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# *CRLF2* overexpression results in reduced B cell differentiation and upregulated E2F signaling in the Dp16 mouse model of Down syndrome

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## Abstract

Children with Down syndrome (DS) are 10-fold more likely to develop B-cell acute lymphoblastic leukemia (B-ALL), with a higher frequency of rearrangements resulting in overexpression of cytokine receptor-like factor 2 (*CRLF2*). Here, we investigated the impact of *CRLF2* overexpression on B-cell progenitor proliferation, immunophenotype, and gene expression profile in the Dp(16)1Yey (Dp16) mouse model of DS compared to wild-type (WT) mice. *CRLF2* overexpression enhanced immature B-lymphoid colony development and increased the proportion of less differentiated pre-pro-B cells, with a greater effect in Dp16 versus WT. In *CRLF2*-rearranged (*CRLF2*-R) B-ALL patient samples, cells with higher *CRLF2* expression exhibited a less differentiated B-cell immunophenotype. *CRLF2* overexpression resulted in a gene expression signature associated with E2F signaling in both Dp16 B-progenitors and in DS-ALL patient samples, and PI3K/mTOR and pan-CDK inhibitors which reduce E2F-mediated signaling demonstrated cytotoxicity in *CRLF2*-R B-ALL cell lines and patient samples. *CRLF2* overexpression alone in Dp16 stem and progenitor cells did not result in leukemic transformation in recipient mice. Thus, *CRLF2* overexpression results in reduced B cell differentiation and enhanced E2F signaling in Dp16 B-progenitor cells and DS-ALL patient samples. These findings suggest a functional basis for the high frequency of *CRLF2*-R in DS-ALL as well as a potential therapeutically targetable pathway.

## GRAPHICAL ABSTRACT

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Authorship Contributions

J.J.J. designed and conducted experiments, analyzed and interpreted data, and wrote the manuscript. V.U.G. and J.M. conducted experiments. B.Z. and P.S. analyzed and interpreted data. H.D.L. designed experiments and analyzed and interpreted data. K.R.R. designed experiments, analyzed and interpreted data, and wrote the manuscript. All authors reviewed and approved the manuscript.

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Conflict of Interest Disclosures

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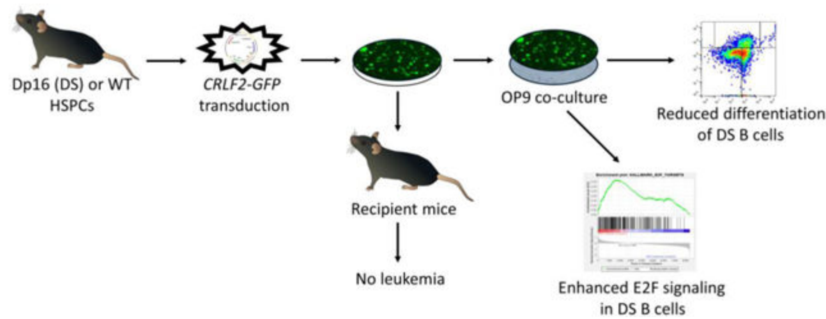


**Keywords**

Leukemia; Down syndrome

## Introduction

Children with Down syndrome (DS) have a 10-fold increased risk of developing B cell acute lymphoblastic leukemia (B-ALL), and have poorer outcomes due to both increased relapse and treatment-related mortality [1, 2]. The spectrum of cytogenetic alterations is very different in DS compared to non-DS ALL. Approximately half of DS-ALL cases have cytokine receptor-like factor 2 rearrangements (*CRLF2*-R), compared to only 5-10% of non-DS ALL cases, with half of these also having Janus Kinase 2 (*JAK2*)-activating point mutations [3-8].

We sought to identify functional effects to explain the increased frequency of *CRLF2*-R ALL in individuals with DS, and identify upregulated signaling pathways in this subtype, which could be targeted therapeutically. Alternative therapeutic approaches offer benefit in patients with DS, due to their increased vulnerability to chemotherapeutic toxicity [1, 9, 10]. We utilized the Dp(16)1Yey (Dp16) mouse model of DS, which has triplication of ~115 genes with orthologues on human chromosome 21 (Hsa21), including the Down syndrome critical region (DSCR) [11]. Since none of the existing mouse models of DS spontaneously develop ALL, we evaluated *ex vivo* effects of *CRLF2* overexpression in Dp16 versus WT hematopoietic stem and progenitor cells (HSPCs), including colony formation, lymphoid differentiation, and gene expression. We also transplanted *CRLF2*-overexpressing Dp16 or WT cells into recipient mice. See Supplementary Data for Methods.

