

# BCL6 inhibition ameliorates resistance to ruxolitinib in *CRLF2*-rearranged acute lymphoblastic leukemia

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## Abstract

Philadelphia chromosome-like acute lymphoblastic leukemia (Ph-like ALL) is an intractable disease and most cases harbor genetic alterations that activate JAK or ABL signaling. The commonest subtype of Ph-like ALL exhibits a *CRLF2* gene rearrangement that brings about JAK1/2-STAT5 pathway activation. However, JAK1/2 inhibition alone is insufficient as a treatment, so combinatorial therapies targeting multiple signals are needed. To better understand the mechanisms underlying the insufficient efficacy of JAK inhibition, we explored gene expression changes upon treatment with a JAK1/2 inhibitor (ruxolitinib) and found that elevated BCL6 expression was one such mechanism. Upregulated BCL6 suppressed the expression of TP53 along with its downstream cell cycle inhibitor p21 (CDKN2A) and pro-apoptotic molecules, such as FAS, TNFRSF10B, BID, BAX, BAK, PUMA, and NOXA, conferring cells some degree of resistance to therapy. BCL6 inhibition (with FX1) alone was able to upregulate TP53 and restore the TP53 expression that ruxolitinib had diminished. In addition, ruxolitinib and FX1 concertedly downregulated MYC. As a result, FX1 treatment alone had growth-inhibitory and apoptosis-sensitizing effects, but the combination of ruxolitinib and FX1 more potently inhibited leukemia cell growth, enhanced apoptosis sensitivity, and prolonged the survival of xenografted mice. These findings provide one mechanism for the insufficiency of JAK inhibition for the treatment of *CRLF2*-rearranged ALL and indicate BCL6 inhibition as a potentially helpful adjunctive therapy combined with JAK inhibition.

## Introduction

Although recent progress in intensified chemotherapy, coupled with risk stratification, has substantially improved the outcomes of patients with acute lymphoblastic leukemia (ALL), the prognosis of patients with some ALL subsets remains poor.<sup>1-4</sup> ALL harbor chromosomal abnormalities and genetic alterations that affect the differentiation and proliferation of lymphoid precursor cells. These abnormalities serve as significant prognostic factors.<sup>1-4</sup>

Philadelphia chromosome (Ph)-like ALL has a gene expression signature similar to that of Philadelphia chromosome-positive ALL, but does not have the *BCR-ABL1* fusion gene.<sup>5,6</sup> Ph-like ALL accounts for about 10%-15% of childhood and approximately 20% of adult cases of B-cell pre-

cursor ALL, with a peak of 25%-30% in adolescents and young adults. Ph-like ALL is associated with a poor prognosis<sup>1-3,7-11</sup> and affected patients are less likely to achieve minimal residual disease-free remission than those with other types of ALL.<sup>10</sup>

Genomic alterations in Ph-like ALL affect cytokine receptors, tyrosine kinase signaling, and transcription factors, leading to disease heterogeneity. However, the kinases or pathways affected allow the identification of various Ph-like ALL subtypes, including those with *CRLF2* rearrangement (*IGH-CRLF2* and *P2RY8-CRLF2*), rearrangement of ABL class tyrosine kinase genes, *JAK2* rearrangement, *EPOR* mutations, and activating mutations of genes involved in JAK/STAT and RAS (*NRAS*, *KRAS*, and *PTPN11*) pathways.<sup>1,2,7,9,11</sup> The subtype with *CRLF2* gene rearrangement (hereafter

CRLF2-ALL) accounts for up to about 60% of cases of Ph-like ALL, depending on ethnicity. Rearrangement of the *CRLF2* gene located on Xp22.3 and Yp11.3 results in CRLF2 receptor overexpression and is frequently accompanied by *JAK* mutations.<sup>1,2,7,9,12,13</sup> Interestingly, about 50% to 60% of children with Down syndrome-associated ALL have *CRLF2* rearrangements (usually *P2RY8-CRLF2* fusions) and *JAK2*<sup>R683G</sup> mutations.<sup>12</sup>

Although CRLF2 receptor engagement can activate the JAK-STAT pathway, CRLF2-ALL shows variable responses to type I JAK inhibitors, such as ruxolitinib.<sup>4,9,14,15</sup> Adjunctive or alternative treatment approaches are therefore required to improve clinical outcomes.

One such approach is the development of a type II JAK2 inhibitor.<sup>16</sup> Heat shock protein 90 inhibition could be an alternative to target JAK2 by inducing its degradation.<sup>17</sup> Other approaches include combinatorial inhibition targeting JAK2 together with BCL2 and BCL-xL<sup>18</sup> or phosphatidylinositol 3-kinase/mammalian target of rapamycin.<sup>19,20</sup> Recent studies have indicated that GSPT1,<sup>21</sup> CRKL, MEK1/2,<sup>22</sup> HMGN1,<sup>23</sup> and B-cell receptor-like signaling<sup>24</sup> are all important targets for better treatment. Additional options include combinations of ruxolitinib with chemotherapeutic drugs.<sup>25</sup> These measures have improved CRLF2-ALL treatment in experiments, but their efficacy in patients is unknown as yet.

In this study, an alternative approach was taken to provide an additional adjunctive option under the hypothesis that gene expression changes elicited by JAK inhibitors might mitigate the efficacy of treatment with such inhibitors. This study focused on *BCL6* as such a gene.

*BCL6* is a DNA-binding protein and predominantly functions as a transcriptional repressor.<sup>26</sup> *BCL6* plays an essential role in germinal center development. In germinal center B cells, *BCL6* represses the transcription of genes involved in DNA damage response elicited by physiological DNA breaks required for immunoglobulin class switch recombination and somatic hypermutation.<sup>27,28</sup> The constitutive expression of *BCL6* is caused by chromosomal translocations, somatic mutations in DNA sequences involved in silencing *BCL6*, activating mutations of transcription factors driving *BCL6* expression, and deletion/mutation of the gene encoding *FBXO11* (a component of a ubiquitin ligase that degrades *BCL6* protein) in diffuse large B-cell lymphoma, contributing to development of the lymphoma.<sup>28,29</sup> *BCL6* is also implicated in oncogenesis and maintenance of other cancers.<sup>27</sup>

## Methods

### Reagents

The reagents used in this study are listed in *Online Supplementary Table S1*.

### Cell lines

The cell lines used in this study are listed in *Online Supplementary Tables S1* and *S2*. YCUB5<sup>30</sup> and KOPN49<sup>22,31</sup> were provided by Hiroaki Goto (Kanagawa Children's Medical Center) and Takeshi Inukai (University of Yamanashi), respectively. Cells were authenticated using the GenePrint 10 System (Promega) with Expassy Cellosaurus short tandem repeat references, except for YCUB5 and KOPN49, which lacked such references. Clinical samples of CRLF2-ALL were obtained, with written informed consent to participation in the study, from patients at Nagoya University Hospital (case 1) and National Hospital Organization Nagoya Medical Center (case 2). These patients' clinical information is described briefly in the *Online Supplementary Methods*. The study protocol was approved by the ethics committees at Nagoya University, National Hospital Organization Nagoya Medical Center, and Aichi Medical University. Gene mutations in CRLF2-ALL cells used in this study are summarized in *Online Supplementary Table S2*.

### Ex vivo drug sensitivity assays

Cells were cultured in triplicate in the presence of the indicated concentrations of drugs or a vehicle. Viable cells were enumerated using a CellDrop BF automated cell counter (DeNovix, Wilmington, DE, USA) or by inspection under a microscope with the trypan blue dye exclusion method. The fraction of surviving cells relative to the control was calculated for each condition. ZIP synergy scores were calculated using the R package synergyfinder (version 2.2.4).

