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REARRANGEMENT OF *CRLF2* IN B-PROGENITOR AND DOWN SYNDROME ASSOCIATED ACUTE LYMPHOBLASTIC LEUKEMIA

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DATABASE ACCESSION

Aglient array-CGH data has been deposited in the NCBI's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), accession GSE16724. The *P2RY8-CRLF2* mRNA sequence has been deposited in Genbank, accession GQ280263. SNP array data is available from the authors upon request.

AUTHOR CONTRIBUTIONS

CGM designed and coordinated the study, designed assays, performed experiments, analyzed data and wrote the manuscript
JRC-U generated retroviral vectors and performed Ba/F3 assays
LAAP performed JAK sequencing and quantitative PCR assays
MLL performed PAR1 deletion genomic PCR
WL performed statistical analysis
JZ analyzed sequencing data
Jing Ma analyzed microarray data
EC-S performed flow cytometry and analyzed data
RCH and CLW developed FISH assays
Julia Meyer performed experiments and analyzed data
FMM, AJC and NAH performed FISH assays and analyzed cytogenetic data
RTW provided luciferase vectors
JC designed subcloning vectors
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SUMMARY

Aneuploidy and translocations are hallmarks of B-progenitor acute lymphoblastic leukemia (ALL), but many patients lack a recurring chromosomal alteration. Here we report a recurring interstitial deletion of the pseudoautosomal region 1 of chromosomes X and Y in B-progenitor ALL that juxtaposes the first, non-coding exon of *P2RY8* to the coding region of *CRLF2* (which encodes cytokine receptor like factor 2, or thymic stromal lymphopoietin receptor). The *P2RY8-CRLF2* fusion was identified in 7% of B-progenitor ALL cases, and was identified in over 50% of ALL cases arising in patients with Down syndrome (53% of 75 cases). *CRLF2* alteration was associated with the presence of activating JAK mutations, and expression of *P2RY8-CRLF2* together with JAK2 mutants resulted in constitutive Jak-Stat activation and cytokine-independent growth of Ba/F3-IL7R cells, indicating that these two genetic lesions together contribute to leukemogenesis in B-progenitor ALL.

Chromosomal alterations are a hallmark of acute lymphoblastic leukemia (ALL), the commonest malignancy of childhood, and include aneuploidy (hyperdiploidy and hypodiploidy) and recurring chromosomal translocations, such as t(12;21) [*ETV6-RUNX1*], t(1;19) [*TCF3-PBX1*], t(9;22) [*BCR-ABL1*] and rearrangement of *MLL*[1]. These alterations are important events in leukemogenesis and influence response to therapy. However, up to one-quarter of childhood ALL cases lack a recurring chromosomal alteration, and the genetic basis of these cases is poorly understood.

To identify submicroscopic genetic alterations contributing to the pathogenesis of ALL, we previously performed high resolution profiling of DNA copy number alterations and loss of heterozygosity (LOH) using single nucleotide polymorphism (SNP) microarrays, and identified multiple recurring genetic alterations targeting key cellular pathways including lymphoid development, cell cycle regulation and tumor suppression[2,3]. These alterations included a novel deletion involving the pseudoautosomal region 1 (PAR1) of Xp22.3/Yp11.3 in 15 B-progenitor ALL cases lacking common chromosomal translocations. Notably, six of eight ALL cases associated with Down syndrome (DS-ALL) harbored this deletion.

To further characterize the PAR1 deletion, we expanded our analysis of DNA copy number alterations and LOH to include 329 ALL cases, including 272 B-progenitor (22 DS-ALL) and 57 T-lineage childhood ALL cases (see Supplementary Table 1 online for patient characteristics and Supplementary Table 2 online for a full listing of all DNA copy number alterations). The PAR1 deletion was identified in 19 (7%) B-progenitor ALL cases, including 12 (54.5%) of the 22 DS-ALL cases, all of which lacked recurring translocations commonly associated with non-DS ALL (Supplementary Table 3 online). Notably, all 10 DS-ALL cases with cytogenetic abnormalities commonly observed in DS-ALL[4–6] (+X in 8 cases, and del(9)(p22) in 2 cases) had a PAR1 deletion.

