



Published in final edited form as:

*Cancer Res.* 2010 October 1; 70(19): 7347–7352. doi:10.1158/0008-5472.CAN-10-1528.

## CRLF2 and JAK2 in B-Progenitor Acute Lymphoblastic Leukemia: A Novel Association in Oncogenesis

J. Devon Roll and Gary W. Reuther

Department of Molecular Oncology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, 33612

### Abstract

Expression of cytokine receptor-like factor 2 (CRLF2) has recently been shown to be upregulated as well as mutated in populations of B-ALL, including Down syndrome (DS-ALL) patients, which lack recurring chromosomal translocations. Increased CRLF2 expression associates with JAK2 mutation, a combination that transforms hematopoietic cells, suggesting that mutant JAK2 and CRLF2 may cooperate to contribute to B-ALL formation. Importantly, elevated CRLF2 expression correlates with poor outcome in high-risk B-ALL patients. Therefore, CRLF2 may provide a new prognostic marker for high-risk B-ALL and inhibition of CRLF2/JAK2 signaling may represent a therapeutic approach for this population of ALL patients.

### Keywords

CRLF2; JAK2; B-ALL; oncogenesis; leukemia

### Introduction

B-ALL is a heterogeneous disease of which a number of different subtypes have been identified. While each subtype tends to progress to an aggressive disease in both children and adults, the various forms of B-ALL can be differentiated by their gene expression profiles, which are highly associated with specific chromosomal alterations (1). For example, chromosomal rearrangements involving the *MLL*, *TCF3*, and *ETV6* genes, as well as *BCR/ABL*, constitute important disease markers and help physicians select treatments to which patients are most likely to respond (1,2). However, about one-quarter of B-ALL patients lack characteristic chromosomal rearrangements, representing a subset of B-ALL that is not well understood (3). Recently, several research teams have utilized techniques such as fluorescent in situ hybridization, single nucleotide polymorphism (SNP) microarrays, array-based comparative genomic hybridization, gene expression profiling, and cDNA library screens to further identify genetic alterations that may contribute to this subset of B-ALL (4–7). Remarkably, the results of each of these studies converged on a single gene that appears to have a novel role in B-ALL carcinogenesis.

### Chromosomal Rearrangements Lead to CRLF2 Overexpression in ALL

The gene identified by these recent studies is *CRLF2*, which encodes for cytokine receptor-like factor 2 (CRLF2). CRLF2 is a type I cytokine receptor also known as thymic stromal

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Address correspondence to: Gary W. Reuther, Moffitt Cancer Center, MRC3E, 12902 Magnolia Dr., Tampa, FL, 33612, (813) 745-6608 (Phone), (813) 745-6817 (Fax), gary.reuther@moffitt.org.

We apologize to our colleagues whose work we were not able to cite due to space limitations.

lymphopoietin (TSLP) receptor (TSLPR) (8). The CRLF2 subunit forms a heterodimeric complex with IL7RA to generate the functional receptor for TSLP (8). TSLP ligand is produced by epithelial cells in order to activate dendritic cells, and is involved in inflammation and allergic responses (9,10). While this cytokine also mediates B-cell precursor proliferation and survival, neither it nor its receptor components, until now, has ever been implicated in tumorigenesis (11).

The first reports of CRLF2 overexpression in leukemia came from four groups in late 2009 and early 2010 (4–7). Russell et al. utilized FISH analyses on leukemic cells of B-ALL patients and identified two chromosomal abnormalities involving the pseudoautosomal region 1 (PAR1) region of the sex chromosomes (4). These abnormalities included small deletions within PAR1 as well as translocations of this area with the *IGH@* locus of chromosome 14 (4). PAR1 deletions were first described in ALL by Mullighan et al. (12,13) who further refined the boundaries of this region using array-based comparative genomic hybridization (5). These deletions appear to be the result of aberrant use of recombination signals. Importantly, each of these chromosomal abnormalities was found to result in overexpression of CRLF2. PAR1 deletions juxtapose the first non-coding exon of the *P2RY8* gene to the first exon of *CRLF2*. Thus, CRLF2 expression from this chimeric locus is driven by the *P2RY8* promoter (5). Similarly, translocations of the *CRLF2*-containing PAR1 region with the *IGH@* locus leads to expression of CRLF2 via *IGH@* enhancer elements. Similar results were obtained by Hertzberg et al., who utilized gene expression profiling and identified high expression of CRLF2 in DS-ALL (6). This report also confirmed the mechanistic basis of overexpression as the aforementioned *IGH@* translocations and PAR1 deletions. Finally, Yoda et al. identified CRLF2 involvement in B-ALL through a functional screen of patient-derived cDNA designed to identify genes whose expression could lead to the transformation of progenitor-B cells in culture (7). This work suggested aberrant V(D)J recombination signals in PAR1 lead to translocation with the *IGH@* locus and subsequent overexpression of CRLF2. While both mechanisms are common, the frequency of translocations versus deletions that lead to CRLF2 overexpression appears to be dependent on the cohort of samples studied (14).

