

## ORIGINAL ARTICLE

Genomic and transcriptional landscape of *P2RY8-CRLF2*-positive childhood acute lymphoblastic leukemia

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Children with *P2RY8-CRLF2*-positive acute lymphoblastic leukemia have an increased relapse risk. Their mutational and transcriptional landscape, as well as the respective patterns at relapse remain largely elusive. We, therefore, performed an integrated analysis of whole-exome and RNA sequencing in 41 major clone fusion-positive cases including 19 matched diagnosis/relapse pairs. We detected a variety of frequently subclonal and highly instable JAK/STAT but also RTK/Ras pathway-activating mutations in 76% of cases at diagnosis and virtually all relapses. Unlike *P2RY8-CRLF2* that was lost in 32% of relapses, all other genomic alterations affecting lymphoid development (58%) and cell cycle (39%) remained stable. Only *IKZF1* alterations predominated in relapsing cases ( $P=0.001$ ) and increased from initially 36 to 58% in matched cases. *IKZF1*'s critical role is further corroborated by its specific transcriptional signature comprising stem cell features with signs of impaired lymphoid differentiation, enhanced focal adhesion, activated hypoxia pathway, deregulated cell cycle and increased drug resistance. Our findings support the notion that *P2RY8-CRLF2* is dispensable for relapse development and instead highlight the prominent rank of *IKZF1* for relapse development by mediating self-renewal and homing to the bone marrow niche. Consequently, reverting aberrant IKAROS signaling or its disparate programs emerges as an attractive potential treatment option in these leukemias.

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## INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the predominant malignancy in children and adolescents and one of the leading causes of death in these age groups. It comprises multiple entities with gene fusions, such as *ETV6-RUNX1*, *BCR-ABL1*, *TCF3-PBX1* and those with *KMT2A* that are associated with distinct clinical features and prognosis.<sup>1</sup> Recently, a novel subgroup has been described whose defining characteristic is the deregulated expression of the cytokine receptor-like factor 2 (*CRLF2*) gene, which is located in the pseudoautosomal region 1 on the short arm of the X and Y chromosome. The two most common causative genetic defects are a small interstitial deletion that fuses the first non-coding exon of the purinergic receptor P2Y, G-protein coupled, 8 (*P2RY8*) to the entire coding region of *CRLF2* and occurs in 5–8% of childhood B-cell precursor (BCP) ALL cases and a more rare translocation that places *CRLF2* under the control of the *IGH* enhancer.<sup>2,3</sup> Activating mutations of the *CRLF2* or *IL7R* genes are far less common.<sup>4–6</sup> In contrast to *IGH-CRLF2* translocations, which occur in a hematopoietic stem/precursor cell and are initiating events, *P2RY8-CRLF2* fusions are caused by illegitimate V(D)J-mediated recombination in a B precursor cell, and are, therefore, likely secondary events and define a more heterogeneous group of leukemias.<sup>7</sup> As such, they accompany *bona fide* primary abnormalities, such as intrachromosomal amplification of chromosome 21 (iAMP21) and, albeit less frequently, hyperdiploidy, but are present only in

small subclones in the vast majority of cases and these fusions are often lost at relapse.<sup>8,9</sup> Further, they show a strong affinity for constitutional as well as acquired numerical chromosome 21 abnormalities.<sup>3,4,8</sup> *P2RY8-CRLF2* fusions often carry additional alterations in JAK/STAT pathway genes and they may cooperatively activate downstream pathways.<sup>4,8,10,11</sup> They are associated with a significantly increased relapse risk in AIEOP/BFM protocols, which is independent of the size of the *P2RY8-CRLF2*-positive clone. Respective cases are primarily classified as non-high risk by clinical and molecular response criteria and relapses occur predominantly late.<sup>9,12–14</sup>

*IKZF1* encodes the lymphoid transcription factor IKAROS, which is a key regulator in early lymphocyte development.<sup>15</sup> Alterations mainly comprise deletions and only rarely sequence alterations.<sup>16–18</sup> Irrespective of the type of *IKZF1* alteration, they prevail in poor responding cases in major treatment protocols.<sup>5,11,17,19–23</sup> They are a hallmark of high-risk BCP ALL, especially those, which carry a *BCR-ABL1* and other cytokine- and kinase-activating fusions, including *IGH-CRLF2* and *P2RY8-CRLF2*.<sup>11,24</sup> The common characteristic of such cases is a gene expression signature that resembles that of genuine *BCR-ABL1*-positive cases and which are, therefore, also referred to as either '*BCR-ABL1*-like' or 'Ph-like'.<sup>11,24</sup> *IKZF1* alterations still confer a dismal prognosis in such cases, even when treated on high risk or kinase inhibitor-containing protocols.<sup>1,16,17,24–28</sup>

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*IKZF1* deletions were also reported in small cohorts of *P2RY8-CRLF2*-positive leukemia cases recruited to various treatment protocols. It, therefore, seems likely that their presence may contribute to relapse development in this particular subgroup, a notion that has so far not been systematically investigated in large and well-characterized cohorts. Notably, hardly any detailed information about concurring alterations, their subclonal or clonal nature as well as whether any or which of them are preserved at relapse, is available. To address these issues we performed whole-exome sequencing (WES) and determined the genome-wide mutational pattern in a quantitative manner in 41 relapsing and non-relapsing *P2RY8-CRLF2*-positive cases that were treated primarily according to BFM protocols. Special emphasis was put on defining the size, mutation patterns and distribution of clones at diagnosis and relapse, which enabled us to deduce the kinetics and indirectly also the *in vivo* selection of particular clones. Together with the transcriptional profiling of these cases, this approach allowed us not only to gain new insights into the mechanisms of relapse development but also to infer the biological impact of genomic alterations, their potential role in resistance mechanisms as well as their applicability as biomarkers and drug targets.

## MATERIALS AND METHODS

### Patients and samples

Selection of BCP ALL cases was based on the presence of a *P2RY8-CRLF2* fusion in the major clone at initial diagnosis, the absence of a *KMT2A*, *BCR-ABL* or *ETV6-RUNX1* rearrangement, and on the availability of DNA or frozen viable cells with a blast count of  $\geq 85\%$  at diagnosis or  $\geq 70\%$  at relapse. In total, 52 children (including 23 individuals with Down syndrome (DS)) with a median age of 3.8 years (range, 1.5–13.7) were included. Cases were initially treated on BFM ALL 2000/09 ( $n = 32$ ), CoALL-08-09 ( $n = 9$ ), FRALLE, EORTC58951 and EORTC58081 protocols ( $n = 11$ ; [www.ClinicalTrials.gov](http://www.ClinicalTrials.gov): NCT00411541, NCT00005603, NCT00343369 and NCT0114348). Leukemic samples from 41 cases, including 19 with matched relapses, were available for WES. From the remaining 11 cases (from FRALLE and EORTC studies) only multiplex ligation-dependent probe amplification (MLPA)-derived copy number alterations (CNA) were available. Remission samples served as germline control in all instances. All non-relapsing cases were in long-term remission (median, 7 years; range, 4–14 years). Relapsing cases had higher white blood cell count at initial diagnosis (Table 1) but did not differ in any other clinical or response criteria from the non-relapsing cohort. The study cases are representative of an unselected cohort of 20 respective ALL cases consecutively recruited to the BFM ALL 2009 study in Austria (Table 1). The detailed clinical data of cases with matched diagnosis and relapse samples are summarized in Table 2. The median time to relapse was 35 months (range 4–97). At relapse, the majority of cases (9 of 16 with available data) had a poor molecular response.<sup>29</sup>

Samples were obtained from the respective study centers upon institutional review and ethics committees' approval. Informed consent for tissue banking and research studies was obtained from patients, their parents or legal guardians in accordance with the Declaration of Helsinki.