## Results and Discussion

Utilizing the Dp16 mouse model of DS, we compared effects of *CRLF2* overexpression in Dp16 versus WT BM cells. We transduced Dp16 and WT BM HSPCs with a *CRLF2-GFP* or control *GFP* vector, and confirmed 70-95% of cells expressed GFP or hCRLF2 and GFP by flow cytometry prior to experiments (Supplemental Figure 1A). *CRLF2* overexpression increased B-lymphoid colony production in both genetic backgrounds. Colonies persisted for only one replating (data not shown), indicating *CRLF2* overexpression was insufficient for transformation. Colony production was lower, but the fold increase in colony formation with *CRLF2* overexpression was significantly greater, in the Dp16 background (mean fold

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change 10.8 vs 6.4 p = 0.004, Figure 1A). These findings are consistent with prior studies reporting reduced B-lymphoid progenitors in DS models, including trisomy 21 fetal liver cells [12] and the Ts65Dn [13, 14] and Ts1Rhr [13] mouse models of DS.

To characterize the effects of *CRLF2* overexpression on B-lymphoid differentiation in the Dp16 and WT backgrounds, we performed Hardy fraction flow cytometric analysis [15] on *CRLF2-GFP* and *GFP*-transduced HSPC-enriched BM following one week co-culture on OP9 cells, which promote B cell differentiation [16]. Overexpression of *CRLF2* significantly increased the percentage of cells in the less differentiated Hardy Fraction A (pre-pro-B) (mean of 3.9% vs 1.7% in Dp16, p=0.0002; and 2.8% vs 1.6% in WT, p=0.0002). The effect of *CRLF2* overexpression was significantly greater in Dp16 versus WT cells (3.9% vs 2.8%, p=0.012) (Figure 1B).

We further evaluated the effects of *CRLF2* overexpression on B-lineage differentiation by examining *CRLF2*-R B-ALL cases that exhibited both a less differentiated pro-B ($CD34^+/CD19^+$) population and a more differentiated pre-B ($CD34^-/CD19^+$) population. We identified two cases with these features, one DS-ALL (839) and one non-DS ALL (105127-R) (Supplemental Table 1). B-ALL cells with the highest CRLF2 staining demonstrated a significantly higher proportion of pro-B cells (78.2% versus 51.4%, p=0.024, Figure 2). We also assessed the correlation of CRLF2 and CD34 staining in non-DS *CRLF2*-R ALL patient samples, which only had $CD34^+$ pro-B cells. We observed a significant (p<0.0001) positive correlation between CRLF2 and CD34 in each B-ALL patient sample. We observed the strongest correlation (by r value) between CRLF2 and CD34 in the DS-ALL patient sample (839, Supplemental Figure 2). Thus, higher levels of *CRLF2* were associated with reduced B progenitor differentiation, similar to that observed in Dp16 and WT HSPCs.

These effects of *CRLF2* overexpression in reducing B cell differentiation are concordant with prior studies [3, 4]. Our study is unique in demonstrating a significantly greater effect in the Dp16 versus WT background. The greater effect of *CRLF2* overexpression in Dp16 versus WT HSPCs suggests that *CRLF2* rearrangements provide a greater competitive advantage in the DS background, which may explain why this lesion occurs in a greater proportion of DS-ALL cases.

To further evaluate *CRLF2* overexpression in the Dp16 and WT backgrounds, we compared transcriptional signatures associated with *CRLF2* overexpression in sorted $GFP^+$ Dp16 and WT HSPCs after one week of OP9 co-culture, which promotes B cell differentiation [16, 17]. We performed RNA sequencing and gene set enrichment analysis (GSEA) using the Hallmark gene expression sets [18] for these comparisons: (1) Dp16 *CRLF2* versus Dp16 *GFP*; (2) Dp16 *CRLF2* versus WT *CRLF2*; and (3) WT *CRLF2* versus WT *GFP*. We compared three independent replicates per group, and confirmed *CRLF2* overexpression (Supplemental Figure 1B). We found that *CRLF2*-mediated decreases in B cell differentiation in the Dp16 background are associated with upregulated E2F signaling (Figure 3). This was the only gene set with a familywise error rate p value <0.05 (Supplemental Table 2). To assess the relevance to human DS-ALL, we performed GSEA using publicly-available data from three cohorts containing *CRLF2*-overexpressing and *CRLF2*-WT DS-ALL patient samples. We observed significant upregulation of E2F

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signaling in two of three cohorts (GSE20910 and GSE17459-BFM) [7, 19], providing further support for the potential relevance of the E2F pathway as a therapeutic target for *CRLF2*-overexpressing DS-ALL (Supplemental Figure 3 and Supplemental Table 3).