The region of PAR1 deletion appeared identical in all cases (Fig. 1*a*), involved at least three genes (*P2RY8*, *SLC25A6* and *IL3RA*, Fig. 1*b*), and was confirmed by genomic quantitative PCR (Supplementary Table 4 online). The deletion was adjacent to the cytokine receptor genes *CSF2RA* and *CRLF2*, but due to sparse probe coverage on the SNP array platform used (Fig. 1*b*), it was not possible to precisely define the extent of deletion using SNP array data alone.

To map the boundaries of deletion, we performed array-based comparative genomic hybridization on two samples using a one million feature oligonucleotide array with dense coverage of the region. This showed that the PAR1 deletion extended from immediately centromeric (upstream) of *CRLF2* exon 1 to intron 1 of *P2RY8* (Fig. 1*b*). *CRLF2* encodes cytokine receptor like factor 2 (also known as thymic stromal lymphopoietin receptor, TSLPR), a lymphoid signaling receptor molecule that forms a heterodimeric complex with interleukin 7 receptor alpha (IL7RA) and binds thymic stromal lymphopoietin (TSLP)[7,8]. *P2RY8* encodes a purinergic receptor (P2Y, G-protein coupled, 8) that is expressed at high levels in many tissues, including leukemic cells[9]. A single case of rearrangement of *P2RY8* to *SOX5* has been reported in primary splenic follicular lymphoma[10]. These observations suggested that the PAR1 deletion may result in a novel rearrangement involving *P2RY8* and *CRLF2*.

RT-PCR confirmed the presence of chimeric transcripts juxtaposing the first, non-coding exon of *P2RY8* to the entire coding region of *CRLF2* in all cases with PAR1 deletion that had available material for analysis, but none of 50 cases that lacked the deletion (Fig. 1*c*, Supplementary Fig. 1 online). The fusion junction was identical in each case (Fig. 1*d*). Quantitative RT-PCR analysis demonstrated increased *P2RY8-CRLF2* expression in cases with the PAR1 deletion (Supplementary Fig. 2 online). Moreover, elevated cell surface expression of CRLF2 was also detected by flow cytometric analysis of cryopreserved leukemic cells (Supplementary Fig. 3 online). A single DS-ALL case (DS-ALL-#14) was found to have elevated *CRLF2* mRNA levels by real time PCR but lacked the deletion, suggesting an alternative mechanism of *CRLF2* dysregulation. Rearrangements of the immunoglobulin heavy chain locus (*IGH@*) at 14q32.33 to *CRLF2* have recently been reported in B-progenitor ALL[11], and fluorescence in situ hybridization (FISH) analyses of interphase nuclei confirmed *IGH@-CRLF2* rearrangement in this case (Supplementary Fig. 4 online). Thus, 13 (60%) of DS-ALL cases had genomic rearrangements resulting in dysregulated *CRLF2* expression.

We next characterized the genomic breakpoints of the PAR1 deletion by long template PCR of genomic DNA extracted from leukemic cells (Supplementary Figs. 5 and 6 online). The deletion breakpoints were highly conserved, and were located 3.4kb upstream of *CRLF2* exon 1, and 0.3–1kb distal to *P2RY8* exon 1 (Supplementary Fig. 5c online). Notably, we observed partly or fully conserved heptamer recombination signal sequences (RSSs) immediately internal to the deletion breakpoints, and a variable number of non-consensus nucleotides between the aligning sequences in each case (Supplementary Figs. 5c and 6 online). These data suggest that the PAR1 deletion may arise as a result of aberrant activity of the antigen receptor recombinases encoded by the RAG genes (recombinase activating genes), a mechanism that has been implicated in the generation of other sentinel chromosomal rearrangements and deletions in ALL[3,12].

