in HRS cells was missing.[39] XPO1 (alias CRM1) shuttles outside the nucleus over 200 cargo proteins harboring a NES, including known tumor suppressors involved in cHL lymphomagenesis and functioning in the nucleus (eg, TP53 and NFKBIA).[2,36,40-43] *XPO1* is overexpressed in various cancers, and inhibition of its activity appears more toxic to tumor cells than normal cells.[42-44] We thus tested the antitumor effect of selinexor, a clinical compound that inhibits XPO1 function by covalently binding to the C528 residue in its NES-binding groove,[45] in 6 cHL cell lines with wild-type or mutated XPO1 alleles. Interestingly, selinexor reduced growth and caused apoptosis in 2 cHL cell lines carrying the E571K mutation (SUPHD1 and UHO1), but not in 4 cell lines

that lacked this mutation (L428, L540, HDLM2) or had a low mutant allele burden (L1236) (Figure 7B; supplemental Figure 9).

Other recurrently mutated genes

Genes encoding inhibitors of the NF-κB pathway (in particular *NFKBIE*, *NFKBIA*, and *TNFAIP3*) are known to be recurrently targeted by inactivating mutations and/or deletions in cHL.[6,15,46-50] Accordingly, disruptive somatic mutations of the NF-κB pathway inhibitor *NFKBIE* (encoding IκBε) were identified in 5/34 (15%) cases (supplemental Figure 10; supplemental Tables 3 and 5). Although we observed monoallelic deletions of *TNFAIP3* in 12/33 (36%) evaluable cases by FISH, no mutations were detected in this gene by WES, and targeted deeper sequencing (performed on 28/34 cases) only identified a non-sense mutation in 1 case
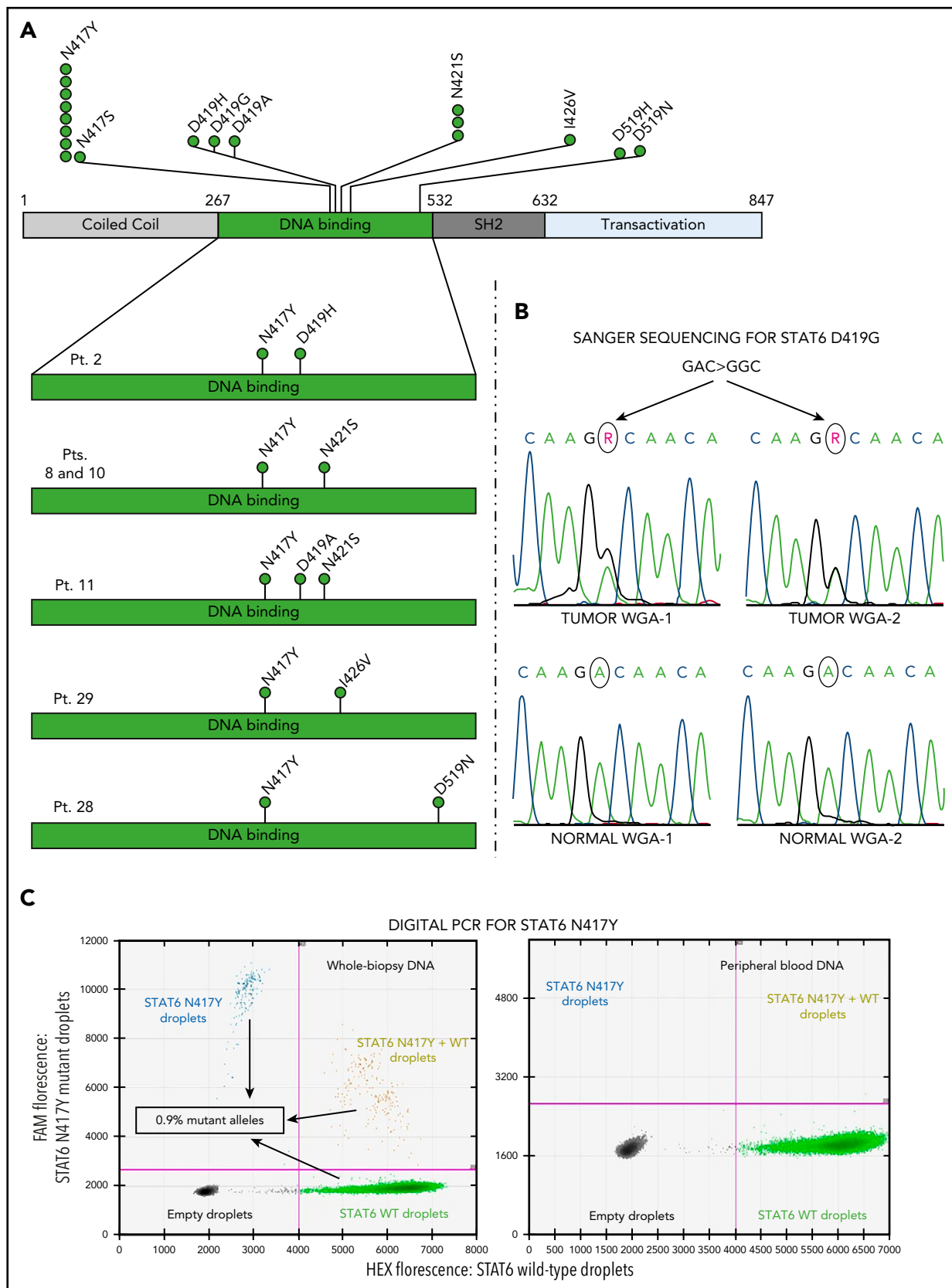
