Suppressors and activators of JAK-STAT signaling at diagnosis and relapse of acute lymphoblastic leukemia in Down syndrome

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Children with Down syndrome (DS) are prone to development of high-risk B-cell precursor ALL (DS-ALL), which differs genetically from most sporadic pediatric ALLs. Increased expression of cytokine receptor-like factor 2 (CRLF2), the receptor to thymic stromal lymphopoietin (TSLP), characterizes about half of DS-ALLs and also a subgroup of sporadic “Philadelphia-like” ALLs. To understand the pathogenesis of relapsed DS-ALL, we performed integrative genomic analysis of 25 matched diagnosis-resmission and -relapse DS-ALLs. We found that the CRLF2 rearrangements are early events during DS-ALL evolution and generally stable between diagnoses and relapse. Secondary activating signaling events in the JAK-STAT/RAS pathway were ubiquitous but highly redundant between diagnosis and relapse, suggesting that signaling is essential but that no specific mutations are “relapse driving.” We further found that activated JAK2 may be naturally suppressed in 25% of CRLF2pos DS-ALLs by loss-of-function aberrations in USP9X, a deubiquitinase previously shown to stabilize the activated phosphorylated JAK2. In terrore of large ALL genomic databases extended our findings to up to 25% of CRLF2pos Philadelphia-like ALLs. Pharmacological or genetic inhibition of USP9X, as well as treatment with low-dose ruxolitinib, enhanced the survival of pre-B ALL cells overexpressing mutated JAK2. Thus, somehow counterintuitive, we found that suppression of JAK-STAT “hypersignaling” may be beneficial to leukemic B-cell precursors. This finding and the reduction of JAK mutated clones at relapse suggest that the therapeutic effect of JAK specific inhibitors may be limited. Rather, combined signaling inhibitors or direct targeting of the TSLP receptor may be a useful therapeutic strategy for DS-ALL.

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Children with Down syndrome (DS) are at a markedly increased risk for B-cell precursor acute lymphoblastic leukemia (BCP-ALL) (1). The poor survival of DS-ALL compared with ALL in children without DS (“sporadic” ALL) is related to increased treatment toxicity and to increased incidence of relapse (2). Thus, better therapy is needed for these patients.

Previous studies by our group and others revealed differences between the genetics of DS-ALLs and of sporadic ALLs (3–6). The typical cytogenetic subgroups, ETV6-RUNX1 and hyperdiploid ALLs, are less common in DS-ALLs. Acquired somatic activation of the thymic stromal lymphopoietin (TSLP) pathway are present at diagnosis in about half of DS-ALLs. Aberrant expression of cytokine receptor-like factor 2 (CRLF2) in these leukemias is caused by chromosomal rearrangements consisting either of a microdeletion on chromosome X, juxtaposing the promoter of P2RY8 with the coding region of CRLF2, or by a translocation of CRLF2 into the Ig heavy chain (Igh) locus. CRLF2 heterodimerizes with IL7 receptor-a (IL7R) to form the receptor to TSLP (reviewed in ref. 7). TSLP receptors signal by activation of the JAK-STAT pathway. Interestingly, in the majority of DS-ALLs, JAK-STAT signaling may be beneficial to leukemic B-cell precursors. This finding and the reduction of JAK mutated clones at relapse suggest that the therapeutic effect of JAK specific inhibitors may be limited. Rather, combined signaling inhibitors or direct targeting of the TSLP receptor may be a useful therapeutic strategy for DS-ALL.

Significance

Children with Down syndrome are at increased risk for B-cell acute lymphoblastic leukemia (DS-ALL), often expressing cytokine receptor-like factor 2 (CRLF2). Here we studied matched diagnosis and relapse DS-ALLs to understand the pathogenesis of relapse. We confirm that enhanced JAK-STAT signaling frequently drives CRLF2pos DS-ALL at diagnosis, but discovered that clones with JAK mutations are unstable, suggesting that they also endowed the transformed cells with vulnerabilities. We find USP9X loss in up to 25% of CRLF2pos ALLs, and demonstrate that its ablation decreases the toxic effect of JAK2 hypersignaling. Thus, in CRLF2pos ALLs JAK-STAT signaling is often buffered by loss of USP9X. These results have therapeutic implications because they suggest that ALL cells can tolerate a limited range of JAK-STAT signaling.


Reviewers: S.D., Pennsylvania State University; and V.S., Veterinary University of Vienna. The authors declare no conflict of interest.

Data deposition: Genomic data included in this study were deposited in the European Genome-phenome Archive (accession no. EGAS00001002410).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1702489114/-/DCSupplemental.