DS-ALL is characterized by the presence of activating JAK mutations in up to 28% of cases, most commonly in the JAK2 pseudokinase domain at or around R683[13–16]. *JAK1*, *JAK2* and *JAK3* mutations have also been identified in 10% of high-risk B-progenitor ALL patients without Down syndrome[16]. In this cohort, we identified previously described and novel JAK mutations in 11 (4%) B-progenitor ALL cases, including six (27.2%) DS-ALL cases (Supplementary Fig. 7 online). These included mutations in the JAK2 pseudokinase domain (R683G, N=5, R683S, N=2 and IR682RG, N=1), JAK2 kinase domain (T875N and G861W, 1 each) and JAK1 pseudokinase domain (V658F, the homolog of JAK2 V617F). Moreover,

we observed significant association between *CRLF2* alterations and JAK mutations. Nine (45%) patients with *CRLF2* alterations had JAK mutations, compared to two (0.6%) patients lacking *CRLF2* lesions (Fisher exact $P<0.0001$; Table 1, Supplementary Table 1 online). Although we previously identified an association between outcome and the presence of JAK mutations in high risk pediatric ALL[16], no association was observed between *CRLF2* and/or JAK mutation status and outcome in DS-ALL (data not shown).

To validate the high frequency of *CRFL2* and JAK mutations in DS-ALL, we examined a second cohort of 53 B-progenitor DS-ALL cases (Supplementary Table 5 online). Twenty-eight (52.3%) had the PAR1 deletion detected by genomic PCR (Supplementary Fig. 8*a* online). In cases with available material, the deletion was associated with elevated *CRLF2* expression on microarray analysis (Supplementary Fig. 9 online) and expression of *P2RY8-CRLF2* (Supplementary Fig. 8*b* online). Moreover, eight (27.6%) patients with the PAR1 deletion had *JAK2* mutations, compared to one patient (4.2%) without the PAR1 deletion (Fisher exact $P=0.02$), again highlighting the remarkable association between *CRFL2* overexpression and activating JAK2 mutations.

The co-occurrence of CRLF2 alteration and JAK mutations suggested that these events likely cooperate in leukemogenesis. To examine this hypothesis, we assessed the effect of *P2RY8-CRLF2* and JAK mutations on the ability of the cytokine-dependent murine B-progenitor Ba/F3 cell line to grow in the absence of exogenous cytokine. JAK mutations are usually alone insufficient to transform Ba/F3 cells, but render the cells growth factor-indepedent when coexpressed with erythropoietin or thrombopoietin receptors13,15,[16]. As CRLF2 normally forms a heterodimeric complex with the interleukin 7 receptor alpha chain (IL7RA), we expressed IL-7 receptor alpha in Ba/F3 cells (Ba/F3-IL7R cells). Expression of *P2RY8-CRLF2* or JAK2 mutants alone (either JAK2 R683G or the kinase domain mutation P933R16) in the Ba/F3-IL7R cells failed to induce cytokine-independent growth. In contrast, co-expression of both *P2RY8-CRLF2* and JAK mutants resulted in constitutive Jak-Stat activation and cytokine-independent growth in Ba/F3-IL7R cells (Fig. 2, Supplementary Figs. 10 and 11 online). Moreover, this transformation was attenuated by pharmacological Jak inhibition (Fig. 2*c*) and knockdown of CRLF2 expression by short hairpin RNAs (Fig. 2*d*, Supplementary Fig. 12 online).