Figure 2. STAT6 mutations in cHL. (A) Secondary structure of the STAT6 protein, with the mutations found in cHL cases (green circles) clustering in the DNA binding domain. The latter is enlarged below to depict individual STAT6 alleles from 6 patients each carrying multiple monoallelic STAT6 mutations. (B) Sanger-sequencing validation in 1 representative case confirms the presence of a heterozygous *STAT6* mutation (D419G) in both tumor WGA-DNA duplicates, but not in matched normal WGA-DNA. (C) Digital polymerase chain reaction analysis allows the backtracking of a somatic N417Y mutation in the unamplified tumor DNA of case 14 extracted from whole-tissue sections of a
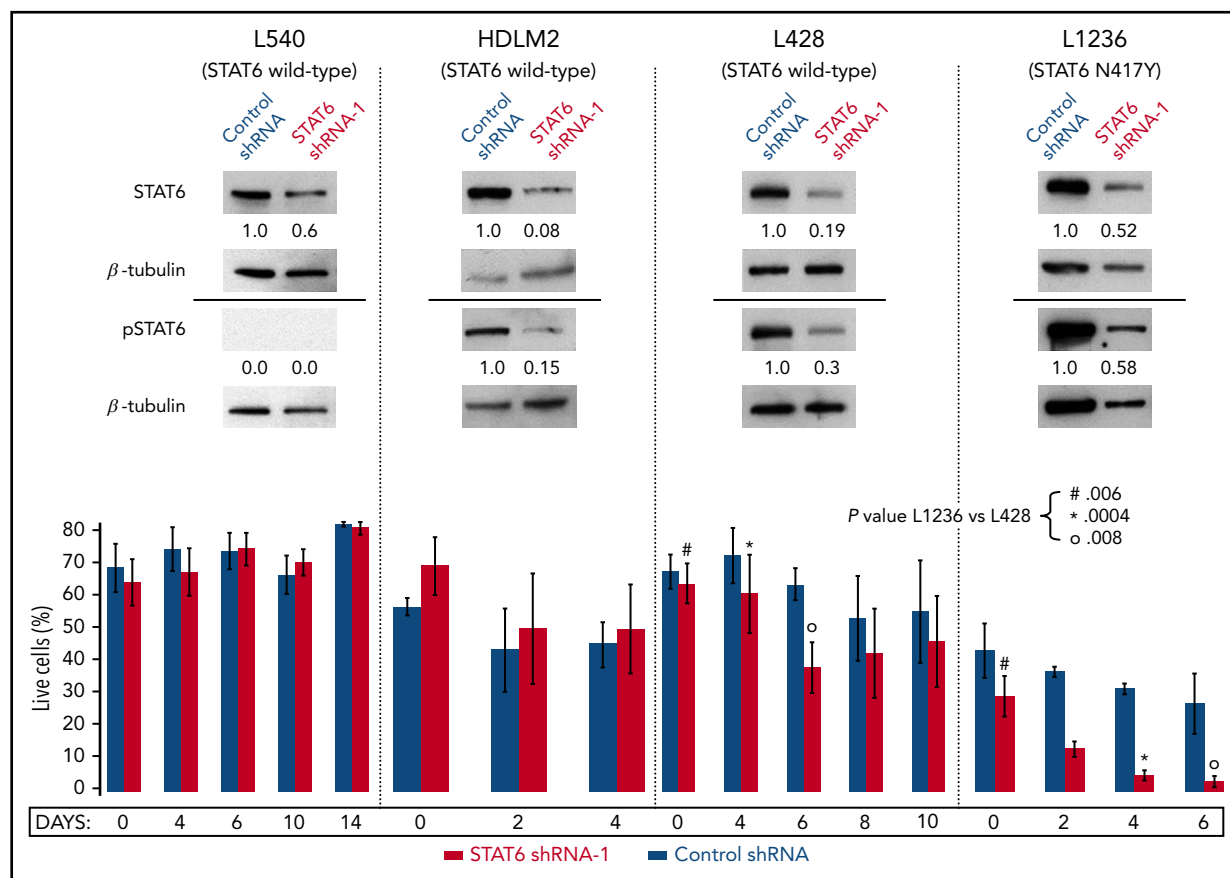
Figure 3. Downregulation of STAT6 triggers apoptosis of *STAT6*-mutated cHL tumor cells. (Top) ShRNA-mediated downregulation of total and phophoSTAT6 by western blotting in 4 cHL cell lines (1 representative experiment per cell line is shown, of 2-4 independently performed, that gave reproducible results); β-tubulin is used as a loading control, and the normalized levels of total and phosphoSTAT6, quantified by densitometry, are shown below the respective blots (solid lines denote distinct gels that were loaded with the same protein lysates and blotted separately). (Bottom) Raw percentage of live cells (AnnexinV-negative by flow cytometry) over time, after transduction of STAT6 shRNA-1 and control shRNA (day 0 = 96 hours after transduction and 48 hours after puromycin selection). Error bars (standard error of the mean) refer to at least 2 or 3 independent experiments per cell line per time point. *P* values refer to unpaired, 2-tailed *t* test. Supplemental Figure 14 shows the same data after normalizing the percentage of live cells in the shRNA-1 sample to the corresponding nontargeting shRNA control sample (set at 100%).