PNAS | Published online May 1, 2017
E0430–E0439
www.pnas.org/cgi/doi/10.1073/pnas.1702489114

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additional activating mutations in this pathway are found. These additional mutations include either activating mutations of the receptors, CRLF2 or IL7R (3, 8), or in the downstream signaling components, most commonly JAK2 (9), but also JAK1 and even RAS (10). Together, these findings suggest that the CRLF2-JAK-STAT pathway is a major “driver” of DS-ALL.

The discovery of the TSLP pathway activation in DS-ALLs proved to be of general significance to sporadic ALLs in children and adults without DS. Indeed, about two-thirds of kinase-driven ALLs, commonly called “Philadelphia like” (Ph-like) ALLs, demonstrate activation of this pathway similarly to DS-ALL (11, 12). The prognosis of these leukemias is worse (11–19) and a clinical trial incorporating ruxolitinib into the chemotherapy backbone for newly diagnosed patients has been recently opened (NCT02723994).

However, two recent studies from the same laboratory questioned the role of CRLF2 in driving therapeutic resistance and relapse of ALL (20, 21). These authors reported that in about a third of the patients, the major CRLF2-positive (CRLF2^pos) clone at diagnosis is lost at relapse. Moreover, in many patients CRLF2 aberrations were subclonal. If independently confirmed, it would suggest that CRLF2 (and the downstream signaling activating mutations) are not important for relapse.

The goal of the research presented herein was to decipher the pathogenesis of relapse of DS-ALL by integrative genomic analysis of matched leukemia samples from diagnosis and relapse. We envisioned that such analysis could lead to development of targeted relapse-preventing approaches in both patients with DS and in sporadic CRLF2^pos Ph-like ALLs. We report the surprising finding of an intricate balance between lesions promoting and blocking JAK-STAT signaling in these leukemias. These findings have potential implications for targeted therapy for prevention of relapse in Ph-like JAK-STAT mutated BCP-ALLs.

Results

To further understand the genetic make-up of DS-ALL, we performed whole-exome sequencing (WES) of diagnosis (DX) and matched remission bone-marrow samples of 31 patients with DS-ALL selected for the absence of the typical cytogenetic abnormalities of childhood ALL (with, in retrospect, the exception of one patient DSALLB4, who was positive for ETV6-RUNX1) (Dataset S1). We further sequenced matched relapse samples (R1) from 25 of these patients including consecutive relapses (R2) in five patients. WES was complemented by barcoded (22) deep-targeted sequencing and, for selected samples, SNP array, and paired-end RNA-seq for confirmation of copy number changes and detection of chimeric transcripts, respectively (Dataset S2). All patients were enrolled on therapeutic Berlin-Frankfurt-Münster–Associazione Italiana Ematologia Oncologia Pediatrica (BFM-AIEOP) protocols with informed consent and appropriate approvals of ethical committees. None of the patients was under certain circumstances, for example after treatment with chemotherapy. We thus selected likely pathogenic somatic events to either diagnosis-restricted, relapse-restricted, or shared panel). There were four patients with the IGH–CRLF2 translocation and 13 patients with P2RY8–CRLF2 rearrangement. We could determine the allelic frequencies of the P2RY8–CRLF2 rearrangements for most the samples (Fig. S2). Both IGH–CRLF2 and P2RY8–CRLF2 were stable between diagnosis and relapse in 11 of the 12 paired patients carrying these lesions (Fig. 1), raising the possibility that in DS-ALLs CRLF2 aberration represents an early event during leukemia development and may also be important for relapse.

We identified lesions affecting known driver genes in most of our samples (Fig. 1). We grouped recurrently mutated genes or putative driver genes by functional classification. In 46 (75%) samples from 25 (80%) patients, we detected activating mutations in genes whose protein products are involved in signaling, including receptors (CRLF2, IL7R, FLT3) or downstream effector enzymes (JAK1/2, KRAS, and NRAS). Among the other affected pathways that were frequently mutated are genes involved in B-lymphoid development and differentiation, transcription factors and chromatin remodeling proteins, general tumor-suppressor genes, and members of the mismatch repair (MMR) pathway. As expected, in all but one sample with mutations in JAK-STAT genes (CRLF2, IL7R, and JAK1,2), CRLF2 was rearranged. Interestingly, the CRLF2^pos DS-ALLs displayed a similar pattern of mutations that was reported for hyperdiploid ALLs (26), namely association between mutations in RAS signaling pathway and chromatin remodeling genes, such as CREBBPs (Fig. 1).

In nine patients, we observed deletions targeting the histone gene cluster-1 (HIST1) on chromosome 6p22-21 (Fig. S3). These deletions were generally stable between diagnosis and relapse (Fig. 24), and were not associated with CRLF2 status. This finding is consistent with previous genomic studies in DS-ALL (27, 28), and to our knowledge, has not been reported in other cancers, including leukemias.