Together, these data describe a recurrent intra-chromosomal deletion of the pseudoautosomal region 1 of Xp22.3/Yp11.3 that results in the generation of a chimeric *P2RY8-CRLF2* mRNA, and markedly elevated expression of CRLF2. CRLF2 overexpression from *IGH@-CRLF2* rearrangement or PAR1 deletion has also been reported in B-progenitor ALL[11]. However, while *CRLF2* alteration is uncommon in B-progenitor ALL (5% of cases), it is present in over 55% of cases of DS-ALL, in which chromosomal rearrangements characteristic of non-DS-ALL are uncommon[4]. Moreover, *CRLF2* alteration was observed exclusively in cases lacking translocations associated with ALL, suggesting that *CRLF2* alteration is a potent leukemogenic event in the setting of trisomy 21, and may account in part for the up to 20-fold increased risk of developing ALL in children with DS[6,17]. The associations of *CRLF2* alteration with JAK mutations and additional recurring cytogenetic alterations in DS-ALL (gain of chromosome X, and del(9p))[4] are also notable, and suggest that these lesions cooperate in leukemogenesis. Interestingly, three of five cases with PAR1 deletion and gain of chromosome X had evidence of two copies of the PAR1 deletion on FISH analysis (Supplementary Fig. 4*e–g* online), suggesting that gain of X results in duplication and high level expression of the *P2RY8-CRLF2* fusion. Amplification of regions of chromosomal rearrangement resulting in gene fusion has previously been noted in acute leukemia, for example the *NUP214-ABL1* translocation in T-lineage ALL[18].

CRLF2 interacts with IL-7 receptor alpha to form a heterodimeric receptor for the cytokine thymic stromal lymphopoietin (TSLP). TSLP/CRLF2 signaling has important roles in T and dendritic cell development, inflammation and allergic disease[7,19], and promotes B lymphoid proliferation, but may not be required for normal B cell development[20,21]. CRLF2 signaling results in downstream STAT5 phosphorylation[20,22], and for the human receptor, phosphorylation of JAK2[20]. An important observation is that in contrast to myeloproliferative diseases, in which homozygous mutation of JAK2 V617 is common[23], the JAK mutations in B-progenitor ALL are usually heterozygous, and do not occur at JAK2 V617[13,15,16]. The basis of the disease specificity of the different JAK mutations is unknown, and it has been suggested that the different JAK2 pseudokinase domain mutations may facilitate interaction with different substrates and signaling pathways[13]. Detailed analysis of the transforming effect of mutant Jak alleles in Ba/F3 cells expressing *P2RY8-CRLF2* and/or IL-7R indicated that most Jak mutations are not transforming in the absence of *CRLF2* overexpression (the exceptions being Jak2 V617F and the homologous mutation Jak1 V658F; Supplementary Note and Supplementary Fig. 11 online). In contrast, the mutations most commonly observed in B-progenitor ALL - those at or near JAK2 R683 - require coexpression of *P2RY8-CRLF2* for transformation in this assay. Notably, transformation (as measured by the rate of cytokine-independent cell growth) was more marked with concomitant IL7RA expression (Supplementary Fig. 11 online). Moreover, co-immunoprecipitation experiments using 3xFLAG-tagged *P2RY8-CRLF2* demonstrated direct interaction of CRLF2 and phosphorylated Jak2 (Supplementary Fig. 13 online). Thus, our results indicate that aberrant signaling through the CRLF2–IL7R receptor, mediated by *CRLF2* overexpression and Jak mutation, is a key event in B lymphoid transformation. Consistent with this, in a recent study identifying *CRLF2* rearrangement by FISH in B-progenitor ALL, overexpression of CRLF2 promoted transformation and Stat5 phosphorylation of primary murine lymphoid progenitors[11]. This study examined JAK2 exon 14 mutational status in a small cohort of DS-ALL cases (N=24) and also observed an association between *CRLF2* rearrangement and JAK mutations[11], further supporting cooperativity of these lesions in leukemogenesis.