### Sample preparation and WES

WES was performed as described previously.<sup>30</sup> Samples were prepared using Nextera Rapid Capture Exome Kit (Illumina, San Diego, CA, USA). Paired-end sequencing of 100 bp reads was performed on a HiSeq 2000 (Illumina) to obtain at least a 50 $\times$  coverage. More detailed coverage metrics computed by Picard (<https://github.com/broadinstitute/picard>) are provided in the Supplementary Methods, additional file 1. Mutations were validated with Sanger sequencing (Supplementary Results).

### Determination of CNA by PCR, MLPA and single-nucleotide polymorphism array analysis

Genomic breakpoint amplification of *P2RY8-CRLF2* was performed as described previously.<sup>9</sup> The MLPA probe mixes used for detection of *IKZF1* deletions were SALSA MLPA probe mix P202-B1 *IKZF1* and P335 ALL-*IKZF1* (MRC-Holland, Amsterdam, The Netherlands). Single-nucleotide polymorphism array analysis was performed using CytoScan HD arrays

(Affymetrix, Santa Clara, CA, USA) and data analysis was done with the Chromosome Analysis Suite (ChAS) version 3.1 (Affymetrix). Summary of methods and analyzed samples can be found in Supplementary Methods, Supplementary Figure 1 and Supplementary Table 1.

### Mutation and CNA detection from WES

Reads were aligned with BWA-MEM and mutations called with MuTect. SnpEff, dbNSFP and custom Perl scripts were used for variant annotation and filtering. WES-based CNA detection was performed with exomeCopy.

### RNA sequencing and differential gene expression analysis

Total RNA was extracted from frozen primary leukemic samples ( $> 90\%$  blasts) upon Ficoll gradient separation using Quick-RNA MiniPrep kit (Zymo Research, Irvine, CA, USA). Selection of cases was based on the availability of material. Sequencing libraries were prepared using the NEBNext Poly(A) mRNA Magnetic Isolation Module and the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) and sequenced on a HiSeq 2000 (Illumina) at the Genomics Core Facility, EMBL, Heidelberg. RNA-Seq data analysis was performed with a custom pipeline implemented in Anduril using GSNAP for read alignment, htseq-count for read counting and DESeq2 for differential gene expression analysis. Gene set enrichment analysis was performed with GSEA.

### Statistical analyses

Associations between categorical variables were examined using Fisher's exact and Mann–Whitney tests. Event-free survival (EFS) and overall survival (OS) were analyzed according to the Kaplan–Meier method and compared by the log-rank test.

### Data availability

WES and RNA-Seq data have been deposited at the European Genome-phenome Archive (accession number EGAS00001001847). WES data are available for academic purposes by contacting the corresponding author, as the patient/parent consent does not cover depositing data that can be used for large-scale determination of germline variants.

## RESULTS

Somatic sequence and CNAs of 41 *P2RY8-CRLF2*-positive ALL cases are summarized in Figure 1. Including mutations with an allelic frequency of  $\geq 10\%$ , the median number of somatic, predicted deleterious sequence mutations per case was 12 (range, 4–44) at initial diagnosis and 14 (range, 4–474) at relapse (Supplementary Methods, additional file 2). CNAs were detected using a custom pipeline that predicts CNAs/microdeletions with high sensitivity and specificity from WES data. Single-nucleotide polymorphism array, MLPA and *P2RY8-CRLF2* breakpoint analyses served for validation (Supplementary Figures 2 and 3, additional files 3 and 4). Integrated analysis of genomic sequence and CNAs showed that all leukemias had at least one aberration in putative leukemic driver genes that were grouped according to pathways or biological processes (Figure 1). In addition, copy number and sequence mutations are represented according to the constitutional status of cases (Supplementary Results and Supplementary Figure 4).

### Clonal heterogeneity and instability of kinase-activating pathway alterations

Irrespective of the presence of a *P2RY8-CRLF2* fusion, 31/60 leukemic samples harbored mutations in JAK/STAT pathway components: *JAK2* ( $n = 21$ ), *CRLF2* ( $n = 3$ ), *JAK3* ( $n = 3$ ), *JAK1* ( $n = 2$ ), *IL7RA* ( $n = 1$ ) and *SYK* ( $n = 1$ ; Supplementary Table 2). *JAK1* and, more frequently, *JAK2* mutations are known to occur in BCP ALL,<sup>31,32</sup> whereas the recurrent, activating *JAK3* mutation in the pseudokinase domain (Supplementary Figure 5) has only been documented in JMML and T-ALL so far,<sup>33,34</sup> but drives B-cell leukemia in mice.<sup>35</sup> All mutations in *CRLF2* (F232C) and *IL7RA* (S185C) are activating mutations and have been reported previously.<sup>4,6,36</sup>

**Table 1.** Clinical characteristics and response criteria of study and control cohorts of *P2RY8-CRLF2* major clone-positive ALL cases at initial diagnosis