In 16 leukemia samples for which RNA material was available, we performed paired-end RNA-seq, aiming to identify chimeric transcripts of putative drivers (Fig. 1 and Dataset S2). In patient DSALLB3, both in diagnosis and in relapse samples, we observed overexpression of the noncoding RNA MIR100HG, as well as chimeric transcripts mapped to IGH and MIR100HG (Fig. S4). RNA-seq data also demonstrated that the aberrant transcription started upstream to the locus of MIR125B1 (Fig. S4), a known oncogenic microRNA (29).

Temporal Association of Somatic Mutations Between Diagnosis and Relapse

A series of driver events are probably necessary for a cell to become leukemic. However, having acquired this set of lesions might not be sufficient for (or even impair the ability of) the leukemic blasts to propagate relapse after chemotherapy treatment. For example, a driver mutation that is found in both time points could be ubiquitously necessary for proliferation or survival of the leukemia cells. However, it is also possible that this mutation might be important at diagnosis, but redundant in the relapse, and its persistence during relapse merely represents the common cell-of-origin. Conversely, time-point–restricted events might allude to alterations that confer selective advantage only under certain circumstances, for example after treatment with chemotherapy. We thus selected likely pathogenic somatic events that were recurrent in at least two paired patients and classified them to either diagnosis-restricted, relapse-restricted, or shared between time points (Fig. 24).
In agreement with a recent report (30), we found relapse-restricted mutations in MSH6 (n = 4), MLH1 (n = 2), and TP53 (n = 3). In addition, four of five leukemia samples with mutations in CREBBP (all CRLF2neg), and four of six samples with SETD2 mutations were relapses (Fig. 2B). MSH6 and MLH1 are both members of the MMR pathway and three of the four samples with MSH6 or MLH1 mutations, demonstrated a markedly increased mutational burden (≥170 SNVs) (Fig. 2C).

The single case (DSALLG15_R1) with MSH6 mutation (p. T915A) and no increase in the mutational burden carried an amino acid alteration predicted as benign by PolyPhen2 (31). Interestingly, the two cases with MLH1 mutation presented an identical alteration, a splicing mutation (X264_splice; rs267607789) observed in patients with Lynch syndrome (32). In addition, the second relapse of patient DSALLG11 displayed an exceptionally high number of SNVs (1225), possibly because of a synergistic effect of mutations both in MSH6 and SETD2 (33). Although recent reports described enrichment of NT5C2 mutations in up to 19% of relapsed ALLs (34, 35), none were detected in our cohort.

Aside from the CRLF2 rearrangements, we found alterations that were predominantly shared between diagnosis and relapse in IKZF1 (12 of 14), HIST1 (7 of 9), ETV6 (7 of 10), RB1 (5 of 5), USP9X (3 of 3), and EBF1 (2 of 2). Contrary to our expectations, JAK mutations, highly enriched in DS-ALL, were often lost at relapse: four of seven of JAK2 mutations and two of four JAK1 mutations were restricted to diagnosis (Fig. 2). This finding

Fig. 1. General distribution of somatic events in 31 patients with DS-ALL. The main panel lists recurrent SNV/indels and copy number variants (CNVs, represented by arrows). The rows correspond to the genes; the columns correspond to leukemia samples. Only SNVs/indels with VAF ≥ 5% are shown. Notice paired samples (i.e., diagnosis and relapse) are adjacent. The bars to the right indicate the number of patients harboring each alteration. The bars at the bottom indicate the total number of exonic somatic SNVs/indels in each sample (log2 scale).
prompted us to examine in more depth the dynamics of ALL subclones with signaling mutations.

The Dynamics of Signaling Mutations in DS-ALL. As been observed by us and others, the majority (12 of 17, 70%) of CRLF2pos ALLs had an activating mutation in components of the JAK-STAT signaling pathway. We used the improved sensitivity (SI Materials and Methods) achieved by targeted sequencing to delineate the dynamics between diagnosis and relapse of major and rare mutated alleles of signaling genes. In the eight paired patients where mutations in JAK1/2 were present at diagnosis, the dominant clone with the mutation was either undetected after therapy or, in a single case (DSALLG8), was diminished significantly [variant allele frequency (VAF) 0.8%] (Fig. 3A). Conversely, in the three patients with JAK2 mutations at relapse, we detected the surviving clone at diagnosis as a minor clone (VAF 1–5%). An exception was patient DSALLB9, in whom JAK1 was present in the first relapse as a subclone (VAF 5.2%) and subsequently disappeared at the second relapse (Fig. 3A). IL7R and CRLF2 SNVs and insertion/deletions (indels), which activate the receptors by mediating ligand independent dimerization (3, 8, 36), are also common in CRLF2pos ALLs, and in our cohort we found two patients with the IL7R mutation (p.S185C) and two patients with the CRLF2 mutation (p.F232C) (Fig. 1). These mutations were shared between diagnosis and relapse in two patients (DSALLG11, DSALLB5), or relapse-restricted in one patient (DSALLB1). Thus, the data suggest