CRLF2 appears to be overexpressed at different frequencies in B-ALL depending on the subtype, but it has not been found aberrantly expressed in other lymphoid malignancies (7). CRLF2 overexpression appears to occur exclusively in cases where recurring ALL-associated chromosomal translocations are absent (5). Aberrant expression of CRLF2 was found in 12.5%–15% of B-ALL that lack typical chromosomal rearrangements but was not overexpressed in BALL cases that have recurring rearrangements (4–7,14). *CRLF2* alteration is seen at low rates (5%–7%) when all B-ALL cases are grouped together, however, it is seen in a striking 50%–60% of DS-ALL, suggesting CRLF2 overexpression is especially relevant to carcinogenesis in patients with trisomy 21 (4–6). Importantly, rearrangement of CRLF2, associates with *IKZF1* alterations as well as poor treatment outcomes in high-risk pediatric B-ALL (6,7,14). However, correlation of CRLF2 rearrangement and patient outcome appears to be cohort and regimen dependent, as no association was found in a separate study that utilized DS-ALL patients (5). Nonetheless, overexpression of CRLF2 in ALL provides a cell surface marker amenable to detection by flow cytometry for clinical diagnostic purposes.

## CRFL2 Overexpression Associates with JAK2 Mutations in ALL

Initially, Russell et al. observed that CRLF2 could enhance the growth of early B-cell precursors in culture (4). In addition, shRNA knockdown of CRLF2 in B-ALL cell lines only partially inhibited cell growth. These data suggested CRLF2 overexpression alone is not sufficient to transform cells. The researchers hypothesized that cooperating mutations may be involved. B-ALL cell lines with CRLF2 overexpression as well as CRLF2-overexpressing primary lymphoid progenitor cells from mouse fetal liver showed evidence of increased JAK2/

STAT5 signaling (4). The frequency of *JAK2* mutations in ALL has been reported to be about 10% in pediatric high-risk ALL and about 20% in DS-ALL (13,15–17). Investigation of *JAK2* mutation status demonstrated association of *JAK2* mutations (most notably *JAK2*-R683G) in about half of cases with *CRLF2* overexpression in DS-ALL (4). Mullighan et al. demonstrated that in 41 *CRLF2*-rearranged DS-ALL individuals, 14 (34%) also had *JAK2* mutations while only one of 34 individuals (3%) without the *PAR1* deletion had a *JAK2* mutation (5). Similarly, of 26 high-risk pediatric B-ALL patients with overexpressed *CRLF2* analyzed by Harvey et al., 18 (69%) of these had *JAK* mutations (14). Again, this association was rarely observed in patients without *CRLF2* overexpression as the rate of *JAK* mutations in these patients was only 2/161 (1.2%), both of which were mutations in *JAK* family members other than *JAK2*. Hertzberg et al. reported that in ten *JAK2* mutant DS-ALL samples for which *CRLF2* expression was available, all ten exhibited *CRLF2* overexpression (6). These observations combine to suggest that *JAK2* mutations strongly associate with *CRLF2* overexpression in B-ALL, including DS-ALL.