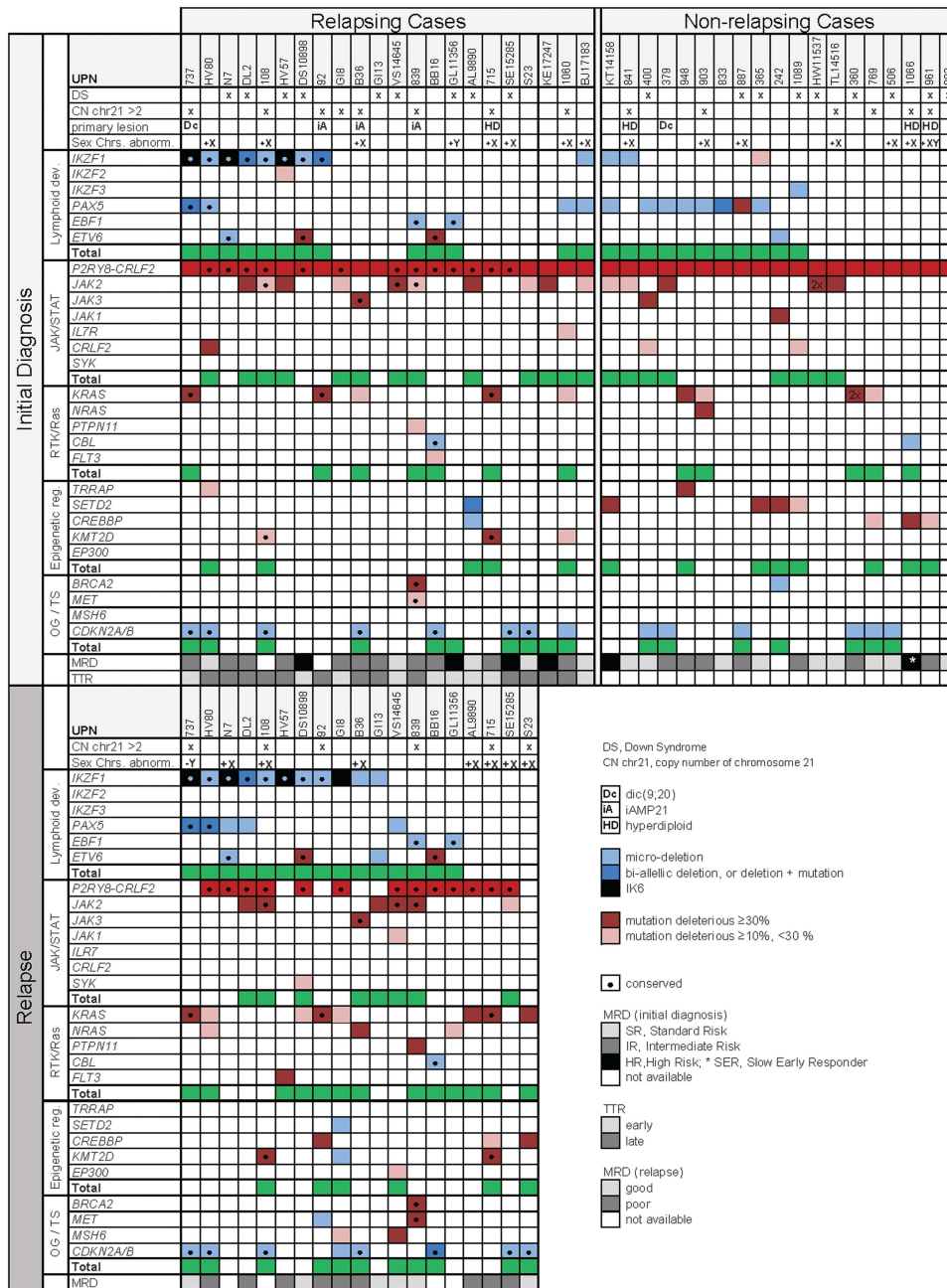
Cohort variable/category	Non-relapsing	Relapsing	P-value	This study	Control BFM AT <sup>a</sup>	P-value
Total number of cases	19 (46%)	22 (54%)	—	41 (100%)	20 (100%)	—
Down syndrome	9 (47%)	9 (41%)	0.76	18 (44%)	9 (45%)	1.00
Age at dx (median, range; yrs)	3.8 (1.8–6.6)	4.2 (1.5–9.6)	0.42	4.0 (1.5–9.6)	4.0 (2.2–18.2)	0.22
Sex						
Female	6 (32%)	12 (55%)	0.21	18 (44%)	7 (35%)	0.59
Male	13 (68%)	10 (45%)		23 (56%)	13 (65%)	
WBC 10 <sup>9</sup> /l	9.2	21.5		9.6	7.8	
Median (range)	(1.7–114)	(3.6–425)		(1.7–452)	(0.5–222)	
< 50	17	11	0.0007	28	18	0.1
> 50	2	11		13	2	
Pred response						
Good	18 (95%)	21 (95%)	—	39 (95%)	20 (100%)	—
NA	1 (5%)	1 (5%)		2 (5%)	0	
Complete remission						
Yes	18 (95%)	22 (100%)	—	40 (97%)	20 (100%)	—
No	1 (5%)	0		1 (3%)	0	
MRD RG						
SR	9 (47%)	5 (23%)	0.50	14 (34%)	3 (15%)	0.36
IR	7 (37%)	11 (50%)		18 (44%)	13 (65%)	
HR	2 (11%)	4 (18%)		6 (15%)	2 (10%)	
NA	1 (5%)	2 (9%)		3 (7%)	2 (10%)	
Treatment arm						
SR	9 (47%)	5 (23%)	0.30	14 (34%)	3 (15%)	0.21
IR	8 (42%)	13 (59%)		21 (51%)	15 (75%)	
HR	2 (11%)	4 (18%)		6 (15%)	2 (10%)	

Abbreviations: ALL, acute lymphoblastic leukemia; FISH, fluorescence *in situ* hybridization; HR, high risk; IR, intermediate risk; NA, not available; SNP, single-nucleotide polymorphism; SR, standard risk; WBC, white blood cell count. Observation time is too short for assessment of relapse incidence, but was found to be ~35% in the previous study<sup>9</sup>. Statistical significance (P-value) according to Fisher's exact test (Down syndrome, sex, minimal residual disease-based risk group (MRD RG), treatment arm) or Mann-Whitney test (age, WBC). <sup>a</sup>The control group comprises cases recruited to the current BFM 2009 study in Austria who were identified as major clone *P2RY8-CRLF2*-positive (by FISH and/or SNP array, genomic breakpoint and transcript analysis).

**Table 2.** Clinical and biological data of relapsing *P2RY8-CRLF2*-positive ALL cases

UPN	Sex	Age Dx (yrs)	Risk Dx	Rel. site	TTR	MRD Rel	Second rem (mo)	Outcome	<i>P2RY8-CRLF2</i> conserved
BB16	M	6.1	IR	BM	4	NA	7	†	Yes
715 <sup>a</sup>	M	8.9	IR	BM	17	Poor	12	†	Yes
BJ17183	F	7.6	SR	CNS	24	NA	78	CCR2	NA
G18	M	2.6	IR	BM	24	Poor	1	†	Yes
B36 <sup>a</sup>	M	4.6	IR	BM	33	Poor	112	CCR2	No
KE17247	M	1.8	HR	BM	40	Good	40	†	NA
HV80 <sup>a</sup>	F	2.7	SR	BM	42	Poor	76	CCR2	Yes
92	F	9.4	NA	BM	43	Poor	203	CCR2	No
839	F	8.1	IR	BM	43	Good	20	CCR2	Yes
108	M	4.1	NA	BM	56	NA	20	†	Yes
1060	F	3.3	IR	CNS	69	Poor	1	CCR2	NA
S23 <sup>a</sup>	F	1.5	SR	BM	97	Good	27	CCR3	No
<b>GL11356<sup>b</sup></b>	<b>M</b>	<b>4.3</b>	<b>HR</b>	<b>BM</b>	<b>24</b>	<b>NA</b>	<b>98</b>	<b>NA</b>	<b>No</b>
<b>VS14645<sup>b</sup></b>	<b>F</b>	<b>3.0</b>	<b>SR</b>	<b>BM</b>	<b>27</b>	<b>Good</b>	<b>130</b>	<b>CCR2</b>	<b>Yes</b>
<b>AL9890<sup>b</sup></b>	<b>F</b>	<b>2.6</b>	<b>SR</b>	<b>BM</b>	<b>27</b>	<b>Poor</b>	<b>13</b>	<b>†</b>	<b>Yes</b>
<b>DS10898<sup>b</sup></b>	<b>F</b>	<b>7.9</b>	<b>HR</b>	<b>BM</b>	<b>29</b>	<b>Good</b>	<b>2</b>	<b>†</b>	<b>Yes</b>
<b>SE15285<sup>b</sup></b>	<b>F</b>	<b>3.8</b>	<b>HR</b>	<b>BM</b>	<b>34</b>	<b>Poor</b>	<b>12</b>	<b>†</b>	<b>Yes</b>
<b>DL2<sup>b</sup></b>	<b>M</b>	<b>4.6</b>	<b>IR</b>	<b>BM</b>	<b>36</b>	<b>Poor</b>	<b>14</b>	<b>CCR2</b>	<b>Yes</b>
<b>GI13<sup>b</sup></b>	<b>F</b>	<b>2.0</b>	<b>IR</b>	<b>BM</b>	<b>62</b>	<b>Good</b>	<b>100</b>	<b>CCR2</b>	<b>Yes</b>
<b>HV57<sup>a,b</sup></b>	<b>F</b>	<b>4.1</b>	<b>IR</b>	<b>BM</b>	<b>65</b>	<b>Poor</b>	<b>15</b>	<b>†</b>	<b>No</b>
<b>N7<sup>a,b</sup></b>	<b>M</b>	<b>8.7</b>	<b>IR</b>	<b>BM</b>	<b>67</b>	<b>NA</b>	<b>0</b>	<b>†</b>	<b>Yes</b>