### In vivo drug sensitivity assays

Cells ( $5 \times 10^6$ ) were transplanted intravenously into NSG mice (obtained from The Jackson Laboratory, Bar Harbor, ME, USA) that had been pre-conditioned using two intraperitoneal injections of busulfan (20 mg/kg) at a 24-hour interval. Mice were given enrofloxacin (170 mg/L) in drinking water *ad libitum* and randomly assigned to the treatment group with ruxolitinib (100 mg/kg/day) by gastric gavage twice daily or FX1<sup>32</sup> (30 mg/kg/day) by intraperitoneal injection once daily, or their combination, 5 days on, 2 days off for 4 weeks. Animals administered only vehicle served as controls. A Kaplan-Meier analysis of survival was performed using the R package survival (version 3.2-7), and the survival curve was generated by the R package survminer (version 0.4.8). All animal experiments were performed according to protocols approved by the Institutional Animal Use and Care Committee of Aichi Medical University.

### Other methods

Additional methods are described in the *Online Supplementary Methods*.

**Data availability**

The RNA-sequencing data have been deposited in ArrayExpress under accession number E-MTAB-10755.

**Results****BCL6 upregulation after treatment with JAK1/2 inhibitors in CRLF2-ALL**

We first examined the growth-inhibitory effects of a class I JAK1/2 inhibitor, ruxolitinib. This drug is clinically used to treat JAK-activated myeloproliferative neoplasms and is being tested for its efficacy in CRLF2-ALL in clinical trials.<sup>4</sup> The growth of four CRLF2-ALL and five non-CRLF2-ALL cell lines in 5-day *ex vivo* cultures with graded ruxolitinib concentrations was compared. The growth of two CRLF2-ALL cell lines, MUTZ-5 and YCUB5, was dose-dependently inhibited, but only modestly. The growth inhibition of the other two CRLF2-ALL cell lines, MHH-CALL-4 and NAGL-1, was blunt enough to be indistinguishable from that of the five non-CRLF2-ALL cell lines (Reh, Kasumi-2, Kasumi-7, NALM-1, and NALM-6) (Figure 1A). A similar effect was observed using a class II JAK inhibitor CHZ868<sup>16</sup> (*Online Supplementary Figure S1A*), suggesting that this new type of inhibitor *per se* does not wholly overcome the resistance of CRLF2-ALL subsets to JAK inhibition.

It was hypothesized that gene expression changes elicited by ruxolitinib might mitigate the treatment efficacy of this inhibitor. Gene expression profiles in MHH-CALL-4 and MUTZ-5 cells in the presence or absence of ruxolitinib revealed *BCL6* to be one of the genes most remarkably upregulated by ruxolitinib (Figure 1B). *BCL6* upregulation was confirmed at the protein level by western blot analysis in four CRLF2-ALL cell lines and samples from two patients with CRLF2-ALL (Figure 1C, *Online Supplementary Figure S1B*). Conversely, the five non-CRLF2 ALL cell lines did not show such a response (Figure 1D). CHZ868 treatment likewise upregulated *BCL6* expression (*Online Supplementary Figure S1C*). The upregulation of *BCL6* transcripts by ruxolitinib or CHZ868 treatment was confirmed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (*Online Supplementary Figure S1D*). However, this study did not exclude the possibility that *BCL6* protein is additionally upregulated post-translationally. Enforced expression of an active form of STAT5B somewhat reduced *BCL6* expression and remarkably blunted ruxolitinib's effect of upregulating *BCL6* (Figure 1E, *Online Supplementary Figure S1E*), suggesting a close inverse relationship between STAT5 activity and *BCL6* expression. Consistently, STAT5 inhibitors upregulated *BCL6* in six CRLF2-ALL cells but not in the non-CRLF2-ALL cell line, Reh (*Online Supplementary Figure S1F*). These findings sug-

gest that ruxolitinib upregulates *BCL6* through STAT5 inhibition.

**Synergy between a JAK inhibitor (ruxolitinib) and BCL6 inhibitors in culture**

Given that CRLF2-ALL cells express *BCL6* even before ruxolitinib treatment (Figure 1C), we investigated whether *BCL6* inhibition affects cell growth in the absence of JAK inhibitors. To this end, three distinct *BCL6* inhibitors, BI3802,<sup>33</sup> BI3812,<sup>33</sup> and FX1,<sup>32</sup> were used. BI3802 is a *BCL6* degrader, and BI3812 and FX1 interfere with *BCL6* interactions with its co-repressors BCOR, NCOR, and SMART. These reagents inhibited the growth of four CRLF2-ALL cell lines and Kasumi-2 and Kasumi-7 but not Reh, NALM-1, and NALM-6 cell lines. The growth inhibition of Kasumi-2 and Kasumi-7 was consistent with the essential roles of *BCL6* in the maintenance of *TCF3-PBX1*<sup>34</sup> (Kasumi-2) and *MEF2D*-rearranged<sup>35</sup> (Kasumi-7) ALL (Figure 1F). These findings suggest that *BCL6* may, at least in part, play roles in CRLF2-ALL cell growth. The study of primary CRLF2-ALL cells (cases 1 and 2 in Figure 1C) was not possible, as these cells could not be maintained in culture long enough to assess drug sensitivity fully.

Next, the efficacy of a combination of ruxolitinib and either FX1 or BI3812 for the inhibition of CRLF2-ALL cell growth was investigated. Figure 2 and *Online Supplementary Figure S2A* show a synergy of the combinations. A similar synergy was observed in modeled CRLF2-ALL in Baf/3 cells (*Online Supplementary Figure S2B*). Likewise, *BCL6* knockdown in the CRLF2-ALL cell lines, MHH-CALL-4 and YCUB5, inhibited their growth (*Online Supplementary Figure S2C, D*), which was further inhibited by ruxolitinib (*Online Supplementary Figure S2D*), suggesting that the growth inhibitory effects of the *BCL6* inhibitors were likely specific. Time-course experiments showed that a combinatorial effect of ruxolitinib and FX1 on cell growth was most pronounced more than 5 days after culture initiation (*Online Supplementary Figure S2E*). However, there was mostly negligible synergy in five non-CRLF2-ALL cells (*Online Supplementary Figure S2F*).

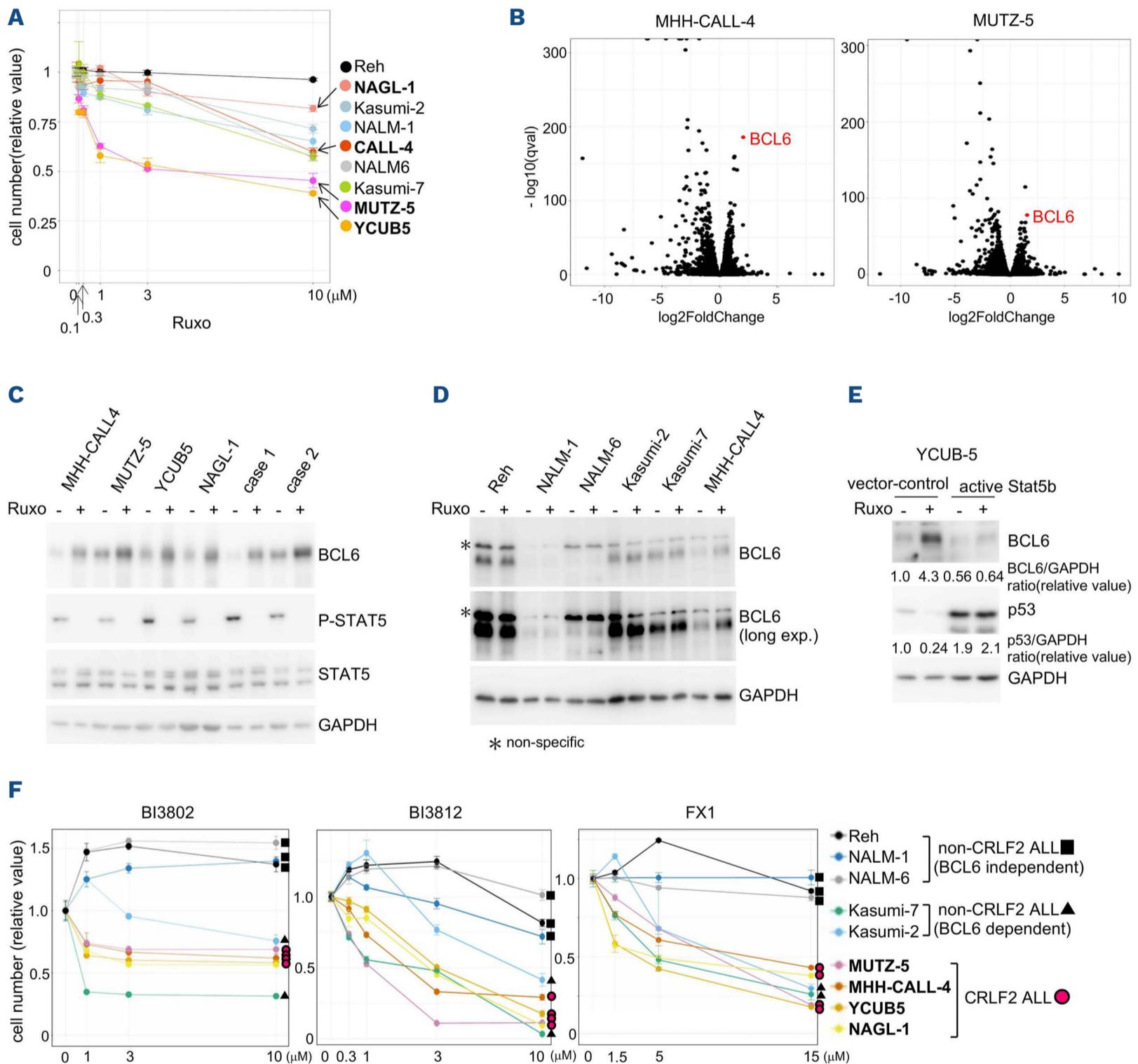
**Combinatorial effect of ruxolitinib and FX1 in vivo**