The majority of the leading edge genes from the E2F gene set are associated with DNA synthesis and cell cycle progression, and have potential as targets for leukemia therapies, including *Wee1* [20] and *Rrm2* [21] (Supplemental Table 4). We did not observe significant enrichment for Hallmark gene expression sets in WT cells overexpressing *CRLF2,* suggesting that *CRLF2* confers a selective advantage in the DS background. In addition, a high percentage of DS-ALL [22] and iAMP21 B-ALL [23] cases have *RB1* deletions, suggesting that higher E2F activity may be selected for with trisomy 21, via *CRLF2* overexpression and/or *RB1* deletion. Studies are needed to decipher the region of triplication contributing to this effect.

Targeting E2F directly is difficult, due to multiple E2F isoforms and limited potency of pan-E2F inhibitors [24, 25]. E2F activation involves signaling inputs from PI3K/mTOR and CDK activity [26-28]. We reasoned that inhibiting these pathways may be cytotoxic in *CRLF2*-overexpressing DS-ALL blasts. We tested a PI3K/mTOR inhibitor AZD2014 and a pan-CDK inhibitor AT7519, which directly reduce expression of E2F-transcribed genes [26, 29], in seven non-DS B-ALL cell lines and nine PDX-expanded B-ALL patient samples (Supplemental Table 1).

Both compounds demonstrated nanomolar-range cytotoxicity in all B-ALL cell lines, irrespective of *CRLF2* mutation status. In the PDX-expanded primary samples, the compounds demonstrated nanomolar-range cytotoxicity in *CRLF2*-overexpressing cases, but demonstrated inconsistent potency in the *CRLF2/JAK2* WT cases, and no clear specificity for *CRLF2*-overexpressing DS-ALL cases (Supplemental Figure 4). These experiments demonstrate preclinical utility of compounds that reduce E2F signaling in *CRLF2*-R DS and non-DS ALL.

Finally, we transplanted *CRLF2*-overexpressing Dp16 or WT HSPCs into recipient mice. We confirmed 70-95% transduction efficiency of *GFP* and *CRLF2-GFP* in Dp16 and WT HSPC cultures before transplantation. Mice engrafted with 2-20% GFP$^+$ cells up to day 100 post-transplant, and the stained GFP$^+$ cells of most recipients were typically B220$^+$ B cells (Supplemental Figure 5), alongside GFP$^+$ myeloid or T cells. However, engraftment was undetectable by day 140 post-transplant. Average engraftment was higher at every time point, and sustained for longer, in mice receiving WT versus Dp16 cells, suggesting an inherent reduction in repopulating capacity of Dp16 cells. No mice transplanted with *CRLF2*-overexpressing Dp16 or WT HSPCs developed leukemia, with at least 7 mice per group monitored for 6 months, which included a minimum of one month after engraftment was undetectable. Interestingly, B220$^+$ *CRLF2*-overexpressing Dp16 cells displayed reduced staining intensity in most engrafted recipient mice relative to other groups and to B220$^+$ GFP$^-$ cells within the same mice. Since B220 expression increases as B cell differentiate [15], *CRLF2*-overexpressing Dp16 cells may have demonstrated particularly dim B220 staining due to reduced B cell differentiation relative to the other conditions. As a control, we transplanted mice with HSPCs overexpressing *NRAS*$^{G12D}$. Mice transplanted with

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*NRAS*$^{G12D}$-overexpressing Dp16 or WT HSPCs developed rapid disease, with 9/24 WT mice and 10/25 Dp16 mice succumbing to CD4$^+$CD8$^+$ T-ALL with median latencies of 92 and 101 days (data not shown). The only current DS-ALL mouse model requires *CRLF2* overexpression, *JAK2*$^{R683G}$, and *Pax5*$^{+/-}$ [13]. However, it utilizes the Ts1Rhr mouse model of DS, which only has 31 triplicated Hsa21 orthologues. We expected the extra triplicated Hsa21 orthologues in Dp16 mice might contribute to B-ALL with only *CRLF2* overexpression, but additional alterations are likely required.