Abbreviations: †, death; ALL, acute lymphoblastic leukemia; BM, bone marrow; CCR, complete continuous remission; CNS, central nervous system; DS, Down syndrome; F, female; HR, high risk; IR, intermediate risk; M, male; NA, not available; second rem, second remission in months; SER, slow early responder; SR, standard risk; TTR, time to relapse in months; UPN, unique patient number death. <sup>a</sup>Included in our previous publication. <sup>b</sup>DS (highlighted in bold).

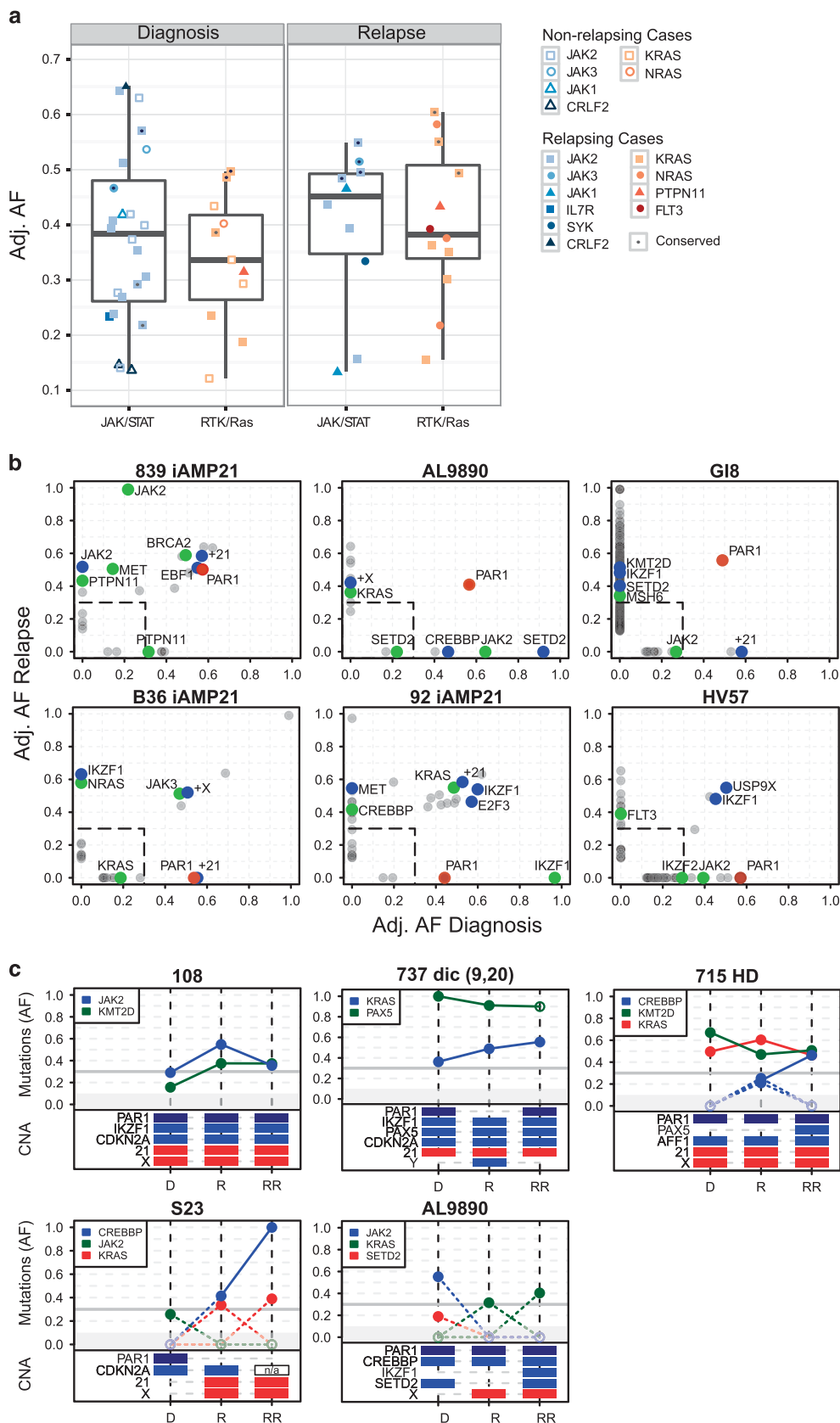


**Figure 1.** Genomic sequence and copy number alterations of 41 *P2RY8-CRLF2*-positive ALL cases. Recurrently altered genes in relapsing cases (left) and non-relapsing cases (right) in columns. Non-silent, predicted deleterious sequence and copy number alterations in genes (rows) are listed according to functional groups in diagnostic (top) and relapse (bottom) samples. Mutations are marked by color codes (as indicated) to show their clonal or subclonal nature based on adjusted allelic frequency (adj. AF), predicted functional effects and conservation from diagnosis to relapse. CN chr21 > 2, somatic gain of chromosome 21; DS, Down syndrome; OG, oncogene; Sex Chrs. abnorm, copy number aberration of sex chromosomes; TS, tumor suppressor; TTR, time to relapse; UPN, unique patient number (columns).

**Figure 2.** Clonal composition and stability of signaling mutations. **(a)** Clonal composition of JAK/STAT (blue) and RTK/Ras pathway (red) signaling gene mutations according to individual genes (symbol code). Dots represent the adjusted allelic frequency (adj. AF) of mutations. Black dots mark conserved mutations at diagnosis and relapse. **(b)** Representative examples of the clonal distribution of selected abnormalities at diagnosis and relapse with either a conserved (top three) or lost (bottom three) *P2RY8-CRLF2* fusion. Blots show the adj. AF of deleterious sequence (green), copy number (blue) and *P2RY8-CRLF2* (pseudautosomal region 1 (PAR1); red) alterations at initial diagnosis (x axis), and at relapse (y axis). Dashed lines indicate the border between subclonal and clonal mutations. **(c)** Mutational patterns of genomic alterations of cases with multiple relapses. Adj. AF of sequence and copy number alterations (y axis) at indicated leukemia occasions (x axis). Dashed lines highlight the loss or gain of distinct mutations. A gray horizontal line separates subclonal from clonal ( $\geq 30\%$  adj. AF) mutations. D, initial diagnosis; R, first relapse; RR, second relapse.