As a substantial proportion of cases with *CRLF2* overexpression lack JAK mutations, mutational analysis of other kinases and mediators of JAK-STAT signaling is warranted. These findings also suggest that detection of increased CRLF2 expression will be a useful diagnostic strategy in ALL, and that JAK-STAT inhibition may be useful in the treatment of patients with B-progenitor ALL with *CRLF2* and JAK mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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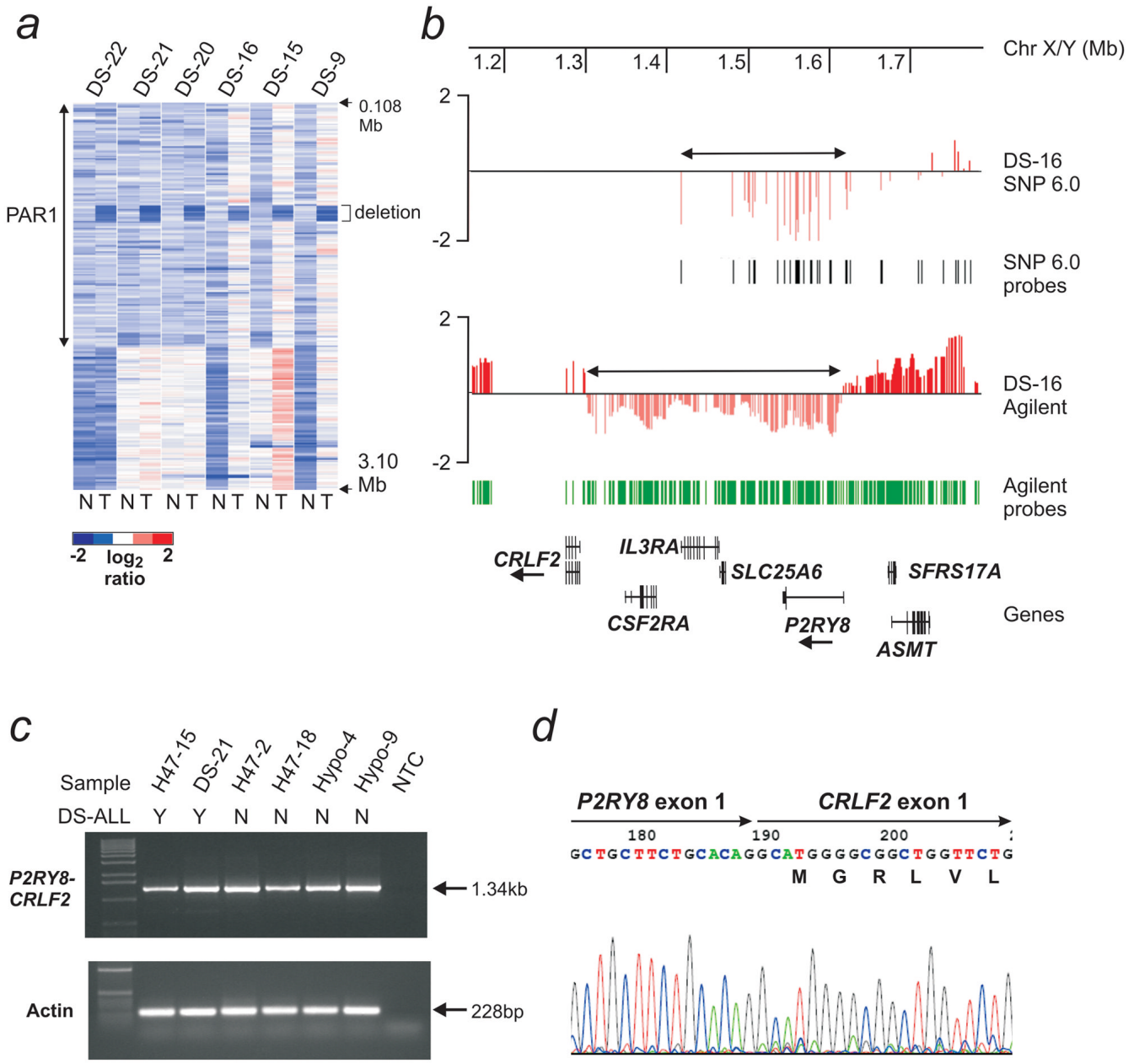