(validated by Sanger sequencing; supplemental Table 5). The low frequency of *TNFAIP3* mutations in our series does not seem to be explained by insufficient coverage depth, as the mean percentage of *TNFAIP3* coding nucleotides with ≥20× coverage in the targeted deep-sequencing data of the tumor samples was 87% (interquartile range, 83%-94%). The *NFKBIA* gene, encoding another NF-κB pathway inhibitor (ie, IκBα) less frequently mutated (17% of cHL cases[48,51-53]), was also not affected in our series, both on WES and on targeted deeper sequencing (average percentage of coding nucleotides with ≥20× coverage in tumor samples, 76%; interquartile range, 63%-95%). Overall, genetic lesions in negative regulators of the NF-κB pathway (*NFKBIE*, *TNFAIP3*, *TNIP1*; Figure 1, supplemental Tables 3 and 5) occurred in 53% of cHL cases (18/34), confirming their important role in the pathogenesis of this disease.

In agreement with a previous study,[6] somatic mutations of *B2M*, encoding a protein indispensable for major histocompatibility complex class I expression on the cell surface, were observed in 9/34 (26%) cases, including 7 with disruptive variants (non-sense, frame-shifting, splice site or start-codon loss) and 2 with missense

variants (Figure 1; supplemental Tables 3 and 5). Monoallelic deletion of *B2M* was observed in 2/29 evaluable cases, 1 of which (no. 2) also carried a splice site mutation of the other allele. One additional case (no. 29) had a heterozygous splice site inactivation of *NLRC5* (supplemental Table 3), which encodes for the master major histocompatibility complex class I transactivator CITA and has been shown to be a target of immune evasion in solid cancers.[54] In cHL, another genetic mechanism of immune escape is represented by relative copy number gain or amplification of 9p24,[55] which we observed by FISH in 11/33 (33%) evaluable cases (Figure 1). The gained/amplified region includes, in addition to *JAK2*, the *PDL1* and *PDL2* genes encoding surface ligands that inhibit activation of T cells expressing the PD1 receptor. Overall, genetic lesions fostering immune evasion (mutations/deletions of *B2M* or *NLRC5*; copy number gain/amplification of 9p24) were observed in 53% (18/34) of cases (Figure 1; supplemental Table 3), confirming their important contribution to cHL lymphomagenesis.

Notably, somatic mutations of the chromatin-modifying genes *KMT2D* (alias *MLL2*), *CREBBP*, *EP300*, and *EZH2*, which are frequent