At initial diagnosis, the overall frequency of JAK/STAT pathway mutations was 51% and equally distributed between relapsing and non-relapsing cases. They were subclonal in 47% of cases,

including two with two mutations each. Notwithstanding of their original extent, JAK/STAT pathway mutation carrying clones were lost at relapse in 60% of the cases. Two subclonal mutations



developed into the dominant clone and four were newly acquired, resulting in an overall relapse frequency of 42% (Figures 1, 2a and 4a; Supplementary Table 2).

Mutations in RTK/Ras pathway genes were seen in 29% of the cases at diagnosis, whereby the majority of them concurred with a somatic gain of chromosome 21, but, in accordance with a recent publication, they were only found in 1/23 DS ALL cases.<sup>37</sup> In line with the high instability of these clones, the frequency of such mutations increased to 68% in relapses ( $P=0.06$ ), including four with DS (Figures 1, 2a and 4a; Supplementary Table 3). Alterations preferably affected *KRAS*, followed by *NRAS*, *FLT3*, *PTPN11* and *CBL*.

Taken together, JAK/STAT (excluding *P2RY8-CRLF2*) and RTK/Ras pathway mutations were found in 78% of all cases at diagnosis and increased to 95% at relapse, but the original mutations were nevertheless only conserved in less than half of the cases (Supplementary Figure 6). In accordance with this finding, *P2RY8-CRLF2* was also lost in 32% of relapses. It is worth noting that RTK/RAS and JAK/STAT pathway alterations were found in three instances, in two of them they obviously even concurred within the same clone.

Recurrent alterations affecting lymphoid differentiation and tumor suppressor genes

The vast majority of alterations in genes that are critical for B-cell development and differentiation, tumor suppression and cell cycle regulation are microdeletions. Including CNA data from additional 11 non-relapsing ALL cases (Supplementary Figure 7), the total evaluable number of cases amounts to 52.

At diagnosis, 58% of them had lesions in lymphoid differentiation genes, which accords with previous observations.<sup>38</sup> They comprise alterations in *PAX5* (30.7%), *IKZF1* (23%), *ETV6* (11.5%), *EBF1* (4%) and *IKZF3* (2%). Deletions in the tumor suppressor/cell cycle regulator genes *CDKN2A/B* were found in 38.5%.

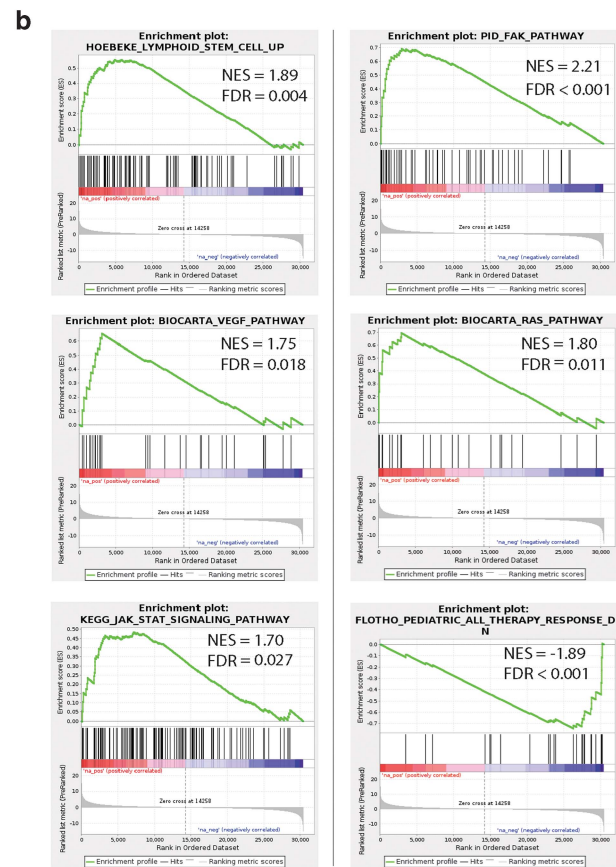
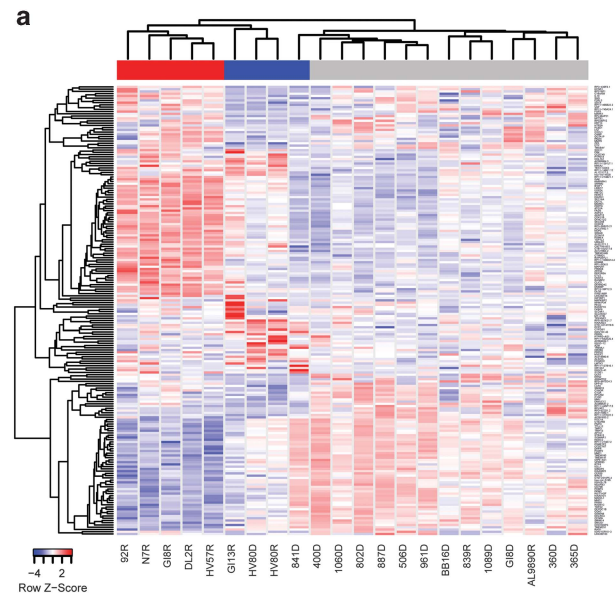
At relapse, respective sequence and CNA were always conserved and only few were additionally acquired in some instances, altogether adding up to 58% *IKZF1*, 42% *CDKN2A/B* and 26% *PAX5* deletions, respectively (Figure 1 and 4a). Similarly, all four *ETV6* alterations (two deletions and two mutations in the ETS domain (E425\* and R369P<sup>39</sup>)) remained conserved. The E425\* mutation is novel in BCP ALL.

Gains or losses of sex chromosomes were seen in 27% of relapsing and non-relapsing cases. Sex chromosome losses at relapse did not concord with that of *P2RY8-CRLF2*, which indicates that at least in the informative cases relapses must have evolved from a fusion-negative ancestral clone.

Relapse-predicting and -associated alterations in *P2RY8-CRLF2*-positive ALL

Except for *IKZF1*, the frequency of alterations in genes implicated in lymphoid development and in tumor suppression/cell cycle regulation did not significantly differ between relapsing and non-relapsing cases. *IKZF1* alterations prevailed in the relapsing cohort with a frequency of 41% versus 10% in non-relapsing cases ( $P=0.001$ ). Two of the latter had deletions and one a subclonal mutation (Figure 1; Supplementary Table 4).