Figure 1. Deletion involving pseudoautosomal region 1 (PAR) and *P2RY8-CRLF2* fusion in B-progenitor ALL

a, representative $\log_2$ratio SNP 6.0 microarray DNA copy number data of six cases with PAR1 deletion. White is normal, blue is deletion, and red is gain. Paired data is shown, with normal (N) on the left, and tumor (T) on the right for each case. DS, Down syndrome. ***b***, mapping of the extent of the PAR1 deletion is shown for a representative case. The sparse SNP 6.0 probe coverage of the region, particularly the lack of coverage of the *CRLF2/CSF2RA/IL3RA* region is shown. Location of SNP 6.0 probes is shown by vertical black lines, and the coverage of the one million feature Agilent array shown as green lines, with the corresponding $\log_2$ratio copy number data shown as red lines. The region of deletion defined by each platform is shown as a horizontal arrow. ***c***, RT-PCR demonstrating *P2RY8-CRLF2* fusion transcripts. NTC, no

template control. ***d***, the fusion junction is identical in each *P2RY8-CRLF2* positive case, and involves the first non-coding exon of *P2RY8* and the entire open reading frame of *CRLF2*.

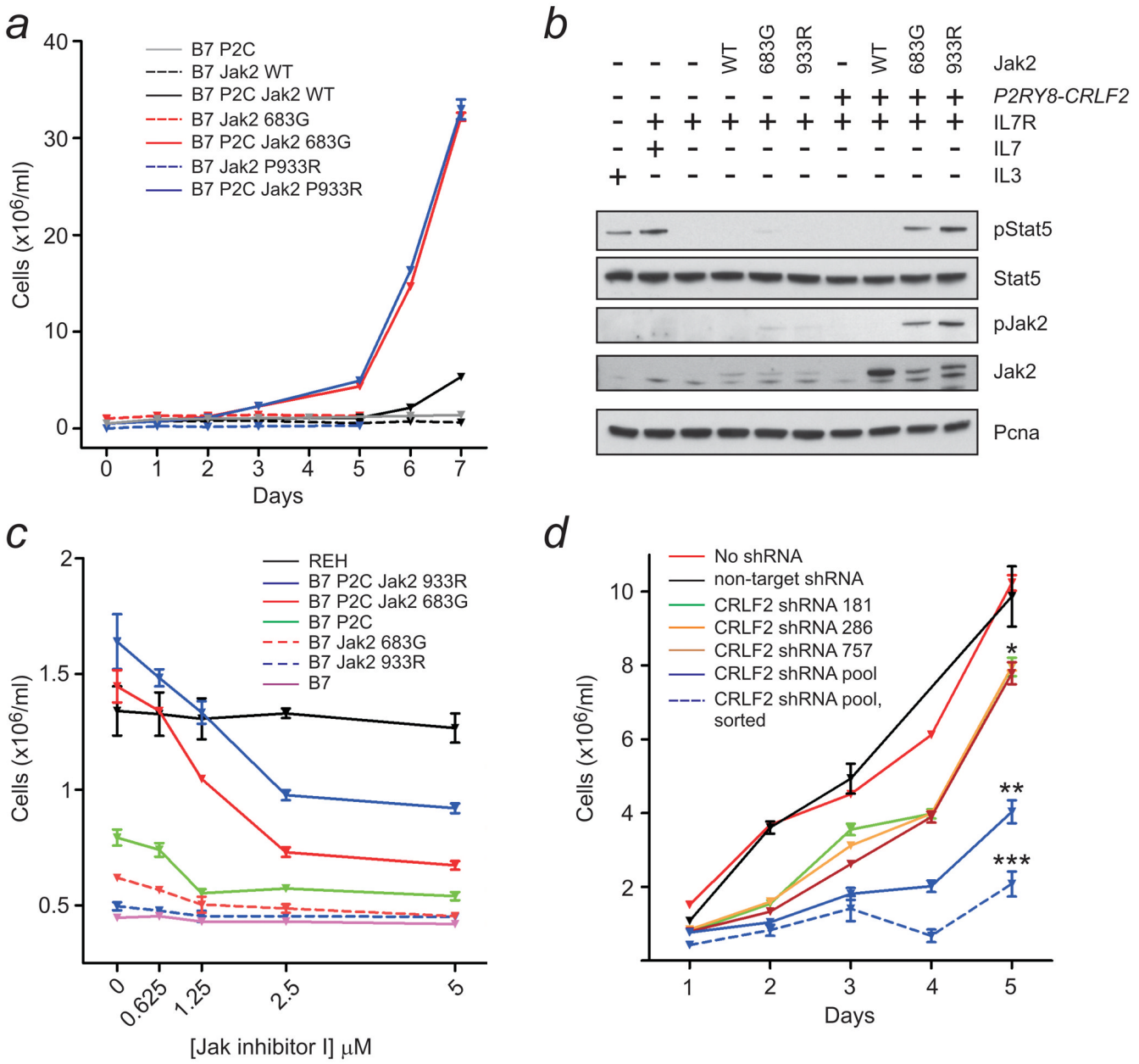

Figure 2. Transforming effects of *P2RY8-CRLF2* and JAK mutations

a, Ba/F3 cells expressing murine IL-7 receptor alpha chain (Ba/F3-IL7R, or B7 cells) were transduced with retroviral constructs expressing *P2RY8-CRLF2* (P2C) and/or Jak2 alleles (wild type murine Jak2, or Jak2 683G and 933R). Cells were washed and cultured in the absence of cytokine. Co-expression of *P2RY8-CRLF2* and Jak mutants resulted in cytokine-independent growth, but not expression of either *P2RY8-CRLF2* or Jak mutants alone. ***b,*** western blotting demonstrating constitutive Jak-Stat activation in Ba/F3-IL7R cells expressing *P2RY8-CRLF2* and Jak2 mutants. Ba/F3 cells grown