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**Fig. 2.** Temporal association of somatic mutations between diagnosis and relapse. (A) The bar plot reports the total number of paired patients (n = 25) with somatic variation in each of the loci listed. Variations were classified as shared regardless whether the identical or different genomic alterations in the same gene were present in both time points. (B) The genomic distribution of protein-changing mutations identified in our cohort in MSH6, MLH1, TPS3, SETD2, and CREBBP. (C) Boxplot showing the mutational burden of each sample grouped by MLH1/MSH6 mutated (n = 4) or wild-type (n = 61).
that mutations in the JAK-STAT genes are secondary events and that clones harboring these mutations are mostly unstable.

RAS mutations displayed different dynamics between diagnosis and relapse. Overall, we spotted mutations in KRAS and NRAS in 11 paired patients, clones harboring mutations in these genes were detected in 6 (24%) patients at diagnosis, in 9 (36%) patients R1 relapse, and in 4 (80%) patients at R2 relapse. Interestingly, all six paired patients with RAS mutations at diagnosis were CRLF2neg and in all but one patient, KRAS/NRAS were also mutated at relapse. Moreover, in all patients with clones harboring RAS mutations that were present at both diagnosis and relapse, the relapse emerged from the same clone that was identified in the diagnosis sample (Fig. 3A). In four CRLF2pos patients and one CRLF2neg patient, either the primary (R1) or the second relapse (R2) emerged from RAS-mutated clones that were undetectable in the preceding time point. This relative durability of clones with RAS mutation could be explained by increased resistance to chemotherapy, as has been suggested by Oshima et al. (25).

Overall, we observed mutations in components of JAK-STAT or RAS pathways in 49 (75%) sequenced exomes from 25 (80%) DS-ALL patients (including both 21 paired and 4 diagnosis-only samples). However, a heterogeneous clonal population, with respect to the mutated signaling effectors, often presented at the same leukemia sample. Because leukemogenesis is assumed to be a process of gradual accumulation of mutations, we asked whether selection of clones with specific signaling oncogenes might reflect the background biology of the tumor. To this end, we designed a three-level graph (Fig. 3B), where the vertices correspond to mutated signaling genes, the edges correspond to co-occurrence in a single patient, and the levels reflect the time point (i.e., DX, R1, R2). Concretely, intralevel edges signify coexistence at the same sample, whereas cross-level edges reflect for each gene (vertex) in level x, what were the observed mutations in the succeeding levels (time points) for each patient. We placed cross-level edges between the same gene regardless whether the relapse emerged from the same clone or from a different clone harboring mutation in that same gene. This graphic representation allows us to evaluate the general landscape of signaling in DS-ALL. It is clear that there are samples in which subclones with mutations in different signaling genes coexists (Fig. 3B). In addition, there are cases where leukemias present with JAK mutations at diagnosis can switch to a RAS mutation, but we have never observed the opposite, suggesting the dominance of RAS signaling.

**USP9X Is Disrupted in CRLF2pos ALLs.** As mentioned above, we found in our cohort several genes with mutations shared between diagnosis and relapse. One of these genes was USP9X, which was mutated in 4 of the 17 patients with CRLF2 rearrangements (Fig. 1). In three of...
the patients, we found focal chromosomal deletions that involved the USP9X locus (Fig. 4A), whereas in the fourth patient we detected a frameshift mutation (p.F1115Lfs) (Fig. 4B). In patient DSALLG14, the deletion extended from exon 32 of USP9X to exon 2 of its nearest downstream gene, DDX3X. This deletion could result in a chimeric transcript USP9X–DDX3X, a fusion that was recently reported in CRLF2pos ALLs with a similar interstitial deletion (28). However, the nature of USP9X deletions in the two other patients was different. In case DSALLA2, the deletion was of 5.3 MB, and stretched until the downstream gene ZNF674, whereas in patient DSALLB7, an upstream deletion of 200 kb that extended to USP9X gene body resulted in reduced USP9X expression at both diagnosis and relapse (Fig. S5). The observations that the VAF of USP9X in the four cases corresponded to the major clone persisting at relapse (Fig. 4C and Fig. S5) are suggestive of an early event in leukemia genesis.