At relapse, 58% of cases harbored an *IKZF1* alteration (including three relapse-specific ones), whereby all initially detected *IKZF1* alterations remained conserved. The only exception was one case (#92) with an originally biallelic alteration, in which the G158S mutation<sup>27,40</sup> was lost but the deletion preserved. Intragenic *IKZF1* deletions result in the expression of the dominant-negative isoform (IK6) in 36% of relapses, whereas deletions of the entire gene presumably lead to haploinsufficiency.<sup>16,17,41</sup> In two cases a dominant-negative acting *IKZF1* mutation concurred with a deletion. In 63% of cases, either *PAX5* and/or *CDKN2A/B* alterations were also present implying that these combinations might confer an evolutionary advantage.<sup>17,27</sup>



**Figure 3.** Transcriptional signature of leukemias according to *IKZF1* status. (a) Cluster heatmap of the top 50 up and downregulated genes in both IKN (*IKZF1* alterations leading to a dominant-negative effect and biallelic alterations) and IKD (*IKZF1* deletions resulting in haploinsufficiency) samples according to fold-change ( $P \leq 1E-8$  for IKN versus IKC,  $P \leq 2E-3$  for IKD versus IKC); IKN cases are indicated in red, IKD ones in blue and controls in gray at the top of the map. (b) Selected gene set enrichment plots showing a significant concordant regulation with IKN and IKD signatures.  $P$ -values are indicated in each analysis. FDR, false discovery rate; NES, normalized enrichment score.

In line with previous reports, we noted also a high number of alterations (32–38%) in epigenetic regulator encoding genes such as *CREBBP*, *KMT2D* and *SETD2*.<sup>30,42–44</sup> However, the frequency of these alterations was similar in relapsing and non-relapsing cases as well as in diagnosis and relapse samples, which indicates that they have little direct relevance in the disease recurrence process.

#### Size and kinetics of mutant clones in relapsing leukemias

Given the instability of *P2RY8-CRLF2*-positive clones,<sup>9</sup> we next compared the mutation patterns in matched diagnosis and relapse samples from the 19 cases in different settings. Figure 2b shows six prototypic cases, three in which the *P2RY8-CRLF2* fusion was conserved (top) and three in which it was lost at relapse (bottom). Other concurring kinase-activating alterations were either retained ( $n=6$ ), replaced by another one ( $n=5$ ), lost ( $n=1$ ) or evolved from an initial subclonal to a clonal one at relapse ( $n=2$ ). Moreover, five cases without an initial kinase-activating mutation acquired one at relapse (Supplementary Figure 8). The respective patterns were similar in cases with conserved or lost *P2RY8-CRLF2* fusions.

In five cases we were also able to study subsequent relapses (Figure 2c). *CDKN2A*, *PAX5* and *IKZF1* were either retained or newly acquired in second relapses (#715, S23, AL9890), whereas kinase-activating alterations were exchanged in the second relapse in three cases.

#### *IKZF1* alterations are associated with dismal outcome

Except for the genomic *IKZF1* status and white blood cell count that were not associated with each other ( $P=0.66$ ), we found no other biological or clinical parameters such as other genetic alterations, age at diagnosis, clinical risk group assignment or morphological and molecular response to treatment to be correlated with the occurrence of relapses. There was no difference between DS and non-DS cases. Of note, only one of eight *IKZF1*-altered relapsing cases had initially a poor MRD response and was, therefore, assigned to high-risk treatment, but none of them had initially a poor response to glucocorticoids

*in vivo*, as one might have expected based on *in vitro* experiments.<sup>45</sup> Yet, *IKZF1*-mutated cases had a significantly poorer outcome than their *IKZF1* wild-type counterparts as evidenced by an adverse pEFS ( $P=0.026$ ) and pOS ( $P=0.051$ ; Supplementary Figure 9).

#### *IKZF1* alterations display distinctive transcriptional signatures

As *IKZF1* alterations are highly associated with relapses in *P2RY8-CRLF2*-positive ALL cases, we profiled 22 leukemia samples and analyzed the RNA-Seq data according to their *IKZF1* status (Supplementary Table 4). We designated IK6 deletions and biallelic alterations as 'IKN', larger deletions of *IKZF1* as 'IKD' and wt*IKZF1* ones as 'IKC'. These analyses revealed specific regulations in the IKN group and, albeit to a much lesser degree, in the IKD group compared with the IKC one (Supplementary Methods, additional files 5 and 6). Unsupervised hierarchical clustering of the 200 most differentially expressed genes in the IKN and IKD cohort grouped them according to their *IKZF1* status (Figure 3a; Supplementary Figure 10). This finding was confirmed in an independent RNA-Seq data set of 20 mainly B-other ALL cases with known *IKZF1* status, where a significant correlation in the log<sub>2</sub>-fold expression of the top 50 up- and downregulated genes from the IKN cohort was observed ( $P=0.0039$ , Spearman correlation; Supplementary Methods, additional file 7).

Consequently, we used the IKN and IKD cohorts for gene set enrichment analysis and found a highly significant correlation of differentially expressed genes with developmental and biological processes (Supplementary Methods, additional file 8). Representative examples are shown in Figure 3b and Table 3. Differentially expressed genes in the IKN group, and less so in the IKD cohort, were significantly enriched for various human hematopoietic and lymphoid stem cells sets, and concordantly for gene sets specifically expressed in immature B cells. This suggests that *IKZF1* alterations lead to impaired B-cell differentiation and the acquisition of stem cell-like features, as described for murine and human *BCR-ABL*<sup>+</sup> leukemias and 'Ph-like' human leukemias.<sup>17,27</sup> Consistent with the adhesive properties of *Ikzf1*<sup>-/-</sup> pro/pre-B cells

**Table 3.** Selected gene sets enriched in *IKZF1*-altered samples

Gene set	IKN			IKD		
	NES	P-value	FDR	NES	P-value	FDR
GAL_LEUKEMIC_STEM_CELL_DN	-2.2	0	0	-1.9	0	2e-3
GENTLES_LEUKEMIC_STEM_CELL_DN	-1.9	0	1e-3	-1.7	0.01	0.04
HOEBEKE_LYMPHOID_STEM_CELL_UP	1.9	0	4e-3	1.9	0	0.04
MORI_IMMATURE_B_LYMPHOCYTE_DN	-2.6	0	0	-2.2	0	2e-5
MANALO_HYPOXIA_DN	-2.1	0	3e-5	-1.5	0	0.14
PID_FAK_PATHWAY	2.2	0	0	1.3	0.08	0.39
PID_VEGFR1_2_PATHWAY	2.1	0	6e-5	0.9	0.78	0.87
REACTOME_CELL_CYCLE	-2.4	0	0	-1.8	0	0.02
KEGG_DNA_REPLICATION	-2.3	0	0	-1.5	0.04	0.13
PID_AURORA_B_PATHWAY	-2.2	0	0	-2.0	0	1e-3
HALLMARK_DNA_REPAIR	-2.1	0	3e-5	-1.4	0.01	0.17
REACTOME_G2_M_DNA_DAMAGE_CHECKPOINT	-1.9	0	4e-3	-1.5	0.05	0.12
ST_JAK_STAT_PATHWAY	1.8	0	8e-3	1.5	0.07	0.25
REACTOME_PI3K_CASCADE	1.9	0	6e-3	1.4	0	0.34
BIOCARTA_RAS_PATHWAY	1.8	2e-3	0.01	0.8	0.75	0.90
BIOCARTA_MAPK_PATHWAY	2.1	0	7e-5	1.5	3e-3	0.27
FLOTHO_PEDIATRIC_ALL_THERAPY_RESPONSE_DN	-1.9	0	6e-4	-1.1	0.34	0.56
RHEIN_ALL_GLUCOCORTICOID_THERAPY_DN	-2.7	0	0	-2.3	0	0
HOLLEMAN_VINCRISTINE_RESISTANCE_B_ALL_UP	1.9	2e-3	3e-3	1.1	0.37	0.59
HOLLEMAN_DAUNORUBICIN_ALL_UP	1.7	0	0.04	0.9	0.58	0.80