in IL-3, and Ba/F3-IL7R cells grown in IL-7 (without starvation and stimulation) are included at the left of the figure as positive controls. A representative blot of three independent experiments is shown. ***c***, pharmacological Jak inhibition (with Jak inhibitor I) inhibits the growth of Ba/F3-IL7R cells transduced with *P2RY8-CRLF2* and Jak2 683G or 933R. Cells were washed three times, plated at 0.5×10^6 cells/

ml in triplicate, and counted after 48 hours. The *ETV6-RUNX1* B-progenitor ALL cell line REH, which does not harbor *P2RY8-CRLF2* or JAK mutations, was used as a control. ***d***, Knockdown of *CRLF2* expression by lentiviral shRNAs attenuates cytokine-independent growth of Ba/F3-IL7R cells expressing *P2RY8-CRLF2* and Jak2 683G. Cells were transduced with non-target (scrambled) shRNA, each of three CRLF2 shRNAs (181, 286 and 757) and a pool of all three shRNAs. This resulted in substantial but incomplete attenuation of CRLF2 expression (see Supplementary Fig. 12 online) and reduced cytokine-independent growth. Cells with near-total down-regulation of CRLF2 expression following shRNA knockdown (isolated by flow sorting for CRLF2) showed marked abrogation of cytokine-independent growth. t-test (compared to cells transduced with non-target shRNA) P-value: *, $0.05<P<0.10$; **$P<0.01$; ***$P<0.001$. All error bars show s.e.m.

TABLE 1

Distribution of PAR1 deletions and JAK mutations in pediatric ALL.

Group	N	PAR1 deletion, no JAK mutation	JAK mutation, no PAR1 deletion	PAR1 deletion and JAK mutation
Non DS-ALL				
High hyperdiploid	43	0	1	0
TCF3-PBX1	17	0	0	0
ETV6-RUNX1	49	0	0	0
MLL-rearranged	24	0	0	0
BCR-ABL1	21	0	0	0
Hypodiploid	10	2	0	0
Other	86	2	1**	3
T-lineage ALL	57	0	0	0
DS-ALL				
St Jude cohort	22	7	0	6*
Validation cohort	53	20	1	8

* Includes one case with *IGH@-CRLF2* translocation.

** JAK2 G861W