USP9X is a deubquitinating enzyme that has been suggested to have a protumorigenic role by preventing the ubiquitin-mediated degradation of prosurvival and growth proteins, such as MCL2 and ERG (37–40). Furthermore, increased expression of USP9X was proposed to mediate resistance to glucocorticoids in ALL (40). Therefore, we were surprised to identify loss-of-function mutations in USP9X. Importantly, even the predicted USP9X–DDX3X in-frame fusion protein loses the deubiquitinating (UCH) domain of USP9X. To further validate these findings, we explored publicly available data. Additional mutations identified in USP9X in several studies (25, 41–44) are depicted in Fig. 4B. Many of these mutations are frameshift or missense mutations within the UCH domain, suggesting loss-of-function. Similar to our findings, the USP9X-mutated BCP-ALLs described by Oshima et al. (25) carried JAK2 and JAK3 activating mutations (Fig. S5E) (and hence, are likely to be CRLF2pos). We further analyzed RNA-seq data from the pediatric cancer (PeCAN) genomic database of childhood cancer (45). We compared USP9X expression to CRLF2 expression in Ph-like ALL (Fig. 4D). We found that a subset of 7 samples of the 28 CRLF2pos ALLs (all classified as Ph-like) demonstrated markedly diminished expression of USP9X, whereas USP9X expression was preserved in all CRLF2neg samples (P < 10−4, Fisher’s exact test). Additionally, four samples in the PeCAN database were annotated with the USP9X–DDX3X chimeric transcript; of these, three were among the CRLF2 rearranged group. Taken together, these data suggest that loss of USP9X by genomic aberrations is a recurrent event in ALL and may be in up to 25% of CRLF2pos ALLs.

**Fig. 4.** USP9X genomic aberrations. (A) Log2R values (Log2 ratio sample/normal) in the USP9X genomic region in the three DS-ALL patients with deletions in the region. Data shown are derived from a high-resolution SNP array (DSALLB7 and DSALLG14), or from exome coverage data (DSALLA2; indicated with “§”). (B) Coding mutations found in USP9X in independent published cohorts of B-ALLs. (C) Scatter plots of the VAF of mutations in diagnosis and in relapse in three patients with USP9X genomic lesions (Und, undetected). (D) CRLF2 and USP9X RNA-seq expression in Ph-like ALLs downloaded from the PeCan database (45). Samples classified as either carrying CRLF2 fusions (n = 28) or negative for the fusions (n = 96). Samples with the known USP9X–DDX3X fusion transcript are indicated with darker color.
Buffering of JAK-STAT Signaling by Loss-of-Function of USP9X. USP9X was recently shown to positively regulate JAK-STAT signaling by binding to JAK2, thereby enhancing JAK phosphorylation via removal of a competing ubiquitin group (46). Consequently, pharmacological inhibition or genetic ablation of USP9X was shown to reduce JAK signaling. Loss of USP9X in CRLF2pos ALLs, thought to be driven by enhanced JAK-STAT signaling, is therefore counterintuitive. Recent studies have suggested that B-lineage lymphocytes have limited capacity to tolerate signaling. For example, up-regulation of phosphatases was reported to be important for lymphoid transformation by BCR-ABL (47). Similarly, experimental ablation of JAK2 was shown to accelerate leukemic transformation by BCR-ABL (48). We thus hypothesized that loss of USP9X promote survival of CRLF2pos cells by restricting JAK signaling.

To test this hypothesis, we simultaneously overexpressed CRLF2 and JAK2R683G in a BCP-ALL cell line (49). Three days after transduction, 96% of the cells expressed surface CRLF2 (Fig. S6A). The cells were then treated with increasing amounts USP9X specific inhibitor BRD0476 (46) in the presence of the human cytokine TSLP (10 ng/mL). After 2 wk of treatment, most of the cells did not express CRLF2, demonstrating a selective advantage for the nontransduced cells. This observation reflects the toxicity of hypersignaling by mutated JAK2. Treatment with USP9X inhibitor increased the fraction of CRLF2pos cells in a dose-dependent manner (Fig. S4), but did not alter the fraction of cells transduced with the control vector (Fig. S6B). Treatment with the inhibitor resulted in a decrease in STAT5 phosphorylation (Fig. S5B) (median fluorescence intensity fold-decrease 1.5 ± 0.2). This response was similar to the decrease in STAT1 phosphorylation observed in other biological systems treated with the same agent (46). BRD0476 did not alter STAT3 phosphorylation, supporting a specific effect of the inhibitor on STAT5 (Fig. S6C).

Although BRD0476 was shown to be highly specific for USP9X, we wished to further rule out nonspecific drug effects by direct genetic disruption of USP9X. Using CRISPR/Cas9, we targeted USP9X at the UCH domain and generated cells lacking USP9X expression (Fig. 5C and Fig. S6D). Similar to the pharmacological studies, the genetic elimination of USP9X rescued the growth of cells expressing CRLF2 and JAK2R683G (Fig. 5D).