Abbreviations: FDR, false discovery rate; NES, normalized enrichment score. MSigDB gene sets enriched in *IKZF1* dominant-negative or biallelic-altered (IKN) and *IKZF1*-deleted (IKD) samples. (positive: enrichment in upregulated genes; negative: enrichment in downregulated genes).

in mice,<sup>46,47</sup> gene sets that are upregulated in the context of microenvironment, focal adhesion kinase and integrin pathways were enriched. We also found an enrichment of genes that are higher expressed in response to hypoxia, downstream VEGF/VEGFR signaling and to EPO signaling.

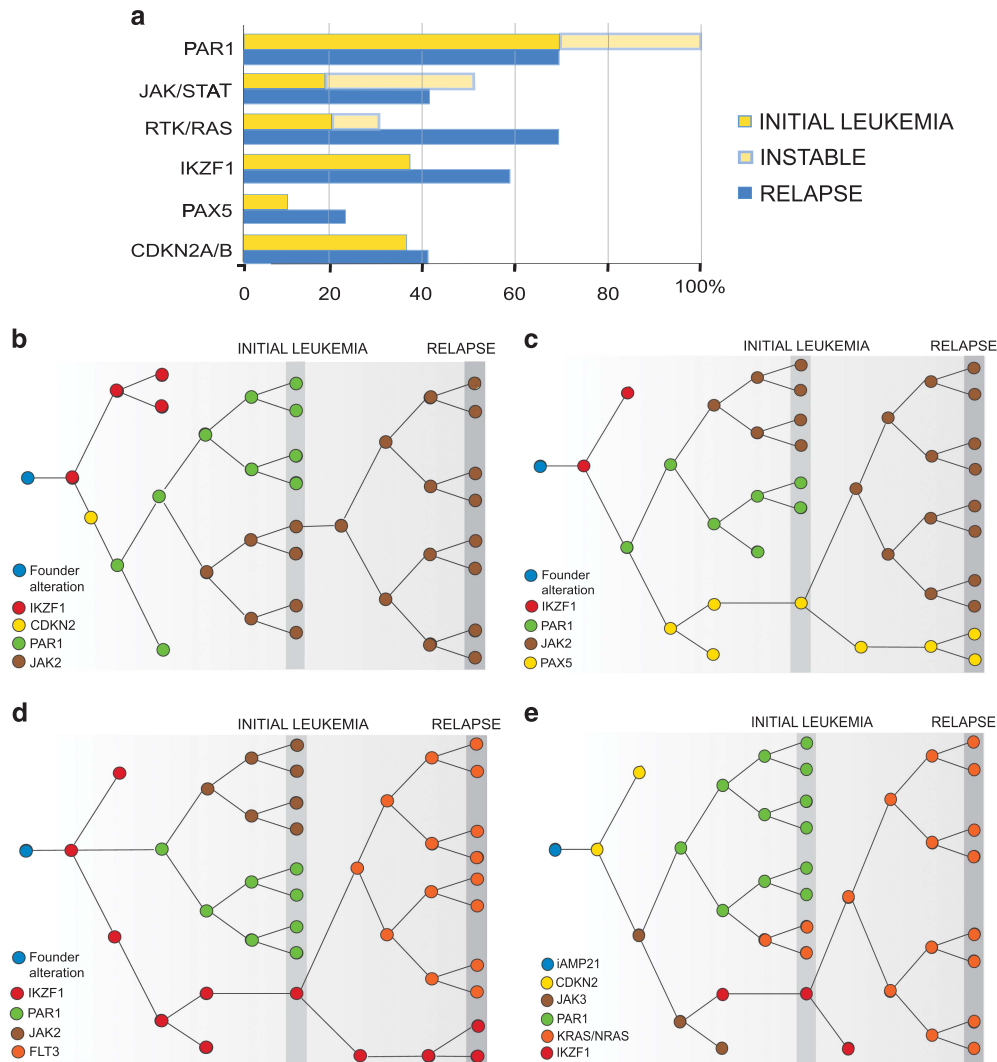
In line with normal murine pro-B cells lacking *Ikzf1*,<sup>46</sup> genes critical for cell cycle regulation were negatively enriched in *IKZF1*-altered compared with wt/*IKZF1* samples and they had lower expression of genes involved in DNA replication and proliferation. Moreover, *IKZF1*-mutant samples were negatively enriched for major DNA damage and repair pathways. By contrast, JAK/STAT, PI3K and mTOR, as well as Ras and MAPK pathway signaling gene sets were positively enriched in the *IKZF1*-mutant samples, a finding that partly might reflect the proposed negative regulation

of STAT5 signaling by *IKZF1*.<sup>48</sup> In concordance with the enrichment of resistance gene sets derived from primary childhood ALL samples, the majority of our relapse cases with *IKZF1* alterations had also a poor response to chemotherapy (Figure 1; Table 2).

Our *IKZF1* signature was further corroborated by comparison with respective data from human leukemias and murine models by revealing a significant enrichment of differentially expressed genes in *IKZF1*-mutant samples (Supplementary Figure 11).

## DISCUSSION

CRLF2 overexpression is the common hallmark of two distinct genetic subtypes of childhood ALL with a high recurrence risk.<sup>2,3,13</sup> Most of the cases with *IGH-CRLF2* translocations belong to the



**Figure 4.** Clonal evolution of leukemia and selection of relapse clones. On the basis of the frequency and stability of genomic alterations, depicted in (a) for the most frequently altered genes and pathways, we envision that RAG-mediated microdeletions affecting B-cell differentiation and tumor suppressor genes follow the occurrence of a leukemia-initiating (founder) alteration. Given their stability and increased frequency at relapse they apparently foster resistance and emergence of relapse. The ensuing continuous emergence of JAK/STAT and/or RTK/Ras pathway mutations eventually leads to the clinical manifestation of leukemia, whereby the type of proliferation-promoting alterations greatly varies. (b) Shown is case 108, which harbors, in addition to alterations in *IKZF1*, *CDKN2A/B* and *P2RY8-CRLF2* (pseudoautosomal region 1 (*PAR1*) deletion), also a *JAK2* mutation in 50% of the population. The latter clone predominates at relapse. (c) Similarly, case DL2 carries a *JAK2* mutation in 70% of the *P2RY8-CRLF2*<sup>+</sup>, *IKZF1*-mutant population at diagnosis. The initial *JAK2* mutation is, however, replaced by another one at relapse, where also a *PAX5* deletion is present. (d) Case HV57, harboring an *IKZF1* alteration, illustrates the loss of an initial predominant *P2RY8-CRLF2*<sup>-</sup> and *JAK2*-mutant clone (the latter affecting ~60% of cells) and the emergence of a fusion negative, *FLT3*-mutant one. (e) In case B36, the *P2RY8-CRLF2* fusion occurs in a *CDKN2A/B*- and *JAK3*-mutant clone and even harbors a *KRAS* mutation in 36% of cells. At relapse, *P2RY8-CRLF2*<sup>-</sup> and *KRAS*-mutant clones are replaced by an *IKZF1*-altered population carrying an *NRAS* mutation. Color code shown as inset of the graph.