We next studied the effects of ruxolitinib and FX1 in combination *in vivo*: FX1 was chosen as the *BCL6* inhibitor since BI3802 and BI3812 were not amenable to *in vivo* treatment.<sup>33</sup> Of the four CRLF2-ALL cell lines tested in this study, YCUB5 and MUTZ5 cells had the highest *in vivo* leukemia-propagating activity (*Online Supplementary Figure S3A*) and were, therefore, used in these experiments. Compared to vehicle-only treatment, FX1 or ruxolitinib treatment alone prolonged the survival of mice transplanted with CRLF2-ALL cells, but the combination of FX1 and ruxolitinib prolonged survival even further, demonstrating its survival benefit (Figure 3A, B left). Consistently, peripheral

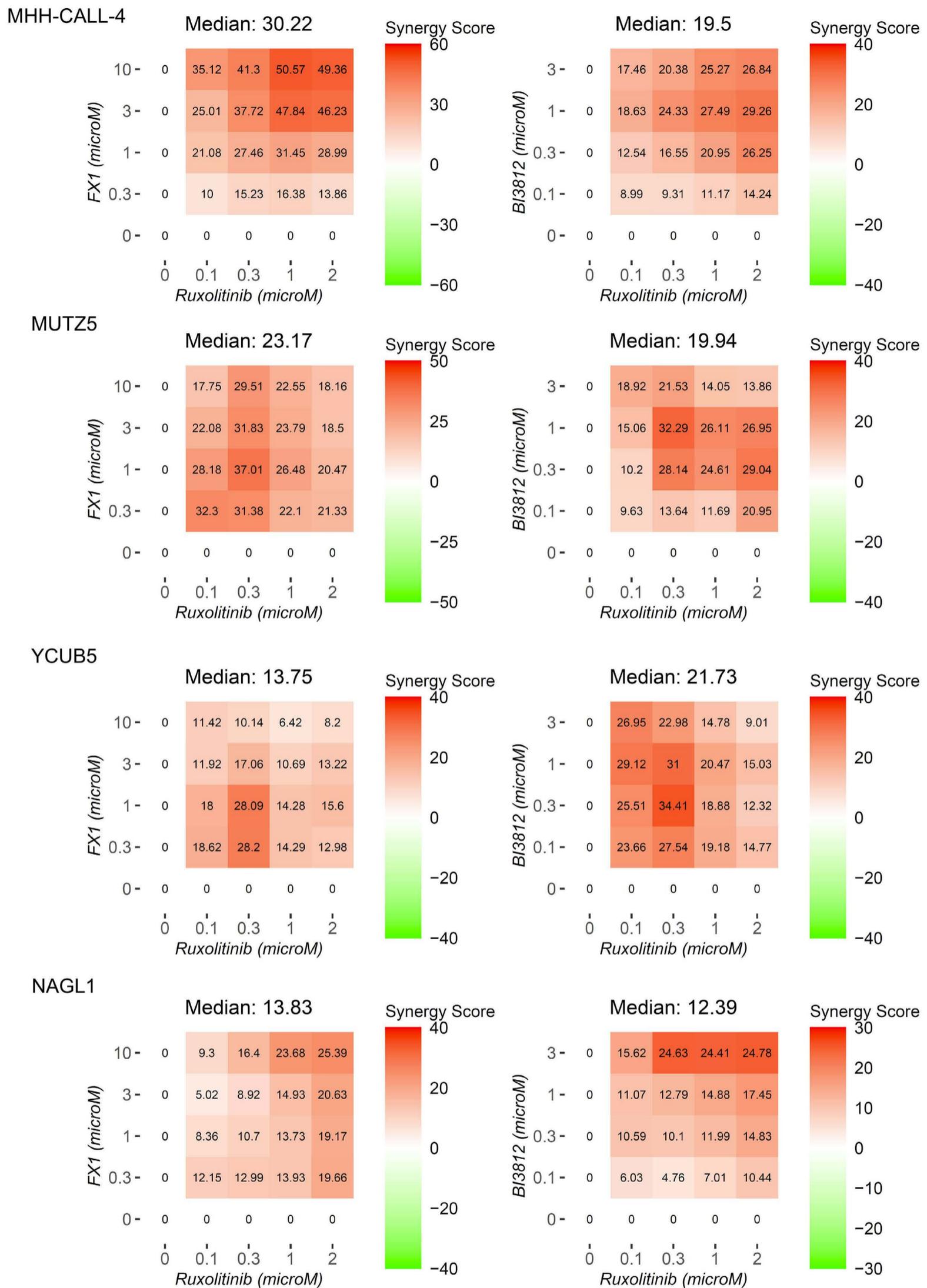
blood collected at the time of treatment completion contained markedly fewer leukemia cells in mice treated with the combination (Figure 3A, B right). A similar observation was made for mice transplanted with leukemia cells from a patient (case 1) (Figure 3C). Addition of FX1 was well tolerated, as suggested by no apparent loss of the treated animals' bodyweight (Online Supplementary Figure S3B).

**Ruxolitinib mitigates TP53-mediated tumor suppression**

To investigate the molecular mechanisms underlying the synergy between ruxolitinib and FX1, the gene expression of MHH-CALL-4 and MUTZ-5 cells in four treatment conditions were compared: ruxolitinib alone, FX1 alone, their combination, and vehicle-only control. Differentially expressed genes between the conditions were extracted



**Figure 1. Upregulation of BCL6 by ruxolitinib and growth inhibition by BCL6 inhibitors.** (A) Growth inhibition of CRLF2-ALL cell lines (MHH-CALL-4, MUTZ-5, YCUB5, and NAGL-1) and non-CRLF2-ALL cell lines (Reh, Kasumi-2, Kasumi-7, NALM-1, and NALM-6) by the JAK1/2 inhibitor, ruxolitinib. Cells were cultured with the indicated concentrations of ruxolitinib for 5 days, and growth relative to that of vehicle-treated cells is shown as the mean  $\pm$  standard deviation (SD). (B) Gene expression changes in MHH-CALL-4 and MUTZ-5 cells upon treatment with ruxolitinib (1  $\mu$ M) for 24 h are presented as a volcano plot. *BCL6* is represented in red. (C) *BCL6* upregulation, at the protein level, with ruxolitinib treatment. The CRLF2-ALL cell lines and clinical samples from two cases were treated with ruxolitinib (1  $\mu$ M, 24 h), and a western blot analysis was performed for *BCL6*, phospho-STAT5, and STAT5. GAPDH served as a loading control. (D) Five non-CRLF2-ALL cell lines treated with ruxolitinib were analyzed for *BCL6* protein expression. The MHH-CALL4 cell line was included as a positive control. Asterisks indicate nonspecific bands. (E) YCUB5 cells overexpressing an active form of STAT5 or control vector were treated with ruxolitinib and analyzed for *BCL6* and TP53 expression. (F) Effects of the *BCL6* inhibitors BI3802, BI3012, and FX1 on cell growth. Cells were treated with graded concentrations of *BCL6* inhibitors for 5 days, and the cell growth relative to that of vehicle-treated cells is shown as the mean  $\pm$  SD. Ruxo: ruxolitinib.