Figure 2 (continued) lymph node biopsy (left), but not in the DNA of a peripheral blood sample analyzed as a negative control (right); the same mutation had been originally identified by WES in the WGA-DNA of microdissected tumor vs normal cells. The low variant allele frequency (0.9%) reflects the known paucity of cHL tumor cells in the involved tissues (see supplemental Table 6 for the full results of digital polymerase chain reaction validation).
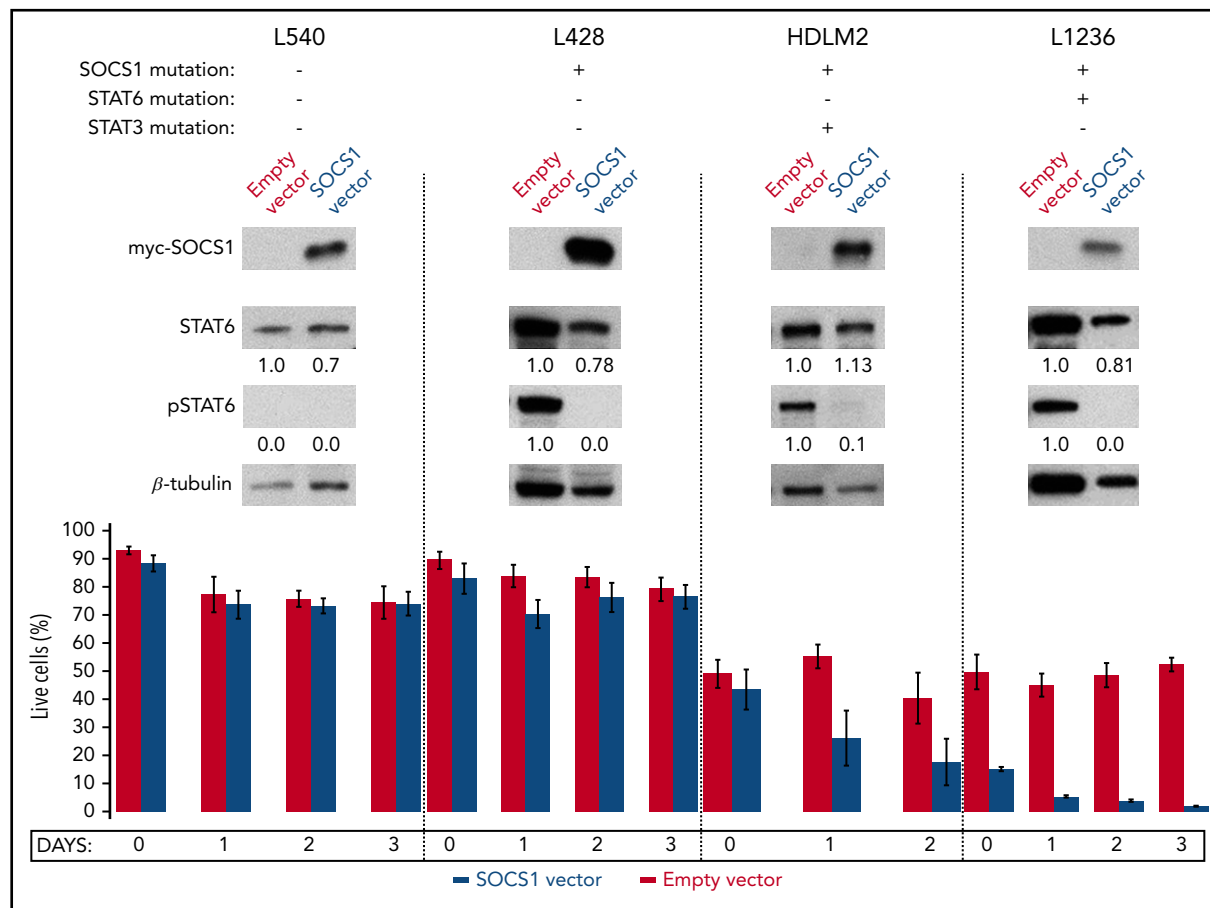
Figure 4. Reconstitution of wild-type SOCS1 causes cell death in cHL lines carrying concurrent mutations of *SOCS1* and *STAT* genes. (Top) Western blot analysis of phosphoSTAT6 after lentiviral transduction of myc-tagged SOCS1 in 4 cHL cell lines (1 representative experiment per cell line is shown, of 2-4 independently performed, that gave reproducible results); β-tubulin is used as loading control, and the normalized levels of total and phosphoSTAT6, quantified by densitometry, are shown below the respective blots. (Bottom) Raw percentage of live cells (based on forward/side scatter parameters by flow cytometry) over time (day 0 = 48 hours after SOCS1 transduction). Error bars (standard error of the mean) refer to at least 3 independent experiments per cell line per time point (except HDLM2/d 1, n = 2 replicates). The induction of cell death on SOCS1 transduction in HDLM2 and L1236 is statistically significant (P values < .05 for each cell line based on 2-way ANOVA). Supplemental Figure 14 shows the same data after normalizing the percentage of live cells in the SOCS1-vector sample to the corresponding empty-vector control sample (set at 100%).

in diffuse large and follicular B-cell lymphoma,[30,56,57] were only occasionally observed in cHL (supplemental Table 3) and included a heterozygous hotspot mutation R1446H affecting the CREBBP acetyl-transferase domain in 1 case (no. 37).

Finally, the tumor suppressor TP53 harbored somatic missense mutations of its DNA binding domain in 3/34 (9%) cases (Figure 1 and supplemental Table 3).[58]

EBV-associated variation in somatic mutation burden

Despite representing a very small subset in our panel, the 4 cHL cases with latent Epstein-Barr virus (EBV) infection of the tumor cells[3] showed a strikingly lower number of somatic mutations (nonsilent, 0-33; silent/noncoding, 0-31) compared with the 30 EBV⁻ cases (median, 50 and 60, respectively; P value < .01) (supplemental Figure 11A). In particular, 3 EBV⁺ cases, all of the mixed cellularity histological subtype (MC; Figure 1), had no (patients 32 and 33) or only 3 (patient 35) somatic mutations in total (nonsilent and silent/noncoding). This finding was not a result of technical differences in the exome sequencing coverage between the 30 EBV⁺ cases and the 4 EBV⁻ cases, as coverage metrics

for these 2 groups were comparable (supplemental Table 2). We confirmed the association between low somatic mutation burden and EBV infection of the tumor cells when we restricted the analysis to the MC histological subtype, as the 6 EBV⁻ MC cases harbored 48 to 144 mutations (median, 111) whereas the 3 EBV⁺ MC cases showed 0 to 3 mutations (P value = .024; supplemental Figure 11B). Conversely, no significant difference was observed when comparing the 6 EBV⁻ MC cases with the 20 EBV⁻ cases of the most frequent histologic subtype (nodular sclerosis; median of total somatic mutations, 107) (supplemental Figure 11B), suggesting that histology is unlikely to be correlated with mutation burden in cHL. Similarly, the 4 EBV⁻ cases previously exposed to chemotherapy had a comparable number of total somatic mutations (79-215) as the 26 EBV⁻ cases studied before chemotherapy (median, 142; supplemental Figure 11C).