high-risk group, whereas those with a *P2RY8-CRLF2* fusion primarily fall into the intermediate-risk group.<sup>8,9,12–14</sup> The genomic components of the latter and especially their role in the disease and particularly in the relapse process remain, to a large extent, elusive. We therefore analyzed the genomic and transcriptional profile of altogether 41 cases, including 21 cases with DS, that had a *P2RY8-CRLF2* fusion in the dominant clone at initial diagnosis. They comprised 22 relapsing cases with 19 matched diagnosis and relapse pairs as well as 20 non-relapsing ones. This largest cohort of its kind reported so far allowed us to determine and examine those genetic lesions that were lost, retained or acquired in relapses.

The *P2RY8-CRLF2* fusion was lost in one-third of the relapses. Together with previous observations—that, for instance, these fusions frequently affect subclones that never evolve into major relapse clones—indicate that it is primarily a secondary change that may potentially supply the respective cells with a certain proliferative but certainly not with an evolutionary advantage.<sup>9</sup>

In line with other types of childhood BCP ALL,<sup>49</sup> B-cell differentiation genes are also commonly deleted in cases with a *P2RY8-CRLF2* fusion. These abnormalities are usually preserved in the corresponding relapses. Consistent with their role in drug resistance, *IKZF1* alterations prevail already in relapse-prone cases at diagnosis but become even more abundant in relapses. *IKZF1*-deleted cases also seem to profit from other associated B-cell development and cell cycle gene defects, particularly those of *PAX5* and/or *CDKN2A/B*, which are found in half of the cases, but also from mutations in specific proliferation-promoting pathway genes.<sup>16,17,50</sup>

Aside from a high white blood cell count, *IKZF1* deletions are also the only relapse-predicting molecular indicator of *P2RY8-CRLF2*-positive ALL cases. As *IKZF1* deletions as well as the vast majority of other genomic abnormalities are similarly frequent in *P2RY8-CRLF2*-positive DS and non-DS ALL at diagnosis and relapse, one can expect that *IKZF1* deletions have a similar role in both cohorts. This seems indeed to be the case, although confounding factors, such as the greater treatment toxicity in DS patients, may influence and bias such outcome results to some extent.<sup>23,51,52</sup>

As primarily reported for DS ALL, our *P2RY8-CRLF2*-positive cases frequently harbor JAK/STAT but also Ras pathway mutations, with those in the first pathway prevailing at diagnosis.<sup>3–5,10,37,53,54</sup> These alterations were usually subclonal, even concurred in three cases, and were lost at relapse in half of the cases. Although almost all relapse cases had mutations in genes that are involved in either signaling pathway, those affecting the Ras pathway predominated. This switch from the JAK/STAT to the Ras pathway was neither related to the preexisting or acquired copy number state of chromosome 21 nor to the leukemias' underlying primary abnormality as reported in DS ALL.<sup>37</sup> As *P2RY8-CRLF2*-positive leukemias not only have JAK/STAT but also PI3K/mTOR signaling activated,<sup>55,56</sup> it is interesting to note that RAS alterations activate both the RAF/MEK/ERK and PI3K/mTOR pathways.<sup>57</sup> Thus, this leukemia subgroup seems to be particularly promiscuous regarding its preference for proliferative driver mutations, which they likely require for the emergence of drug-resistant relapses. Although *IKZF1* deletions might enhance STAT5 signaling in JAK-mutated cases,<sup>48</sup> our findings provide some circumstantial evidence that Ras pathway alterations could interact in a similar fashion and substitute those in the JAK/STAT pathway.

Taken together, we consider the *P2RY8-CRLF2* fusion as one of the secondary proliferative driver alterations that—in line with those activating JAK/STAT and RTK/Ras pathways—are highly unstable at relapse, albeit to a lesser degree if initially present in the major clone,<sup>30</sup> and not as a *bona fide* primary genetic alteration that is always stable at relapse and critical for the maintenance of the leukemia (Figure 4a). We, therefore, illustrate the various scenarios by showing prototypic cases where either the *P2RY8-CRLF2* fusion and/or a JAK/STAT and/or RTK/Ras

pathway mutation is lost at relapse, and instead another proliferative driver mutation in one of the two pathways occurs (Figures 4b–e). As chemotherapy primarily eliminates the bulk of rapidly proliferating cells, it spares those less active, resistant stem cell-like ones that eventually generate relapses.<sup>58</sup> In this scenario, the *P2RY8-CRLF2* fusion is only one of several proliferation-activating alterations and merely serves as a common marker for an otherwise genetically heterogeneous group, whose other and probably more relevant features are *IKZF1* deletions. These deletions are well-known disease drivers in many types of drug-resistant leukemias, such as *BCR-ABL1*-positive ones, which share a similar gene expression signature.<sup>11,24</sup> The transcriptional profile of *IKZF1*-deleted cases reflects their strong homing preference to the bone marrow niche as well as their high repopulation capacity, attributes that also become apparent in mouse models in which *Ikzf1*<sup>−/−</sup> pro/pre-B cells acquire stem cell and adhesion properties including activation of the focal adhesion kinase pathway.<sup>27,46,47,59</sup>

Besides these biological insights, our findings also provide some clues that may become relevant in future treatment decisions. Apart from their prognostic implications, *IKZF1* alterations may eventually serve as markers for specific therapeutic interventions. It appears reasonable to try to restore IKAROS signaling especially in those *IKZF1*-deleted cases that still have retained a functional wild-type allele. For the other 20% of cases with biallelic *IKZF1* alterations,<sup>60</sup> inhibition of the activated focal adhesion kinase pathway may become a viable treatment option. Such approaches might perhaps be combined with a cocktail of other signaling inhibitors given the availability of various JAK/STAT, Ras/MEK/ERK and PI3K/mTOR pathway inhibitors.<sup>55</sup>

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## AUTHOR CONTRIBUTIONS

CV performed wet lab work, analyzed and compiled data, and contributed to manuscript writing; CF performed bioinformatics and statistical analyses, and contributed to manuscript writing; CE, GC, UzS, HC, AA, AvS, MS, MAH, GM provided patient samples and clinical information; AM, FK and SF performed experiments; KN performed SNP array analysis; MS and CB performed WES and primary analysis; OAH is responsible for cytogenetics, MLPA and SNP array analysis, and contributed to manuscript writing; RP-G conceived the study, supervised research, interpreted results and wrote the paper.

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