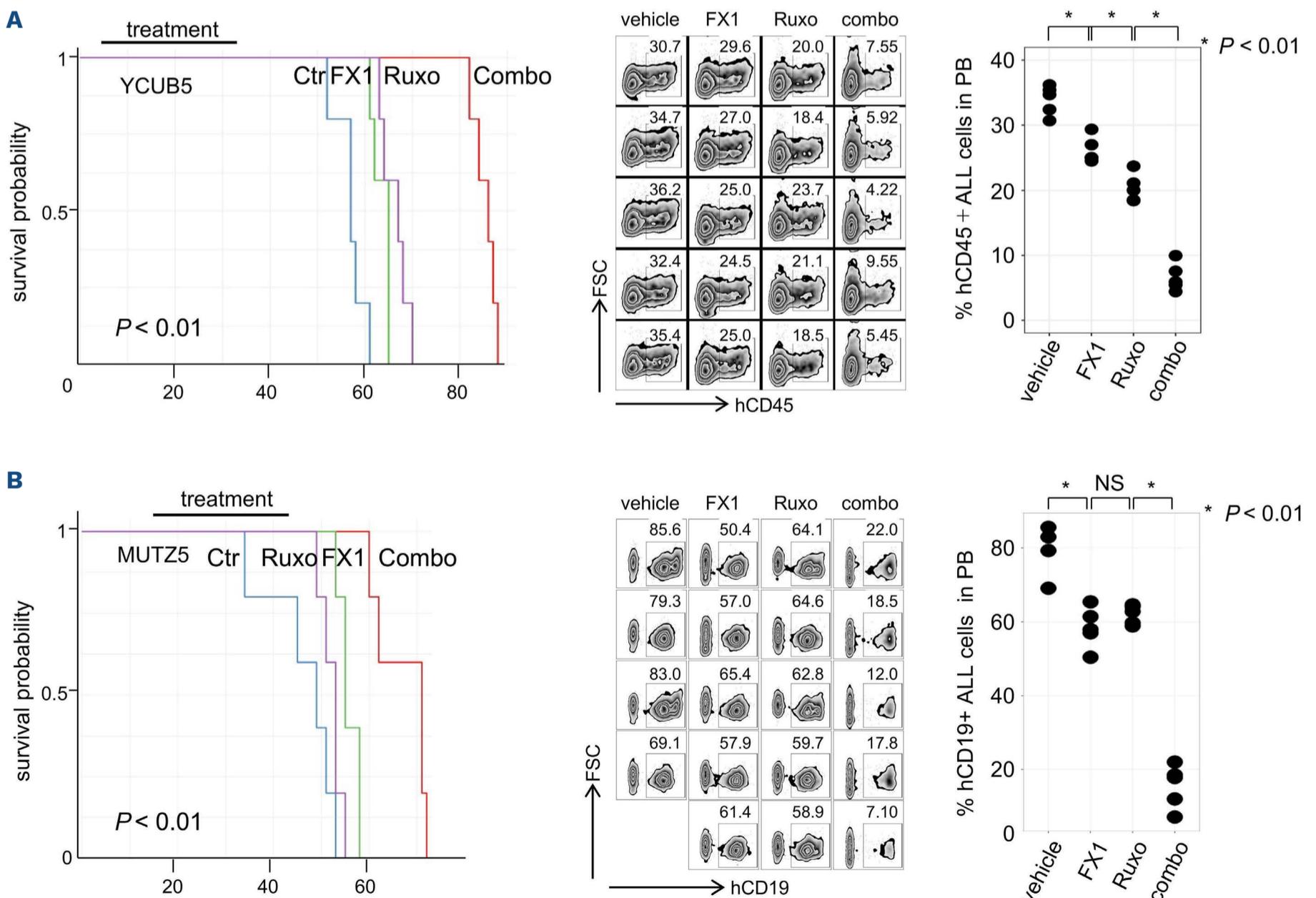
**Figure 2. Ruxolitinib and BCL6 inhibitors synergistically suppress growth of CRLF2-ALL cells in culture.** Combinatorial effects of ruxolitinib and BCL6 inhibitors on cell growth. CRLF2-ALL cell lines (MHH-CALL-4, MUTZ-5, YCUB5, and NAGL-1) were treated with the indicated concentrations of ruxolitinib, FX1, and BI3812 alone, or a combination of ruxolitinib + FX1 (left) or ruxolitinib + BI3812 (right), for 5 days. The ZIP synergy scores are presented. ZIP synergy scores >10 are regarded as representing “strong synergy.”

and subjected to pathway and gene ontology (GO) term analysis (Figure 4A). First, the focus was on genes that ruxolitinib treatment downregulated compared to vehicle control (Ctr > Ruxo). A pathway involving STAT5 activation was enriched for such genes, consistent with the known activities of JAK1/2 in CRLF2-ALL. Intriguingly, TP53 and apoptotic signaling pathways and positive regulation of cell death and cell cycle arrest GO terms, among others, were likewise enriched. The latter findings suggest that ruxolitinib treatment may mitigate tumor-suppressive activity by downregulating genes involved in the TP53 pathway, cell death, and cell cycle arrest.

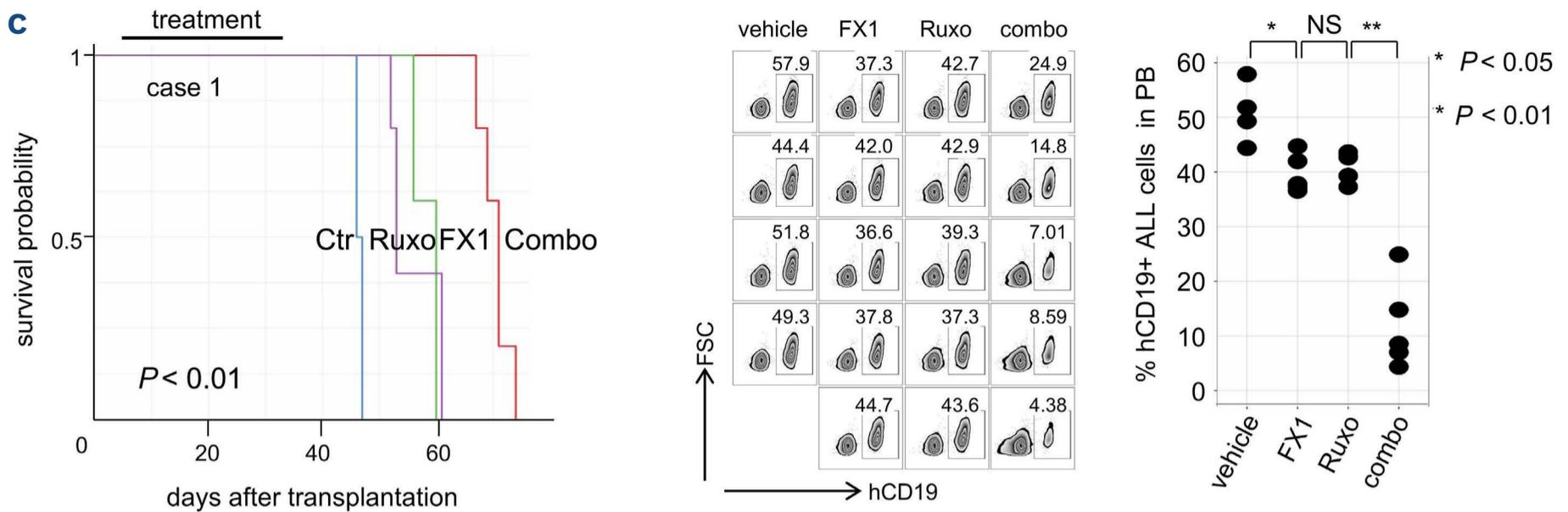
The focus was then set on genes FX1 upregulated in ruxolitinib-treated cells (Ruxo < Combo). Pathways enriched for such genes again included TP53, apoptotic signaling pathways, and positive regulation of the cell death GO term. These findings suggest that FX1 may counteract, at least in part, ruxolitinib-mediated mitigation of tumor-suppressive activity. This notion was corroborated by gene set variation analysis using MSigDB hallmark gene sets (Figure 4B). Heatmap presentations of such gene expression changes are provided in *Online Supplementary*