Although the number of EBV⁺ cases we studied is small (n = 4) and more cases should be analyzed to confirm these findings, these data may suggest a link between latent EBV infection and low somatic mutation burden in HRS cells. Because EBV is known to provide critical signals that promote early cHL lymphomagenesis
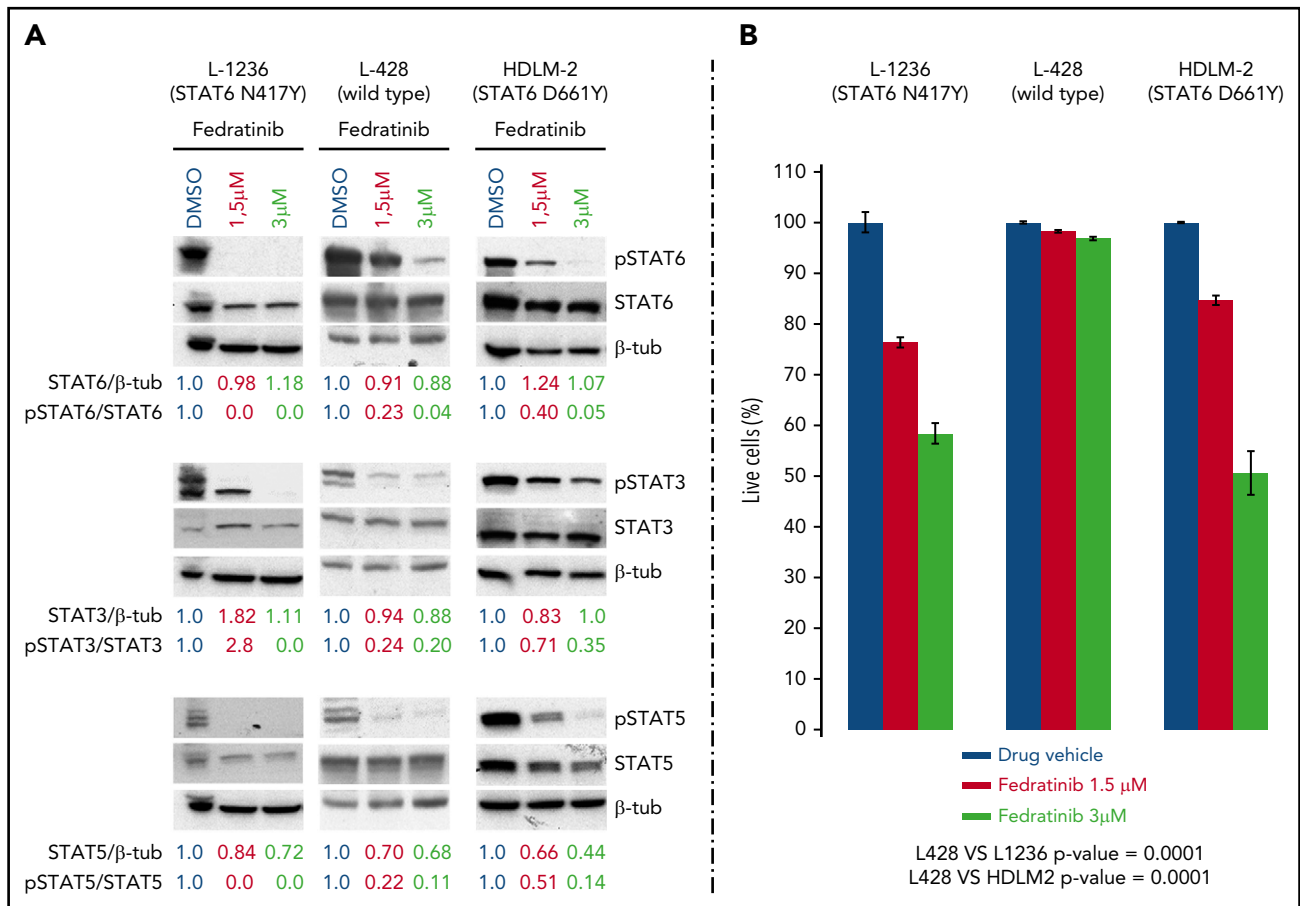
Figure 5. JAK2 inhibition leads to STATs dephosphorylation and apoptosis in cHL tumor cells carrying *STAT6* or *STAT3* mutations. (A) Western blot analysis of STAT6, STAT3, and STAT5 phosphorylation in L1236, L428, and HDLM2 cells analyzed 24 hours after treatment with the JAK2 inhibitor fedratinib (at the indicated concentrations) or with vehicle (dimethyl sulfoxide) as control (1 representative experiment per cell line is shown, of 3 independently performed, that gave reproducible results). Normalized levels of total STATs relative to β-tubulin, and of phospho-STATs relative to the corresponding total STATs, were quantified by densitometry and are shown below the respective blots. (B) Percentage of live cells (AnnexinV-negative by flow cytometry) in the same cell lines, measured 48 hours after treatment with fedratinib, relative to the vehicle set at 100%. Error bars (standard error of the mean) refer to 4 independent experiments, each done in duplicate; *P* values were computed by 2-way ANOVA.

by rescuing pre-apoptotic GC B cells,[3] it is conceivable that its expression may relieve the pressure toward selection for exome-wide mutagenic mechanisms.