Our Dp16 *ex vivo* differentiation model demonstrates the proliferative advantages of *CRLF2* overexpression in Dp16 versus WT cells, which may contribute to the increased susceptibility to *CRLF2*-R ALL in children with DS. The upregulated E2F signaling may provide opportunities for therapeutic intervention. This model will be useful to test cytotoxicity of targeted therapies and combination regimens, and to investigate the leukemogenic effects of other clinically relevant oncogenes. Ultimately, insights from these studies may improve efficacy and reduce toxicity of therapy for *CRLF2*-R ALL, particularly in children with DS.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Data Availability Statement

The datasets generated and/or analyzed during the current study are available in the European Nucleotide Archive repository: https://www.ebi.ac.uk/ena/browser/view/PRJEB43591.

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## Highlights

- High *CRLF2* reduces B cell differentiation in Down syndrome mouse progenitor cells
- High *CRLF2* is associated with less B-cell differentiation in human leukemia cells
- High *CRLF2* is associated with E2F signaling in Down syndrome mouse and human cells
- Inhibitors of E2F signaling are cytotoxic in *CRLF2*+ acute lymphoblastic leukemia
- High *CRLF2* in Dp16 cells is not sufficient to generate leukemia in recipient mice

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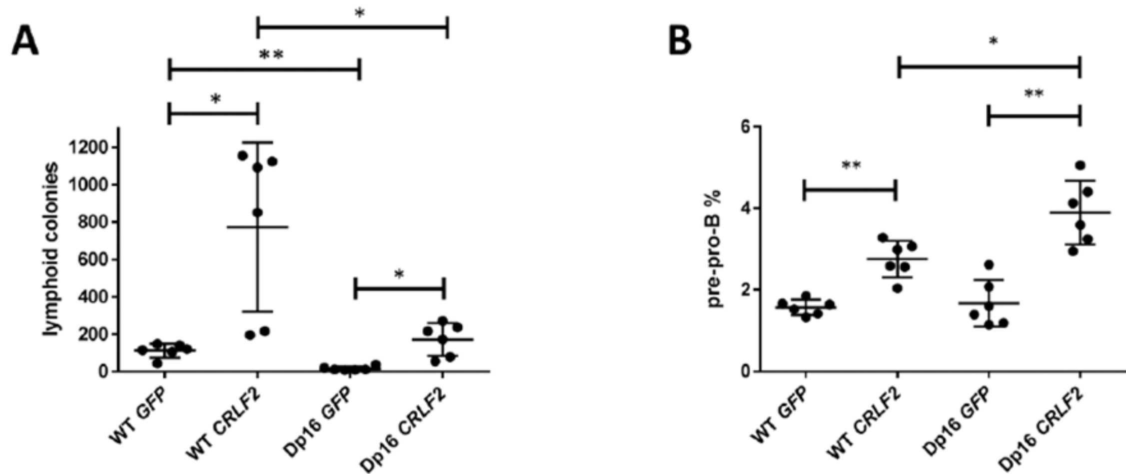


**Figure 1. *CRLF2* overexpression enhances B-lymphoid colony formation and reduces B cell maturation, with a greater fold change in the Dp16 background.**

**(A)** *CRLF2* overexpression in BM HSPCs from both WT and Dp16 mice increased B-lymphoid colony growth compared to *GFP* control. Histogram depicts colony counts from six samples (two technical replicates across three independent experiments, Student's t-test, *p<0.01, **p<0.001). Error bars indicate standard deviation. **(B)** *CRLF2* overexpression in WT and Dp16 HSPCs grown in B cell-differentiation conditions resulted in an increased percentage of the less-differentiated pre-pro-B cells. This effect was greater in the Dp16 versus WT genetic background. Histogram shows percentage of pre-pro-B cells in the GFP$^+$ gate of each experimental group, with mean values from three independent experiments with two biological replicates each (Student's t-test, *p<0.05, **p<0.001). Error bars indicate standard deviation.