### Cooperation of *CRLF2* and Mutated *JAK2*

This strong association of *CRLF2* overexpression and *JAK2* mutation suggested these proteins might cooperate to transform cells, especially since *CRLF2* is a *JAK*-binding, Box 1 motif-containing cytokine receptor (18). This situation would be analogous to *JAK2* interaction with cytokine receptors in MPNs, where MPN-associated *JAK2* mutants require expression of and interaction with a cytokine receptor to induce its transforming signals (19). To test this, several groups expressed *CRLF2* and ALL-associated *JAK2*-R683 mutants alone and in combination in BaF3 progenitor-B cells, and determined the ability of these proteins to transform cells to cytokine-independence. While expression of each protein alone did not induce transformation, co-expression of both *CRLF2* and mutated *JAK2* did transform these cells to cytokine-independence (5–7). This was associated with enhanced activation of *JAK2*-dependent transforming signaling events, including activation of *JAK2* and its downstream signal mediator *STAT5* (5,7). The natural receptor partner of *CRLF2*, *IL7Ra*, was not required for transformation mediated by *CRLF2* and mutant *JAK2* expression, although *IL7Ra* is expressed in B-ALL leaving open the possibility it may contribute to *CRLF2*-mediated transformation (4–7). Signaling downstream of ligand-induced *CRLF2* activation is poorly understood, but is known to involve *STAT5* phosphorylation (11,20). Interestingly, activation of mouse *CRLF2* does not appear to lead to tyrosine phosphorylation of *JAK* family proteins, but activation of human *CRLF2* does result in *JAK2* phosphorylation (21). Consistent with this notion, Mullighan et al. demonstrated co-immunoprecipitation of human *CRLF2* and phosphorylated mutant *JAK2*, suggesting these two proteins physically interact (5). Thus, these data imply that the genetic association of *CRLF2* overexpression and *JAK2* mutation in B-ALL leads to a functional cooperation, presumably through a physical interaction resulting in activation of signaling.

As noted earlier, the MPN-associated *JAK2*-V617F pseudokinase domain mutation requires scaffolding with a cytokine receptor in order to induce activation of transforming signals (19). Similarly, *JAK2*-R683 pseudokinase domain mutations found in ALL are not transforming unless expressed with a cytokine receptor such as *CRLF2* (5,15). However, the ALL-associated *JAK1*-V658F mutation, which is the analogous *JAK1* mutant to *JAK2*-V617F, does not require expression of an exogenous receptor in order to transform cells (5,22). While this may suggest that *JAK1*-V658F does not require cytokine receptor scaffolding to transform cells, we have recently observed that mutation of the *JAK1* FERM domain abolished the activation and transforming properties of *JAK1*-V658F and *JAK1*-S646F, another ALL-associated mutation (manuscript submitted). This suggests that these mutant *JAK1* proteins may in fact be interacting with unknown endogenous receptors. This is consistent with studies by Hornakova et al. who demonstrate the requirement for the FERM domain for ALL-

associated JAK1 mutant-mediated signaling (23). In addition to possible differences in expression levels (19), JAK1-V658F may have a higher affinity for endogenously expressed receptors in BaF3 cells, and this may account for the differences observed with respect to dependence on expression of an exogenous receptor. Alternatively, perhaps there are subtle unknown differences in JAK1 and JAK2 signaling that make JAK2 mutants more dependent on the level of scaffolding cytokine receptor. It is important to note that the reasons underlying the selective nature of the association of specific JAK mutations with myeloid and lymphoid diseases remain unknown. For example, JAK2-V617F is only observed in MPNs and not ALL while JAK2-R683 and JAK1 mutations are seen in ALL and not MPNs. One possibility is that these disease/mutation associations may involve selective downstream signaling manifested by differential interaction of JAK mutants with lineage-specific scaffolding cytokine receptors and/or differential interaction with downstream effectors (15). For example, JAK2-V617F associating with myeloid lineage receptors such as EpoR/TpoR drives MPN formation, while JAK2-R683 mutations associate with CRLF2 to contribute to B-ALL formation. Amino acids 617 and 683 of JAK2 both lie within the pseudokinase domain of JAK2 but are positioned on different surfaces of this domain (15). Thus, alterations of these amino acids could potentially result in different signaling properties.

### CRLF2 Mutation in B-ALL

Importantly, a point mutation changing phenylalanine 232 to cysteine of CRLF2 has also been identified in CRLF2-overexpressing B-ALL patients, including 9% (3 of 33) of DS-ALL patients and 21% (3 of 14) adult B-ALL patients analyzed (6,7). Chapiro et al. also identified this mutation in a single adult B-ALL patient (24). Expression of this mutant CRLF2 in the absence of co-expression of mutant JAK2 enhances activation of downstream targets of JAK2 as well as transformation of cells in culture (6,7,24). The ability of mutant CRLF2 to induce signaling and transformation in cultured cells without the need for mutationally activated JAK2 correlates with data from patient samples where mutated CRLF2 is not associated with mutated JAK2 (6,7). The potential significance of aberrant CRLF2 activation in primary hematopoietic cell transformation was reported by two groups (4,24). Russell et al. demonstrated enhanced growth in culture of mouse primary early B-cell precursors, mediated by CRLF2-overexpression (4). Chapiro et al. demonstrated that expression of CRLF2-F232C in primary mouse bone marrow cells induced a myeloproliferative disorder following transplantation of these cells into recipient mice (24). While these experiments did not lead to a lymphoid disease in mice, they minimally define mutant CRLF2, and presumably aberrantly activated CRLF2, as an activator of signaling pathways that can induce hematopoietic disease in an animal model. As the authors suggest, further investigation into the role of CRLF2 in ALL may require a model in which CRLF2 is specifically targeted to lymphoid progenitor cells.