Because the observed effect of USP9X inhibition might not be directly mediated through attenuation of JAK signaling, we asked whether mild pharmacological inhibition of JAK would produce a similar phenotype. We treated CRLF2/JAK2R683G-transduced cells with increasing doses of ruxolitinib, an inhibitor of JAK2 and JAK1. At low concentrations (0.25–1 μM), ruxolitinib provided growth advantage to the transduced cells in the culture compared with vehicle (DMSO 0.25%), consistent with the results obtained with BRD0476 (Fig. 5E). Ruxolitinib exerted the expected cytotoxic effect on the transduced cells only at higher concentrations. Notably, the molecular effect of reduction in STAT5 phosphorylation (with no effect on STAT3 phosphorylation) was achieved even at low concentration of ruxolitinib (Fig. 5F). Together, these results suggest that loss of USP9X in CRLF2pos ALL cells may promote survival by buffering the intensity of JAK signaling. Because USP9X interacts with both JAK1 and JAK2 (Fig. S7A), it is likely that the observed effects of USP9X loss would not be restricted merely to JAK2 signaling (46). Activating mutations in IL7RA are associated with CRLF2 rearrangements (Fig. 1) and were shown to signal through JAK1 (8, 36). We therefore transduced 018z cells with CRLF2/IL7RINS, a Pro-Pro-Cys-Leu insertion in the transmembrane domain that promotes homodimerization and activation of the receptor (8). Pharmacological and genetic inhibition of USP9X (Fig. S7B–D) and pharmacological inhibition JAK1 (Fig. S7E) resulted in increased survival of transduced cells, similar to our observations following transduction with CRLF2/JAK2R683G (Fig. 5).

Discussion

The association between DS and ALL is intriguing. The genetic makeup of these BCP leukemias differs from the most common cytogenetic variants of childhood ALL. Therapeutically, DS-ALLs pose a significant challenge. Increased toxicity of chemotherapy is coupled with an intrinsic increased risk for relapse to negatively impact the survival of children with DS-ALL (2). By focusing on DS-ALL unique leukemias and on matched diagnosis and relapse samples, our goal was to decipher the pathogenesis of relapse of DS-ALLs aiming at improved therapy. We discovered that the genomic landscape differs significantly between the two major subtypes of DS-ALLs. CRLF2pos DS-ALLs are characterized by enhanced RAS signaling coupled by mutations in chromatin remodeling genes, in particular CREBBP. This genetic architecture is similar to hyperdiploid ALL, in which the cooperation between these two genetic lesions is thought to drive relapse (26, 50). In contrast CRLF2neg DS-ALLs are characterized by high dynamics of proliferative signaling. Enhanced JAK-STAT signaling mediated by the increased expression of CRLF2, the receptor to TSLP, is an early event during the evolution of DS-ALL, as is evident by its high allelic frequency and its stability between diagnosis and relapse. However, it also endows the leukemic cells with vulnerabilities, as evident by the presence of mutations in USP9X that attenuate JAK-STAT signaling, by the subclonal nature of downstream JAK mutations, and by decreased prevalence.
of JAK-activating mutation in relapse. These results have implications to Ph-like ALLs in patients without DS and for the incorporation of JAK inhibitors for treatment of ALL.

Analysis of our cohort and publicly available databases revealed recurrent loss-of-function mutations in USP9X. This finding was surprising because USP9X is perceived mainly as an oncogene in solid tumors and in BCP-ALL and B-cell lymphoblastic lymphomas (37, 40, 51, 52). Because USP9X is a deubiquitinase, its effect on tumor growth should largely be dependent on its substrates, which probably explains its ascribed role as tumor suppressor in pancreatic cancer (53). In a recent report describing 17 children with germ-line mutations in USP9X, one child developed ALL (54); however, this single case is not enough to define USP9X as an ALL tumor suppressor.

USP9X was also reported to enhance JAK2 signaling, as well as signaling of immune receptors (46, 55, 56). Thus, the loss of USP9X activity in up to 25% of CRLF2pos ALLs, a subtype of leukemia believed to be driven by enhanced JAK-STAT signaling, is intriguing. We show here that USP9X loss may promote the survival of leukemic cells by restricting the intensity of JAK2 and JAK1 signaling. Importantly, low-dose ruxolitinib, a JAK2/ JAK1 inhibitor currently in clinical trials for CRLF2pos ALLs, also increased the survival of leukemic cells transduced with CRLF2- and JAK2K648E, the most common JAK2 mutation in ALL (9). This observation suggests the need for high-dose ruxolitinib in these trials.

Our discoveries are consistent with recent findings that B cells, including leukemic B-cell precursors, can tolerate a relatively narrow range of signaling, possibly reflecting the evolutionary important autoimmunity checkpoint (57). Although signaling is required for their proliferation, enhanced signaling could cause their demise. The increased lymphoid sensitivity to hypersignaling may also explain the intriguing observation that the V617F strong-activating JAK2 mutation, characterizing myeloproliferative neoplasms, is never found in ALL (9). The buffering effect of loss of USP9X in CRLF2pos ALL is, therefore, similar to the buffering effect of increased expression of phosphatases observed in BCR-ABL ALL (47). Interestingly, a high expression of the phosphatase PTPRC (CD45) was also recently reported to be associated with P2RY8–CRLF2 ALLs (58).