Discussion

Here we provide a comprehensive analysis of the cHL coding genome based on a relatively large number of primary cases (n = 34), uncovering frequent mutations in several genes that are likely to have relevant pathogenetic functions and could be amenable to targeted therapeutic strategies. Key in our effort was the successful overcoming of 2 technical challenges, that is, the purification of the rare HRS cell from tissue samples through microdissection and an efficient bioinformatics control of the bias introduced by the WGA reaction, when applied to a few cells, through independent sequencing of duplicate WGA reactions. Although this bias was successfully overcome with regard to the correct calling of point mutations and short indels, it did complicate a reliable exome-wide calling of somatic copy number alterations (supplemental Materials and Methods), which represents a limitation of our work.

A main finding of our study is the almost ubiquitous (~90% of cases) genetic targeting of a variety of JAK-STAT pathway members, which goes beyond previous estimates based on the presence of copy number gains of *JAK2* and the mutational disruption of the *SOCS1* and *PTPN1* inhibitors[2,3,10] and includes activating mutations of *JAK1* and of multiple STAT transcription factors (STAT3; STAT5B), together with highly recurrent mutations of STAT6 (32% of cases). Although necessarily limited to cell lines, the observation that genetic and pharmacologic inhibition of STAT6 elicited significant apoptosis in *STAT6*-mutated but not in *STAT6* wild-type HRS cells, even if expressing phosphorylated (ie, activated) STAT6, suggest that *STAT6* variants may promote the growth of the cHL clone in a manner distinct from STAT6 phosphorylation. Because mutations clustered in the DNA binding domain, a possible mechanism by which STAT6 mutants could confer survival advantage to HRS cells might imply an aberrant target gene recognition and transactivation after the phosphorylation-dependent nuclear translocation of mutant STAT6, a hypothesis that will need to be tested in future research. Although the biochemical consequences of STAT6 mutations toward DNA binding and target gene transcription are controversial,[12,13] the fact that both pharmacological blockade of phosphorylation and genetic silencing of STAT6 induced marked apoptosis of L1236 cHL cells,
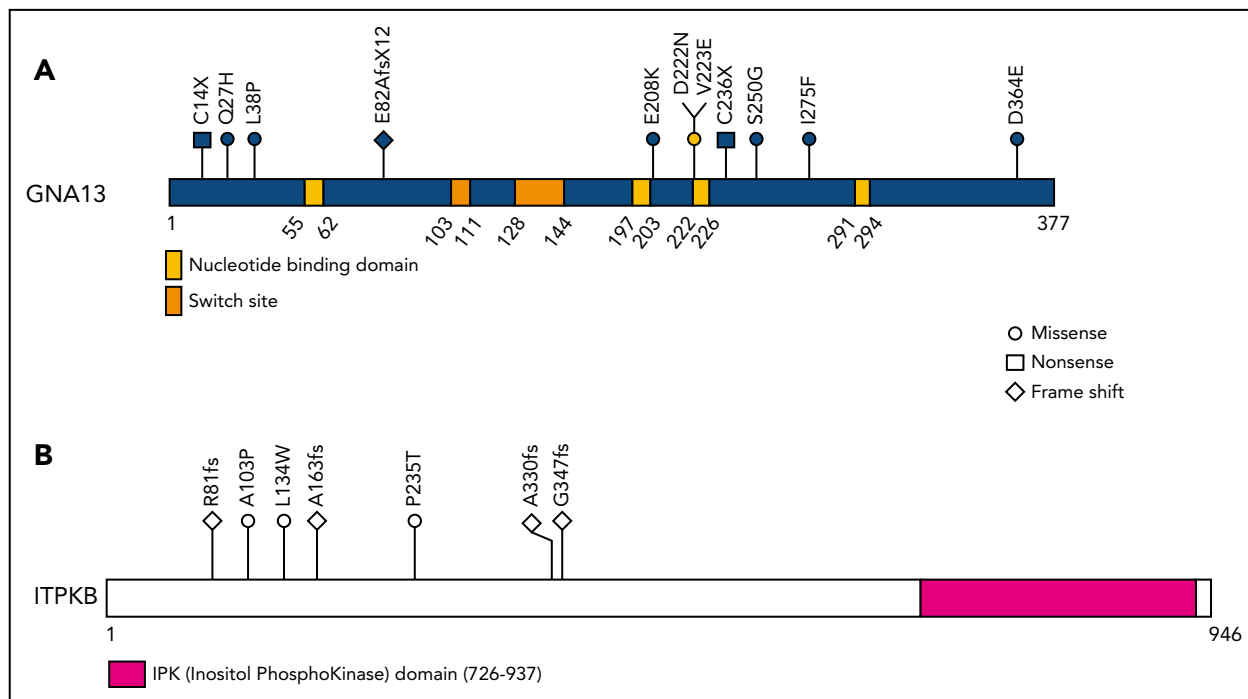
Figure 6. GNA13 and ITPKB mutations in cHL. (A) Schematic representation of the GNA13 protein and its functional domains, with the 11 mutations found in 8 cHL cases depicted above. (B) Schematic representation of the ITPKB protein and its functional domains, with the 7 mutations found in 4 cHL cases depicted above.

which only express the mutant N417Y allele, is consistent with a gain of function of this mutant toward tumor clone growth downstream of JAK-STAT pathway activation.