*Figure S4A*. Indeed, ruxolitinib treatment led to negative enrichment of genes representing TP53 and apoptosis pathways, but the addition of FX1 largely reversed this effect (Figure 4B, *Online Supplementary Figure S4A, B*). Pathway activity evaluated by PROGENy analysis was consistent with the results (*Online Supplementary Figure S4C*). In addition, an inhibitor of TP53 transcription activity, pifithrin- $\alpha$ , largely antagonized the growth-suppressive activity of the BCL6 inhibitors FX1 and BI3812 in culture (*Online Supplementary Figure S4D*), suggesting the involvement of TP53 in BCL6 inhibitor-mediated growth inhibition.

We next investigated whether ruxolitinib, BCL6 inhibitors and their combination affect the levels of TP53 protein (Figure 5). In four CRLF2-ALL cell lines, ruxolitinib treatment diminished TP53 protein and phospho-STAT5 (a substrate of JAK2) levels while upregulating BCL6. This relationship among STAT5 activity, BCL6 expression, and TP53 expression agreed with the observation made in YCUB5 cells forcedly expressing an active STAT5 (Figure 1E). TP53 downregulation coupled with BCL6 upregulation upon STAT5 inhibition was also in accord (*Online Supple-*



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**Figure 3. Ruxolitinib and a BCL6 inhibitor (FX1) synergistically suppress CRLF2-ALL growth *in vivo*.** (A-C) Immunodeficient NSG mice were implanted with the indicated cells, (A) YCUB5, (B) MUTZ5, and (C) a CRLF2-ALL patient's cells, and treated with ruxolitinib or FX1, or their combination. Bars represent treatment periods. Deaths were defined as mice found dead or requiring euthanasia. The Kaplan-Meier survival curves and the differences in survival probability estimated by the log-rank test are presented (left). Peripheral blood drawn at the time of completion of the treatment was evaluated for tumor burden by flow-cytometric analysis after red blood cell lysis, using anti-human CD45 or anti-human CD19 antibodies. Percentages of CD45<sup>+</sup> or CD19<sup>+</sup> human acute lymphoblastic leukemia cells in peripheral blood are presented (middle), with the graphs (right) showing the statistical difference (pairwise comparisons by a *t* test) in tumor burden. Ctrl: control; Ruxo: ruxolitinib; combo: FX1 + ruxolitinib; FSC: forward side scatter; ALL: acute lymphoblastic leukemia; PB: peripheral blood; NS: non-significant.

mentary Figure S1F). In contrast, BCL6 inhibitors BI3812 and FX1 alone increased TP53 levels; the increase of BCL6 expression by these inhibitors probably reflected an auto-inhibitory effect of BCL6.<sup>29</sup> Likewise, a BCL6 degrader, BI3802, increased TP53 protein. In five non-CRLF2 ALL cell lines, there were no appreciable changes in TP53 after treatment with either ruxolitinib or BI3802. However, JAK activity, reflected by phospho-STAT5 level, was lower in the non-CRLF2-ALL cells tested, complicating a direct comparison with CRLF2-ALL cells (Figure 5A).

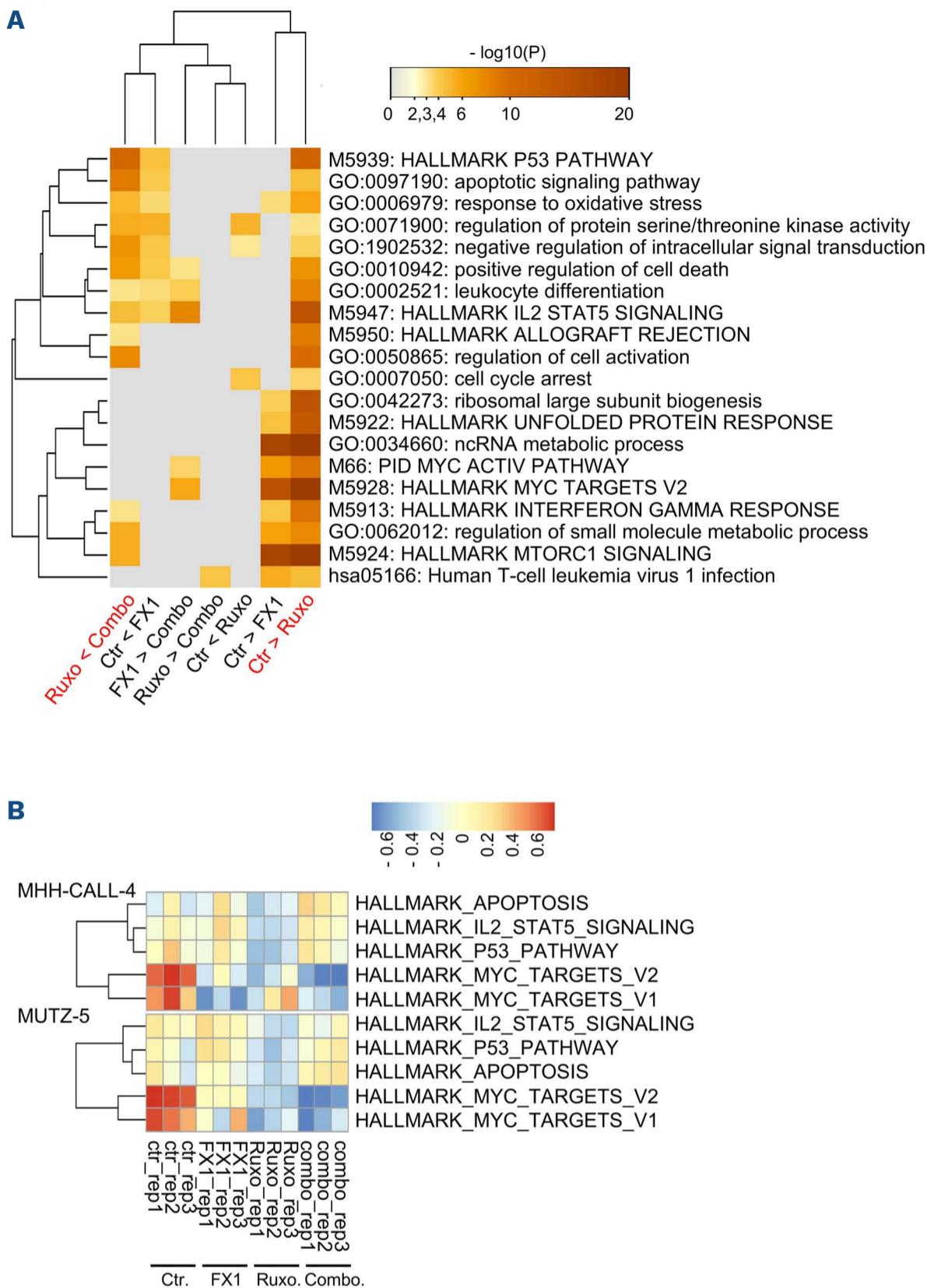
Of particular note, BCL6 inhibitors restored the TP53 protein levels that ruxolitinib treatment had lowered (Figure 5B). p21CIP1 (CDKN1A) expression, a known transcriptional target of TP53, accompanied the changes in TP53 levels (Figure 5C). A similar result was obtained in two patients' leukemia cells (Figure 5D). Consistently, BCL6 knockdown upregulated TP53 and blunted ruxolitinib-mediated TP53 downregulation (Online Supplementary Figure S2C).