Elimination of USP9X may promote ALL through other mechanisms yet to be discovered. The very early occurrence of USP9X loss-of-function mutations and their presence in other subtypes of ALL (including T-ALL (25)) are consistent with additional tumor-suppressive mechanisms. In fact, it is also possible that the selection of activating JAK-STAT mutations may have occurred to compensate for the loss of USP9X. Hematopoietic-specific elimination of Uspx in mice results in differentiation block of both T and B cells (56). Thus, mutations in USP9X may therefore participate in the leukemogenesis process by arresting B-cell differentiation similar to the mutations in other B-cell–differentiation transcription factors. Clearly, additional studies are needed to clarify the leukemogenic role of USP9X loss-of-function mutations.

A major goal of our study was to decipher the mechanisms of relapse of DS-ALL. Relapse reflects two cooperating processes: the primary leukemogenic process driving the growth and survival of leukemic cells and the selection pressure imposed by frontline therapy. Similar to other studies of relapsed ALL (21, 25, 26, 44, 50, 59), we observed increased RAS activation in relapse. Indeed, it has been proposed that RAS may mediate resistance to chemotherapy. This hypothesis is questioned by inspection of the clonal dynamics between diagnosis and relapse. Both in our cohort and in published studies (21, 25, 60) the expanded clones at relapse often carry a different RAS mutation than clones detected in diagnosis. This phenomenon is consistent with a general role of signaling in enhancing growth and proliferation of leukemic cells and not with resistance to chemotherapy.

The subclonal nature and the complex dynamics of CRLF2pos leukemia cells carrying either JAK-STAT or RAS suggest that inhibition of a single signaling pathway will select for leukemic clones driven by signaling of the other pathway. Our genomic data (Fig. 1) also suggest that signaling by either JAK-STAT or RAS is essential for leukemia growth. Therefore, we suggest that JAK inhibitors should be combined with MEK inhibitors for treatment of DS-ALL and CRLF2pos Ph-like ALLs.

Mutations selected by chemotherapy, either from preexisting subclones or by the enhanced genomic instability, are likely to be enriched in relapse specimens. The relapse-enriched mutations found in our DS-ALL cohort are similar to those found in other ALL subtypes (21, 25, 26, 30, 44, 50, 59). Intriguingly, however, we have found no NT5C2 mutations in our cohort. Previous studies identified NT5C2 mutations in 8–45% of the relapses of BCP-ALLs (34, 35, 44). Mutations in NT5C2 were associated with early relapses, whereas in our cohort the median time to relapse was relatively long, 1,071 d (range 375–2,226 d). NTSC2 mutations are thought to provide resistance to 6-mercaptopurine (6MP), a key drug in the treatment of ALL. Instead, we identified relapse-specific mutations in MMR genes in 15% of our patients. The MMR pathway is important for repair of damage caused by drugs, such as 6MP, and changes in MMR gene expression have been reported in association with resistance to 6MP (61). It will be interesting to see if different mutations conferring resistance to 6MP are mutually exclusive in relapse ALL.

Strikingly, most of relapsed ALLs, in our and in published studies, are not characterized by a major clone carrying a relapse-specific chemotherapy-resistant mutation. This finding implies that relapse may be often facilitated by one of the early founding mutations that initiated the primary leukemia. This hypothesis is supported by the strong prognostic significance of certain mutations found already as a major clone at diagnosis. Two such good examples, IKZF1 aberrations and CRLF2 rearrangements that often co-occur in the same leukemias (14, 17, 18, 21, 28, 62), predict worse prognosis and, as clearly shown in our data, are usually shared between diagnosis and relapse samples.

A recent experimental study, modeling diagnosis and relapse ALL, suggests that relapse initiates from dormant leukemic cells that survive by adhering to protective bone-marrow stroma cells (63). According to this model, initial chemotherapy eliminates the bulk of leukemic cells (e.g., JAK and RAS mutated cells). The slow-dying dormant cells are eliminated by the continuous maintenance therapy (6MP and methotrexate). In relapsing patients a few residual cells remain dormant on stroma. Creation of full-blown leukemia depends on proliferative signaling (e.g., RAS).

IKZF1 mutations have been shown to promote adherence to bone-marrow stromal cells and therefore, through this mechanism, may promote dormancy during complete remission and relapse (64, 65). Hence, targeting IKZF1 by, for example, casein kinase II (66) or FAK inhibitors (65), may be useful in targeting this dormancy stage. Similarly, we speculate that leukemic cells carrying CRLF2 rearrangements—and, hence, expressing the TSLP receptor—may survive chemotherapy during complete remission by interacting with TSLP-producing cells in the bone-marrow niche. The experimental examination of this hypothesis is challenging, because mouse TSLP is not reactive with the human TSLP receptor (67). The hypothesis suggests that targeting TSLP [for example, by the novel anti-TSLP antibodies (68)] or the TSLP receptor [by for example, CRLF2 antibodies or CAR-T cells (69, 70)] may be a useful additional therapy—better than JAK inhibition—to prevent relapse in patients with DS and sporadic CRLF2pos ALL.