A potential model representing contributions of CRLF2 and JAK2 mutation in B-ALL development is depicted in Figure 1A. In addition to mutations in JAK2 and CRLF2, this model includes a significant proportion of CRLF2-overexpressing cases that lack JAK2 or CRLF2 mutations. Such samples also exhibit gene expression profiles similar to that found in BCR-ABL-positive ALL and suggest activation of the JAK-STAT pathway (7). Thus it is reasonable to hypothesize that additional unknown mutations, likely in tyrosine kinases, may play a role in these patients. Therefore, to fully understand the molecular basis of this subset of patients it will be important to identify additional mutations that cooperate with CRLF2.

### Hetero- and Homodimeric Cytokine Receptors and JAK2 in Hematopoietic Disease

The ability of CRLF2 to cooperate with JAK2-R683 mutants to transform cells is highly reminiscent of the requirement for homodimeric cytokine receptors for the activation and

transforming properties of JAK2-V617F, an MPN-associated JAK2 mutation (19). However, while JAK2-V617F has been shown to require homodimeric receptors, CRLF2 is a component of a heterodimeric receptor (8). We have recently shown that a component of a heterodimeric receptor can functionally replace a homodimeric receptor to activate JAK2-V617F and JAK2-R683G (25,26). In fact, we previously hypothesized that heterodimeric receptor components may contribute to myeloid disease, either through aberrant expression or through point mutation, resulting in JAK2 activation (27). The recent identification, although in a lymphoid disease, of CRLF2 as a transforming gene supports our hypothesis in both of these respects. Yoda et al. provide evidence that the F232C mutation of CRLF2 enhances homodimer formation and we recently reported data suggesting that aberrant expression of heterodimeric receptor components (specifically IL27Ra) may induce homodimer formation and activate JAK2 (7,26). Additionally, CRLF2 mutation appears to be exclusive of JAK2 mutation in B-ALL patients, mirroring *Mpl* mutations that occur exclusive of JAK2 mutations in a subset of MPNs (6,7). Thus it is logical to suggest that aberrant expression or mutation of additional heterodimeric receptor components, leading to JAK activation, will be found associated with hematopoietic disease. While it remains unclear how CRLF2 activates signaling in B-ALL, and which specific kinases it utilizes, potential scenarios are depicted in Figure 1B.

## Significance

The identification of CRLF2 as a potentially important etiologic genetic factor in B-ALL is a significant finding for multiple reasons. First, aberrant CRLF2 expression is associated with patients who lack recurring ALL-associated chromosomal translocations, thus highlighting a potentially important and previously unknown oncogenic mechanism in a subset of patients. Second, CRLF2 overexpression associates with poor treatment outcomes in high-risk B-ALL patients, thus potentially giving physicians an important marker to stratify risk and thus guide treatment decisions. Third, the identification of aberrant CRLF2 expression associated with JAK2 mutation in ALL provides an exciting example of a genetic and physical/biochemical association of oncogenes/oncoproteins in a single cancer. Fourth, the significant association of CRLF2 overexpression with JAK2 mutation suggests that CRLF2 is the primary scaffold for JAK2 in ALL, which presents a seemingly different paradigm than in MPNs, where multiple receptor scaffolds likely participate in JAK2 activation. Fifth, JAK2 mutant/CRLF2-mediated and CRLF2 mutant-mediated transformation are sensitive to JAK inhibition *in vitro*, suggesting that ALL patients with CRLF2 overexpression may benefit from future kinase inhibitor approaches. In addition, cell surface expressed CRLF2 itself may be a potential target for anti-CRLF2 antibody-based therapies. Finally, the discovery of aberrant CRLF2 activation in ALL suggests that other JAK-activating heterodimeric receptor components may play unidentified roles in hematopoietic disease.