Given that MDM2 inhibitor-mediated TP53 upregulation (Online Supplementary Figure S5A) inhibited the growth of CRLF2-ALL cells synergistically with ruxolitinib (Online Supplementary Figure S5B), TP53 likely mediates the synergistic activity of FX1 co-treatment with ruxolitinib. Consistently, no synergy was found between ruxolitinib and FX1, BI3812, or an MDM2 inhibitor in a biallelically TP53-mutated CRLF2-ALL cell line, KOPN49 (Online Supplementary Figure S5C, D). Notably, however, KOPN49 cells retained the ability to upregulate BCL6 upon ruxolitinib or FX1 treatment like the other four CRLF2-ALL cell lines (Online Supplementary Figure S5E). These findings suggest that BCL6 inhibitors counteract TP53 pathway inhibition

elicited by ruxolitinib.

BCL6 functions primarily as a transcriptional repressor, but ruxolitinib treatment did not appreciably downregulate TP53 mRNA levels (Online Supplementary Figure S5F), as confirmed by qRT-PCR (*data not shown*), which is inconsistent with previous observations made in lymphoma cells.<sup>36</sup> Instead, ruxolitinib downregulated, and FX1 addition restored many mRNA encoding molecules that contribute to stabilizing TP53<sup>37</sup> (Online Supplementary Figure S5F). Furthermore, chromatin immunoprecipitation sequencing data made available by Cistrome Data Browser (<http://cistrome.org.db>) showed that BCL6 could bind to genomic regions near the transcription start sites of these genes (Online Supplementary Figure S5G). These observations suggest that BCL6 may account for gene expression changes that concertedly diminish the amount of TP53 protein.

Finally, we focused on the possible involvement of MYC in CRLF2-ALL cell growth. It was seen that ruxolitinib or FX1 alone downregulated MYC target genes and that the combination of ruxolitinib and FX1 did so even further (Figure 4A, B, Online Supplementary Figure S4), thus the effects of ruxolitinib, FX1, and MDM2 inhibitors on MYC expression were investigated. Indeed, MYC was downregulated by ruxolitinib or FX1 alone at the transcript (Figure 6A) and protein (Figure 6B) levels. The downregulation was most prominent with the combination of the two drugs. A similar observation was made using BI3012 in place of FX1 (Online Supplementary Figure S6A). Since TP53 activation with MDM2 inhibitors downregulated MYC expression (Online Supplementary Figure S6B),<sup>38,39</sup> the ability of BCL6 in-



**Figure 4. Critical involvement of the TP53 pathway in the response of CRLF2-ALL cells to ruxolitinib and FX1.** (A) Two CRLF2-ALL cell lines, MHH-CALL-4 and MUTZ-5, were treated with vehicle, ruxolitinib (1  $\mu$ M), FX1 (20  $\mu$ M), or their combination for 24 h. After global analysis of differentially expressed genes between treatment conditions, pairwise comparisons of gene expression between the treatments were made for pathway analysis and represented as a heatmap showing  $-\log_{10}(P)$  value. The columns in the heatmap indicate the treatment conditions between which the comparison was made. For example, “Ruxo<Combo” means genes elevated by ruxolitinib+FX1 treatment compared with ruxolitinib treatment alone. (B) Gene set variation analysis of genes with MSigDB hallmark gene sets. Enrichment scores of the indicated gene sets in MHH-CALL-4 (top) and MUTZ-5 (bottom) cells are shown as heatmaps. Ruxo: ruxolitinib; Combo: ruxolitinib+FX1; Ctr: control.

inhibitors to downregulate MYC is likely due to TP53 activation. The biallelically *TP53*-mutated CRLF2-ALL KOPN49 cell line did not show downregulation of MYC in response to FX1, despite retaining the ability to downregulate MYC in response to ruxolitinib (*Online Supplementary Figure S6C*). Since MYC knockdown inhibited the cell growth of CRLF2-ALL cell lines proportionately to MYC levels (Figure 6C, D), the concerted downregulation of MYC by ruxolitinib and FX1 likely represents one mechanism underlying the synergy in growth inhibition between the two drugs.

### Ruxolitinib affects TRAIL- and Fas-mediated tumor suppression

Gene expression analysis revealed the downregulation of apoptosis-associated genes in response to ruxolitinib

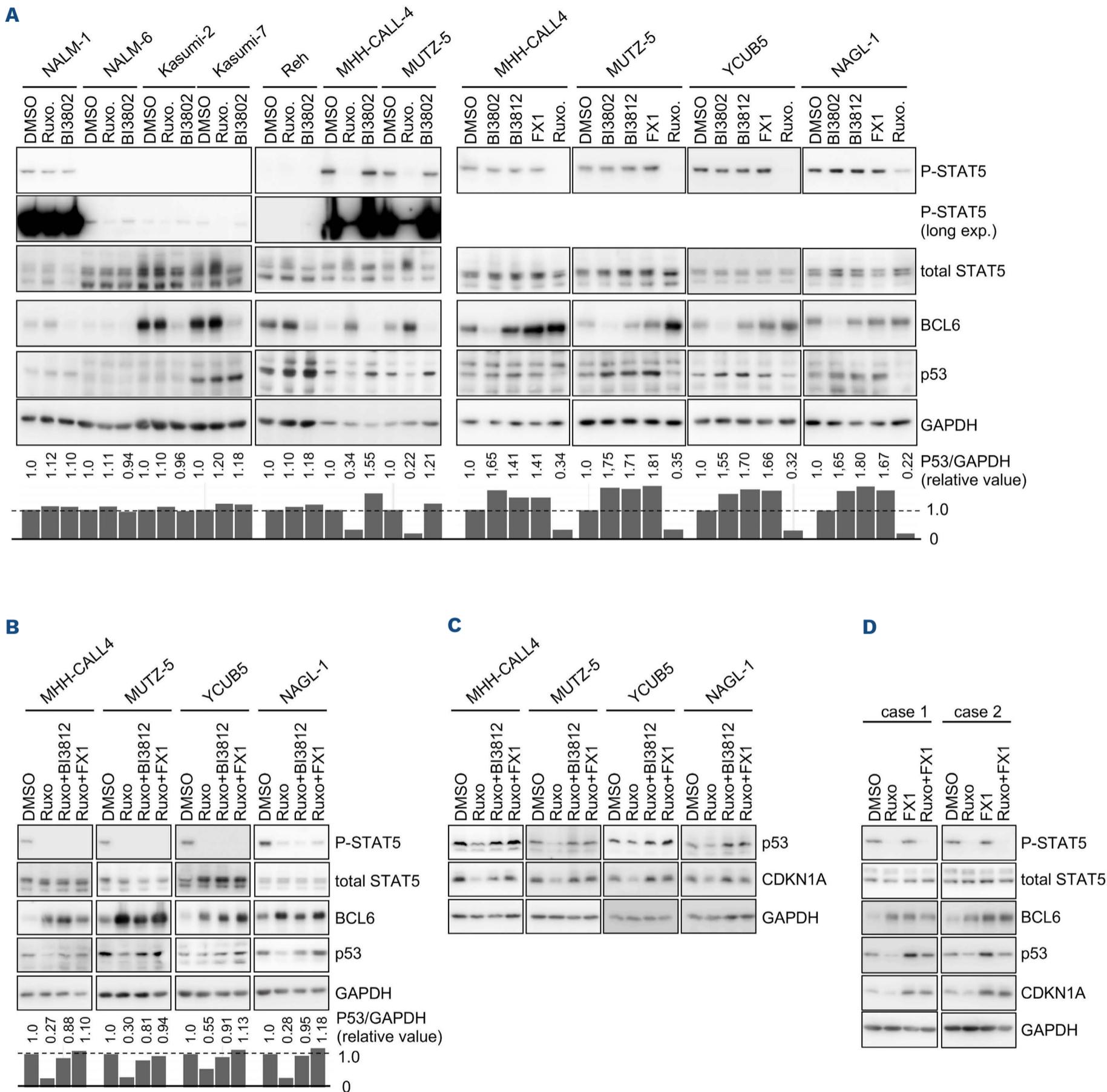
treatment and the reversal of this effect following BCL6 inhibition (Figure 4A, B). As for the “extrinsic” apoptosis pathway-associated genes, TNFRSF10B (TRAIL receptor) and FAS were noticeably downregulated by ruxolitinib treatment and mostly restored by the addition of FX1; both are known transcriptional targets of TP53<sup>40</sup> (Figure 7A).