Interestingly, *STAT6* mutations and activating mutations of *STAT3* and *STAT5B* were enriched in cHL cases harboring disruptive mutations of the *SOCS1* inhibitor. The observation that exogenous SOCS1 restoration, as well as selective JAK2 inhibition downstream of SOCS1, proved selectively toxic to *SOCS1*-disrupted HRS cells that concomitantly carried a mutant *STAT* family member suggests functional dependence between the activities of these mutant alleles in sustaining tumor growth.

The pervasive targeting of JAK-STAT signaling genes in cHL makes clinically available JAK or STAT inhibitors[59,60] an attractive therapeutic approach in this disease in the context of a comprehensive targeted genotyping of patients.

The second most commonly mutated gene was *GNA13* (24% of cases). The G-protein subunit encoded by *GNA13* transduces critical signals that constrain GC B cells within secondary lymphoid follicles but also favor their apoptosis as a balance to the risk for genomic instability posed by the high rate of cell proliferation and DNA damage physiologically occurring during the germinal center reaction to ensure proper affinity maturation and class switch recombination of immunoglobulin genes.[25-27,61] Because the *GNA13* mutation pattern resembles that observed in other GC B-cell-derived lymphomas and is consistent with loss of function,[25,27,29-32] *GNA13* is likely to play a tumor suppressor function in cHL. *GNA13* is also recurrently mutated in primary mediastinal B-cell lymphoma,[14] which shares several clinicopathological, molecular, and genetic features with cHL.[9,62] Conversely, *GNA13* mutations are rare in the ABC-type of diffuse large B-cell lymphoma,[25,27,29-32] suggesting a specific pathogenetic role

in GC B-cell-derived aggressive lymphomas. In the context of cHL, inactivating *GNA13* mutations may facilitate lymphomagenesis by rescuing crippled GC B cells (the proposed HRS cell precursors[2]) from apoptosis and by promoting their dissemination outside the lymphoid follicles, where HRS cells are indeed observed on analysis of lymph nodes with early, partial involvement by cHL.[63]

We also identified missense mutations of the XPO1 nucleo-cytoplasmic shuttling protein recurring in 18% of cases at the hotspot residue E571. Intriguingly, exposure of cHL cell lines to a low dose of the specific, clinically available XPO1 inhibitor selinexor reduced growth and caused apoptosis in a manner correlated with the *XPO1* mutant allele burden. This finding points to XPO1-E571 mutations as potential gain-of-function events on which HRS cells may depend for survival. Although the functional consequences of E571 mutations in cHL need to be mechanistically worked out, our data suggest a vulnerability that could be exploited therapeutically, considering the clinical feasibility of XPO1 inhibition[64,65] and the likely larger therapeutic window afforded in cHL by the greater sensitivity to selinexor shown by *XPO1*-mutated HRS cells.

Finally, *ITPKB* was disrupted in 16% of cHL cases by truncating mutations that eliminate its protein kinase domain and, as a consequence, are predicted to blunt its antagonistic activity toward the AKT kinase.[33] Although the function of ITPKB and the pathways that may be modulated by its activity have not been studied in normal GC B cells, *ITPKB* inactivation may provide a genetic basis to the aberrant PI3K-AKT signaling activity that is known to support HRS cells viability and that is, again, potentially susceptible of pharmacologic inhibition.[34,35]