## Acknowledgments

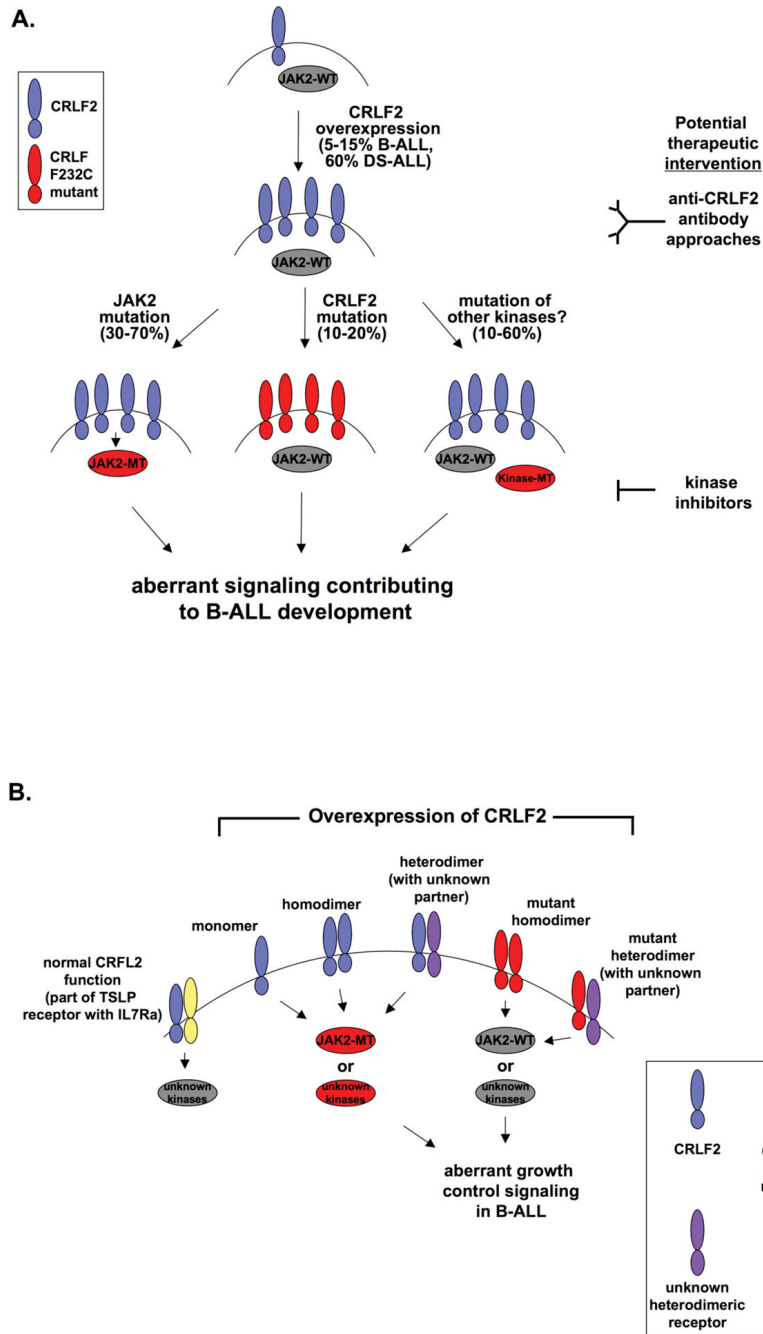
**Grant Support:** This work was supported in part by grants from the American Cancer Society (Research Scholar Grant RSG-07-197-01-LIB), the Lauri Strauss Leukemia Foundation, the Children's Leukemia Research Association, and the National Institutes of Health Grant R01CA127250 (all to G.W.R.).

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**Figure 1. Aberrant CRLF2/JAK2 signaling in B-ALL**

A. CRLF2 overexpression in B-ALL may lead to aberrant signaling through mechanisms involving mutationally activated (red) JAK2, CRLF2, or other unknown kinases. Thus, CRLF2-overexpressing B-ALL patients may benefit from therapeutic use of anti-CRLF2 antibodies or small molecule kinase inhibitors. This model, based on that put forth by Hertzberg et al., includes estimated percentage ranges based on published data for both B-ALL and DS-ALL (6). B. Overexpression of CRLF2 may lead to aberrant signaling through monomeric, homodimeric, or heterodimeric receptor configurations of wildtype (blue) or mutated (red) CRLF2 protein, via activation of mutant (red) or wildtype (grey) JAK2 or other unknown kinases, as indicated.