Indeed, ruxolitinib treatment diminished, but FX1 effectively restored TNFRSF10B and FAS expression in four CRLF2-ALL cell lines and two samples from patients. FX1 alone without ruxolitinib marginally yet reproducibly up-regulated the two molecules (Figure 7B). Consistently, TRAIL diminished the number of viable MHH-CALL-4 and MUTZ-5 cells (albeit to a much lesser degree than MHH-CALL-4), but ruxolitinib treatment blunted this effect of

TRAIL. The addition of FX1 largely restored the responsiveness of the cells to TRAIL (Figure 7C).

Similarly, ruxolitinib treatment blunted, and FX1 addition

restored sensitivity to FAS-mediated apoptosis in MHH-CALL-4 cells. Although MUTZ-5, YCUB5, and NAGL1 cells were minimally sensitive to FAS-mediated apoptosis, FX1

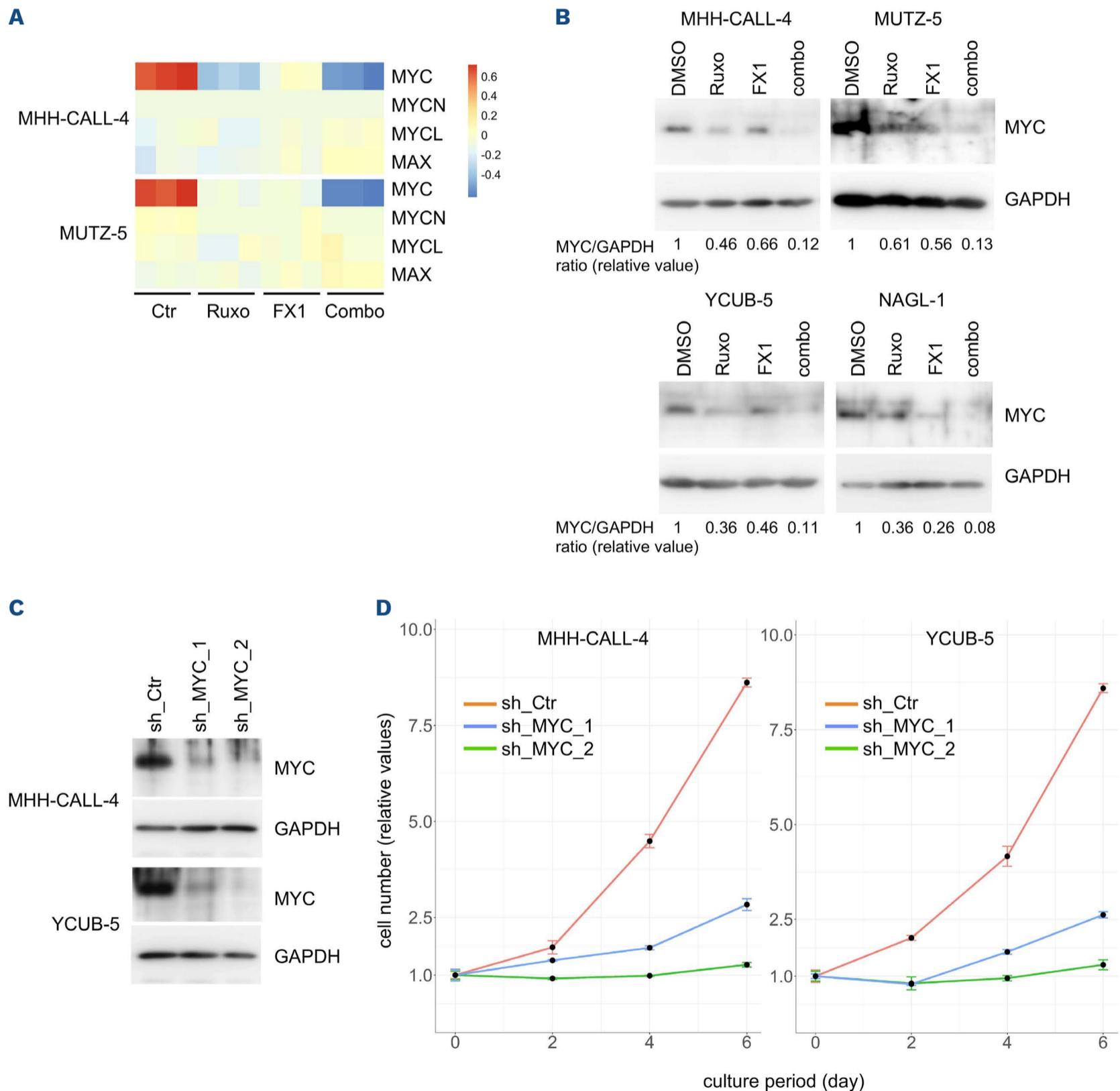


**Figure 5. Alterations of TP53 protein levels in CRLF2-ALL cells in response to treatment with ruxolitinib and BCL6 inhibitors.** (A, left) Five non-CRLF2-ALL cell lines and two CRLF2-ALL cell lines (MHH-CALL-4 and MUTZ-5) were treated with vehicle (DMSO), ruxolitinib (1 μM; Ruxo), or BI3802 (3 μM) for 24 h and western blot analysis was performed for the expression levels of phospho-STAT5, total STAT-5, BCL6, TP53, and GAPDH (a loading control). (A, right) CRLF2-ALL cells were treated with vehicle (DMSO), BI3802 (3 μM), BI3812 (10 μM), FX1 (20 μM), or ruxolitinib (1 μM; Ruxo) for 24 h and subjected to western blot analysis for the indicated proteins. (B) The CRLF2-ALL cells were treated with the indicated drugs for 24 h and subjected to western blot analysis as in (A). In (A) and (B), p53 expression levels were normalized based on GAPDH levels and are presented as bar charts, with controls set to 1.0. (C) The CRLF2-ALL cells were treated with the indicated drugs for 24 h and subjected to western blot analysis, as in (B), for the indicated proteins. (D) Clinical samples from two patients (case 1 and case 2) with CRLF2-ALL were analyzed for the indicated proteins.