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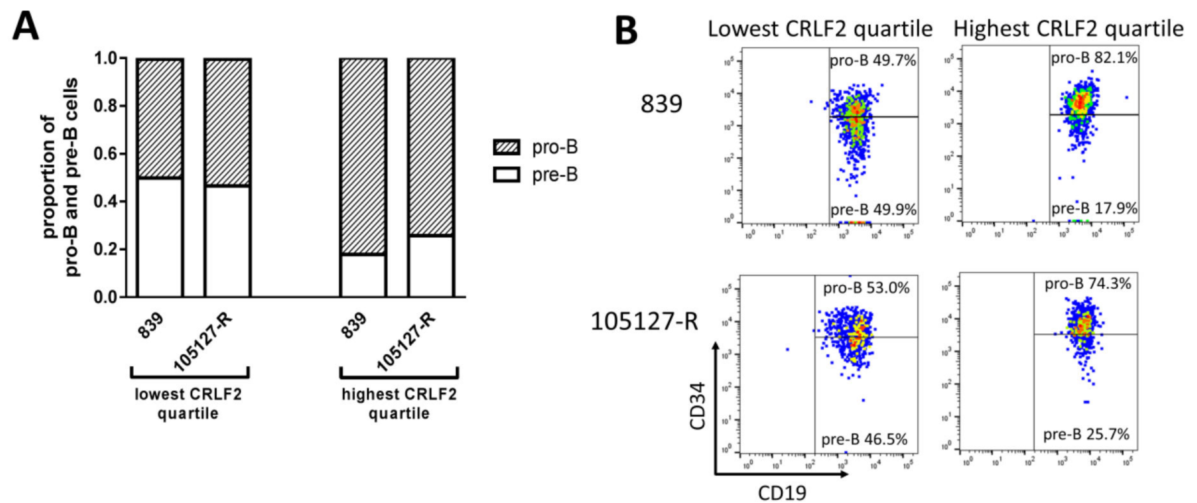


**Figure 2. *CRLF2* overexpression is associated with reduced B cell differentiation in B-ALL patient samples.**

**(A)** In two *CRLF2*-overexpressing B-ALL patient samples (DS-ALL 839 and non-DS ALL 105127-R) demonstrating both pro-B and pre-B populations, the high-CRLF2 mean fluorescence intensity (MFI) quartile demonstrated a significantly higher mean proportion of pro-B cells (p=0.024). **(B)** Representative flow plots showing *CRLF2* overexpression correlates with a less differentiated immunophenotype in B-ALL patient samples 839 (DS) and 105127-R (non-DS).

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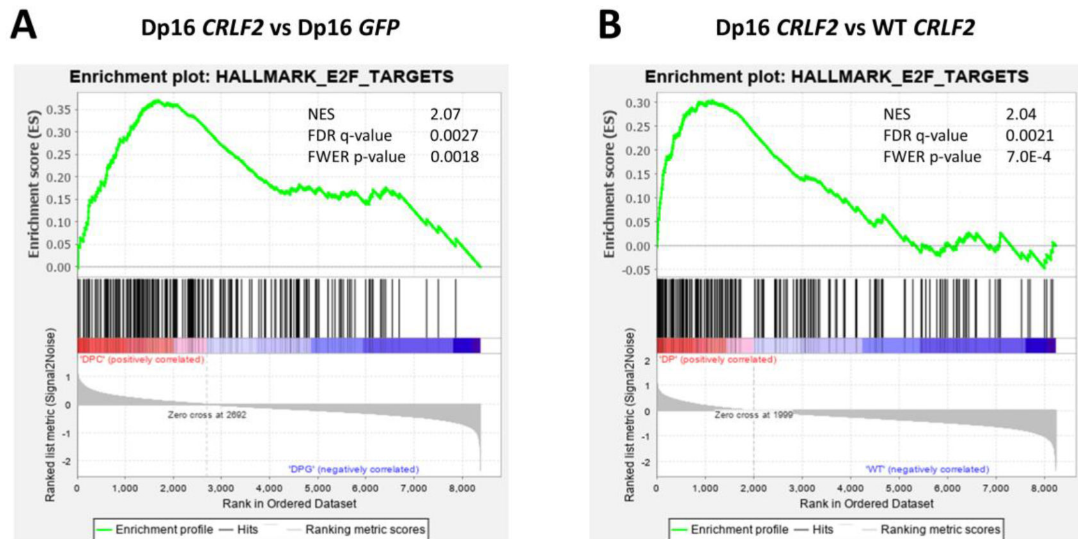


**Figure 3. Dp16 *CRLF2* cells demonstrate enrichment for E2F targets.**

Gene set enrichment analysis plots show upregulation of E2F targets in Dp16 *CRLF2* cells compared to **(A)** Dp16 *GFP* cells and **(B)** WT *CRLF2* cells. Normalized enrichment score (NES), false discovery rate (FDR) q-value, and familywise error rate (FWER) p-value are also displayed.