We did not observe any grossly evident enrichment of specific mutated genes in samples analyzed at relapse vs initial diagnosis,
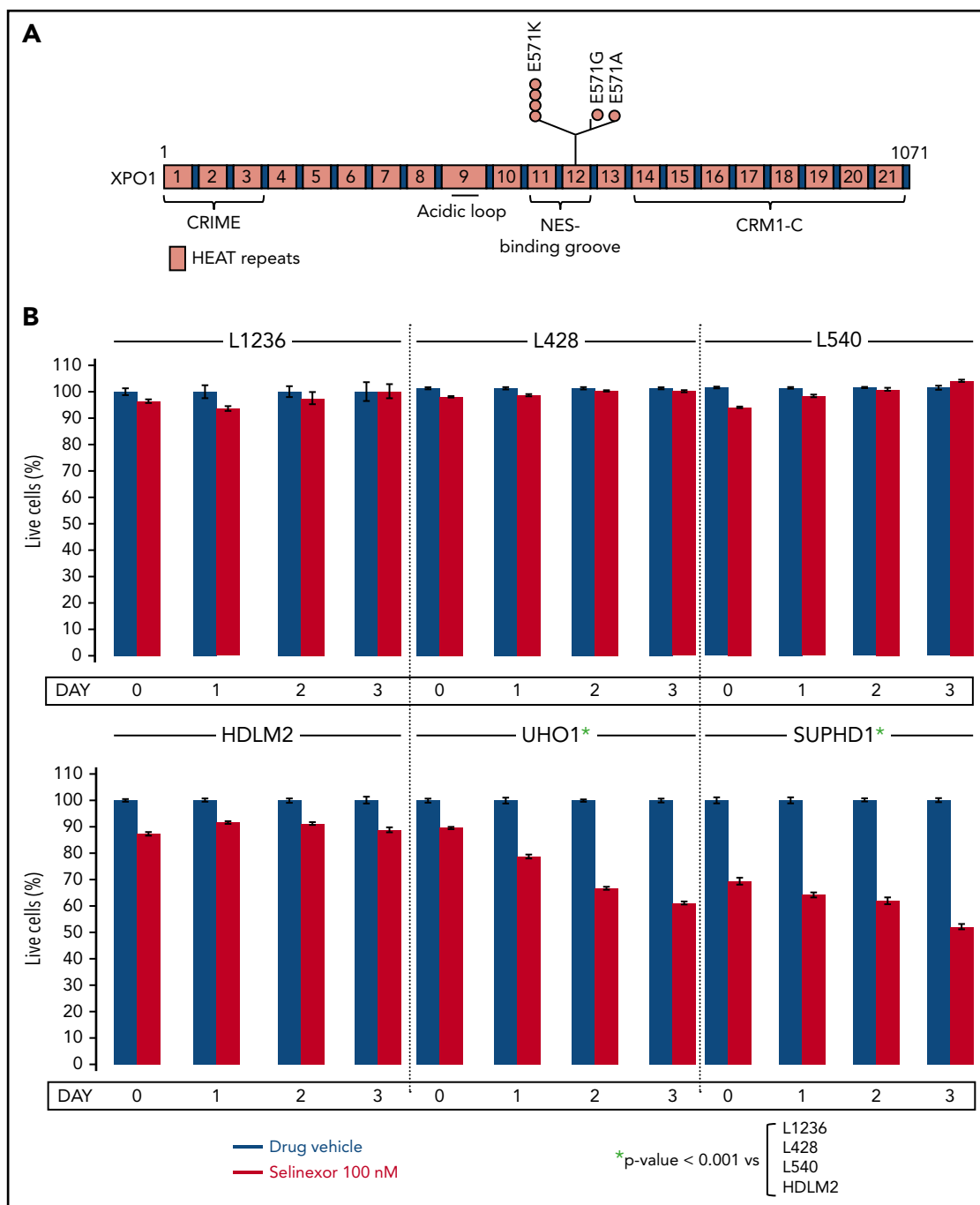
Figure 7. XPO1 mutations in cHL. (A) Schematic representation of the XPO1 protein and its functional domains, with the mutations found in cHL cases shown above. (B) Percentage of nonapoptotic cells (AnnexinV-negative by flow cytometry) assessed in 6 cHL cell lines at the indicated times after exposure to a low dose (100 nM) of the XPO1 inhibitor selinexor, or drug vehicle (dimethyl sulfoxide) as negative control (set at 100%). Day 0 corresponds to 48 hours of culture with selinexor or vehicle. Error bars refer to 3 independent experiments, each performed in triplicate; P values were calculated by 2-way ANOVA.

with the limitation of the small number of relapsed cases analyzed (n = 6). Similarly, although certain genes (eg, *B2M*, *GNA13*, *STAT6*, *SOCS1*) appear more frequently mutated in nodular sclerosis than mixed cellularity cHL tumors, this was not statistically significant. However, the small number of MC cases in our series (n = 9) prevents a robust comparison, and larger studies focused on relapsed and MC cases will need to be performed to reliably determine whether specific genes are preferentially mutated/unmutated in these groups.

In summary, our analysis of the cHL coding genome uncovered recurrent mutations in several genes, most notably *GNA13*, *XPO1*, *ITPKB*, *STAT6*, and multiple other members of the JAK-STAT signaling pathway, pointing to a critical role for these genes in the pathogenesis of cHL. The high frequency of mutations in JAK-STAT pathway genes provides a genetic explanation for the constitutive activation of this cascade in almost 90% of cases. Although additional studies will be needed to mechanistically document the dependency of the mutated cHL cell lines on the

affected genes, the observed association between the presence of specific gene mutations and the susceptibility to targeted pathway inhibition suggests new therapeutic targets in this common lymphoma. Finally, the landscape of recurrently mutated genes we have defined in cHL can be harnessed to develop liquid biopsy strategies for noninvasive monitoring of the response to therapy.[66] This could improve the imperfect prognostic accuracy of PET imaging in guiding treatment escalation or de-escalation,[67] and thereby reduce the overall toxicity burden associated to chemo-radiotherapy.

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Authorship

Contribution: E.T. conceived the study, designed research, analyzed data, and drafted the paper; E.L., G.S., A.P., and E.F. performed most of the research, analyzed the data, and edited the paper; V.P., Y.W., A.R., A.V., S.V., R.P., S.P., A.T., A.P., B.B., A.S., B.C., and C.M. performed research and analyzed data; A.M.G., S.V., A.C., and S.A. contribute vital samples; L.P. designed research, analyzed data, and edited the paper; R.R. contributed vital analytical tools, designed research, analyzed data, and edited the paper; and B.F. contributed vital samples, designed research, analyzed data, and edited the paper.

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Footnotes

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