Materials and Methods

DNA and RNA of bone-marrow–derived DS-ALL blasts were obtained from patients after informed consent and approval of the ethics committees of all participating institutions in the AIEOP-BFM protocol, in accordance with the
Declaration of Helsinki. Our study was also approved by the ethic committee of Israeli Health Ministry and Sheba Medical Center approval #8481-2346. As required by the Hopkins samples were anonymized. Samples were included based on the availability of DNA from matched diagnosis-remission (n = 31) and -relapse (n = 26). The clinical details are listed in Dataset S1. More detailed materials and methods are in the SI Materials and Methods. See Dataset S3 for the high-confidence variant list and Table S1 for 018 cell line short tandem repeat (STR) information.

Genomic Analysis. Whole-exome sequencing was performed using SureSelect Human All Exons–Ultralow V5 kit (Agilent Technologies) and performed on was done with HiSeq2500 platform (Illumina). Somatic variants were detected using MuTeC2 (71). Copy number variations (CNV) were inferred from WES coverage data using EXACVATOR (72), or using Cytoscan HD (Affymetrix). DNA targeted resequencing was done with a UMI-tagged Human All Exons short tandem repeat (STR) information.

Biolegend), LIVE/DEAD Fixable staining (ThermoFisher Scientific), pSTAT5 reagents: 7AAD, anti-TSLPR (CRLF2; clone 1D3 1B4 PE-conjugated; Biologend), LIVE/DEAD Fixable staining (ThermoFisher Scientific), pSTAT5

A minimal threshold of 5% VAF was used for the SNVs and indels presented in SI Materials and Methods based on the availability of DNA from matched diagnosis-remission (n = 31) and -relapse (n = 26). The clinical details are listed in Dataset S1. More detailed materials and methods are in the SI Materials and Methods. See Dataset S3 for the high-confidence variant list and Table S1 for 018 cell line short tandem repeat (STR) information.

In Vitro Studies. All in-vitro studies were performed on ALL 018 BCP-ALL cells (49) transduced with lentiviruses. Flow cytometry analysis was done on Gallios platform and Kaluza software (Beckman-Coulter) with the following reagents: 7AAD, anti-TSLPR (CRLF2; clone 1D3 1B4 PE-conjugated; Biologend), LIVE/DEAD Fixable staining (ThermoFisher Scientific), pSTAT5 antibody (APC-conjugated, E-Bioscience, ThermoFisher Scientific), Counting beads (CountBright, ThermoFisher Scientific). Small molecule inhibitors used were BRD0476 (46) and ruxolitinib (Selleck Chem). Targeting USP9X using CRISPR/Cas9 was done per a previously published protocol (73). Western blot analysis was done with the following antibodies: anti-human USP9X (Bethyl Laboratories) and anti–β-actin (Sigma Aldrich).

Data Availability. Genomic data included in this study were deposited in the European Genome-phenome Archive (accession no. EGASS0001002410). The study protocol’s patient consent did not include permission to make the data publicly available. Researchers wishing to gain access to the raw data must contact the corresponding authors directly.

ACKNOWLEDGMENTS. We thank Inna Muller for excellent technical support, and members of the A.T. and S.I. research groups for fruitful discussions and advice. These studies were funded by an Israel Science Foundation Legacy grant complemented by an Israel Science Foundation-Israel National Center for Personalized Medicine grant (to S.I.); an Israel Science Foundation joint grant with China (to S.I. and S.-J.C.); European Union European Research Area-NET TRANCALL (S.I., G. Cario, M. Stanulla, G.T.K., G. Cazzaniga, and C.E.); the Israel Cancer Research Fund (S.I.); the Waxman Foundation (S.I.); the William Lawrence and Blanche Hughes foundation (S.I.); the German Israel Foundation (S.I. and C.E.); the Israel Absorption Ministry (I.G.); the Israel Cancer Association (S.I.); Children with Cancer UK (S.I.); the Dotan Center for Hematological Malignancies in Tel Aviv University (S.I.); the Dora and Giorgio Shapiro Chair for Hematological Malignancies, Tel Aviv University (S.I.); Fondazione Italiana contro la Ricerca Sul Cancro (A.M.S.); the German Consortium of Translational Cancer Research (A. Borkhardt). This research partially fulfills the requirements for a PhD of Tel Aviv University for O.S., A.R., and I.G.


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