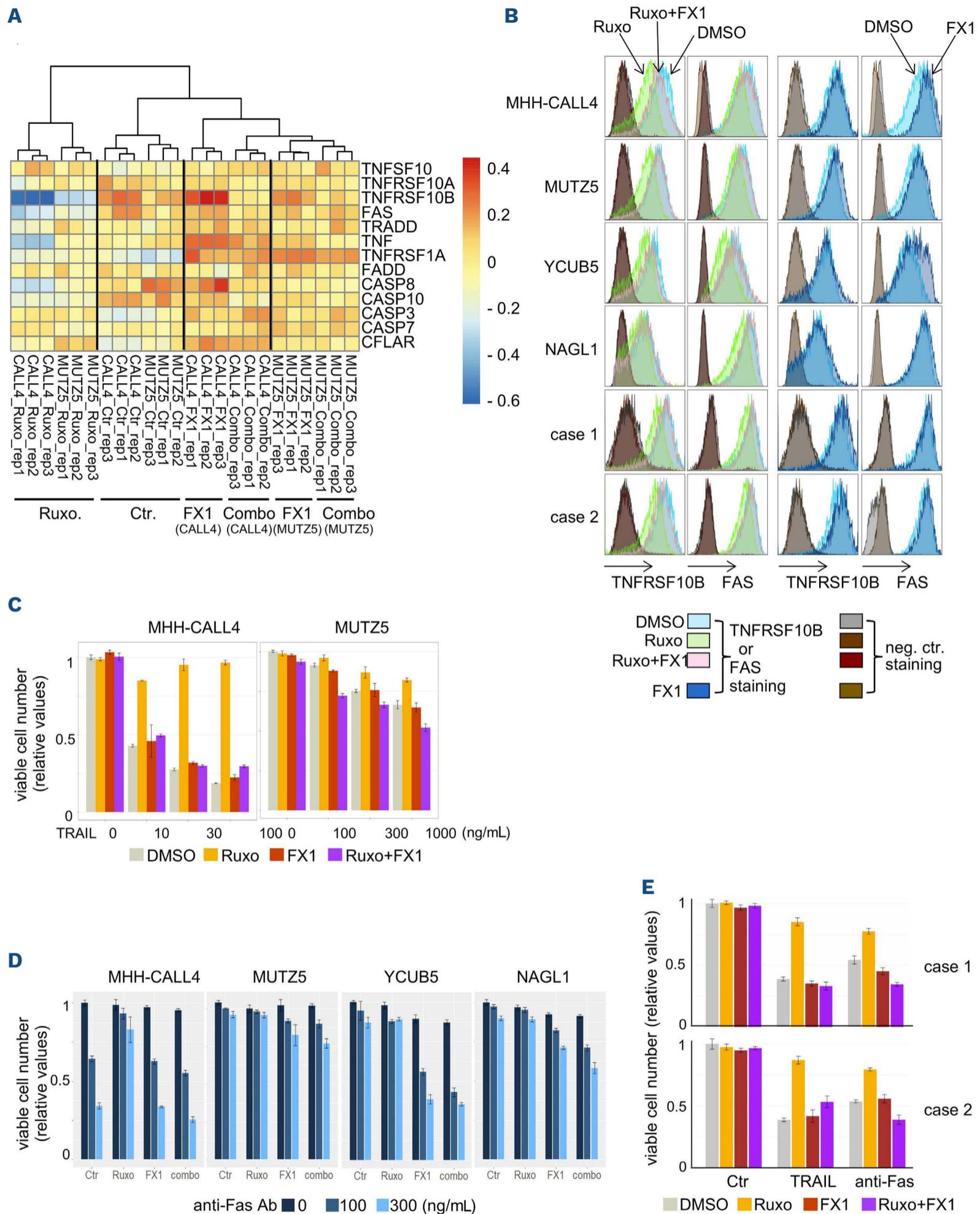
treatment partially sensitized these cells to apoptosis (Figure 7D). Two patients' cells were sensitive to TRAIL- and FAS-mediated apoptosis, which ruxolitinib blunted but FX1 addition restored (Figure 7E). However, the biallelically TP53-mutated KOPN49 cell line was inert to ruxolitinib or FX1 treatment regarding TNFRSF10B/FAS expression and FAS-mediated apoptosis (Online Supplementary Figure S7A). MDM2 inhibitors largely recapitulated the observations made using FX1. The MDM2 inhibitors, NVP-CGM097 and MI-773, restored TNFRSF10B and FAS expression that ruxolitinib treatment had diminished (Online Supplementary Figure

S7B). The addition of NVP-CGM097 restored the sensitivity of MHH-CALL-4 cells to FAS-mediated apoptosis. The combination of ruxolitinib and MDM2 inhibitor sensitized YCUB-5 cells to FAS-mediated cell death (Online Supplementary Figure S7C).

These findings suggest that ruxolitinib allows CRLF2-ALL cells to evade TRAIL- and Fas-mediated immunological elimination. Thus, the use of a BCL6 inhibitor may benefit patients in bolstering such elimination, even while off ruxolitinib treatment. These effects of BCL6 inhibitors likely depend on TP53.



**Figure 6. Ruxolitinib and FX1 treatment downregulates MYC.** (A) Changes in expression levels of the indicated genes in MHH-CALL-4 (upper panel) and MUTZ-5 (lower panel) cells. Cells were treated as in Figure 4A, and gene expression changes are presented as heatmaps. (B) The MYC protein levels in the indicated four CRLF2-ALL cell lines. These MYC levels normalized to GAPDH levels are also depicted. (C, D) The shRNA-mediated knockdown of MYC attenuated the growth of the indicated CRLF2-ALL cells. MYC protein levels (C) and cell growth (D) after the knockdown. Ctr: control; Ruxo: ruxolitinib; Combo: ruxolitinib+FX1.



**Figure 7. Alterations of extrinsic apoptosis pathway gene expression and their relation to sensitivity to TRAIL- and FAS-induced cell death.** (A) Heatmap representation of changes in the expression of genes involved in the extrinsic apoptosis pathway. The HMM-CALL4 and MUTZ-5 cells were treated as in Figure 4A. (B) The CRLF2-ALL cells were treated with vehicle (dimethylsulfoxide, DMSO), ruxolitinib (1  $\mu$ M; Ruxo), FX1 (20  $\mu$ M), and their combination for 24 h, and the expression of TNFRSF10B (TRAIL receptor) and FAS was analyzed by flow cytometry. Ruxolitinib treatment downregulated the two molecules on the cell surface. FX1 treatment counteracted the downregulation (left panel). FX1 treatment alone upregulated the two molecules' expression compared with the control treatment (right panel). (C) Cells were treated with the indicated drugs, as in (A), for 24 h and incubated with the indicated concentrations of TRAIL for an additional 12 h. Numbers of viable cells, relative to those not incubated with TRAIL, are shown as the mean  $\pm$  standard deviation. (D) Cells were treated as in (C) but with the indicated concentrations of anti-FAS antibody instead. Numbers of viable cells, relative to those not incubated with anti-FAS antibodies are presented. (E) Results of clinical sample cells treated as in (C) and (D). Ruxo: ruxolitinib; Ctr; control; Combo: ruxolitinib + FX1; Ab: antibody.

### Ruxolitinib and FX1 co-treatment renders CRLF2-ALL cells hypersensitive to BCL2 inhibition in apoptosis

The next focus was on genes affiliated with the “intrinsic” pathway of apoptosis. Gene expression (Figure 8A) and protein expression (Figure 8B; *Online Supplementary Figure S8A*) analyses suggested that ruxolitinib treatment downregulated, and FX1 addition restored, the expression of the pro-apoptotic molecules PUMA (BBC3), NOXA (PMAIP1), BID, BAX, and BAK. As for anti-apoptotic molecules, ruxolitinib downregulated BCLxL, an effect not modified by the addition of FX1. Ruxolitinib marginally downregulated MCL1 and the addition of FX1 further downregulated this molecule. The ability of FX1 to downregulate MCL1 is likely mediated through the upregulation of TP53, as MDM2 inhibitor-mediated activation of TP53 likewise downregulated MCL1 (*Online Supplementary Figure S5A*). FX1 and its combination with ruxolitinib did not diminish MCL1 in biallelically mutated TP53 KOPN49 cells (*Online Supplementary Figure S8B*). These findings suggest that the ruxolitinib and FX1 combination lowers the threshold in executing apoptosis. However, apoptosis was observed only after 6 days in CRLF2-ALL cells and only to a moderate extent (*Online Supplementary Figure S8C*). BCL2 may be one explanation of the inertness of CRLF2-ALL cells to apoptosis, as the level of this protein did not change appreciably upon treatment with ruxolitinib, FX1, or their combination in the four CRLF2-ALL cell lines tested (Figure 8B; *Online Supplementary Figure S8A*). Indeed, venetoclax (a BCL2 inhibitor)<sup>41</sup> induced striking apoptosis of CRLF2-ALL cells when combined with ruxolitinib and FX1 even at low concentrations ( $\leq 30$  nM), which itself otherwise had only a minor effect (Figure 8C). Venetoclax remarkably diminished the mitochondrial membrane potential in the presence of both ruxolitinib and FX1, whereas S63845 (a MCL-1 inhibitor) or A-1155463 (a BCLxL inhibitor) did so only modestly (*Online Supplementary Figure S8D*). These FX1-mediated apoptosis-promoting effects were again, at least partly, mediated through TP53 activation since NVP-CGM097 produced an effect similar to that of FX1 (*Online Supplementary Figure S8E*); the biallelically TP53-mutated CRLF2-ALL KOPN49 cell line was insensitive to NVP-CGM097 and FX1 (*Online Supplementary Figures S8F, G*) regarding venetoclax-induced apoptosis. Overall, these findings suggest that ruxolitinib treatment *per se* does not favor apoptosis. However, the addition of FX1 tips the balance toward apoptosis, thus rendering CRLF2-ALL cells hypersensitive to venetoclax.

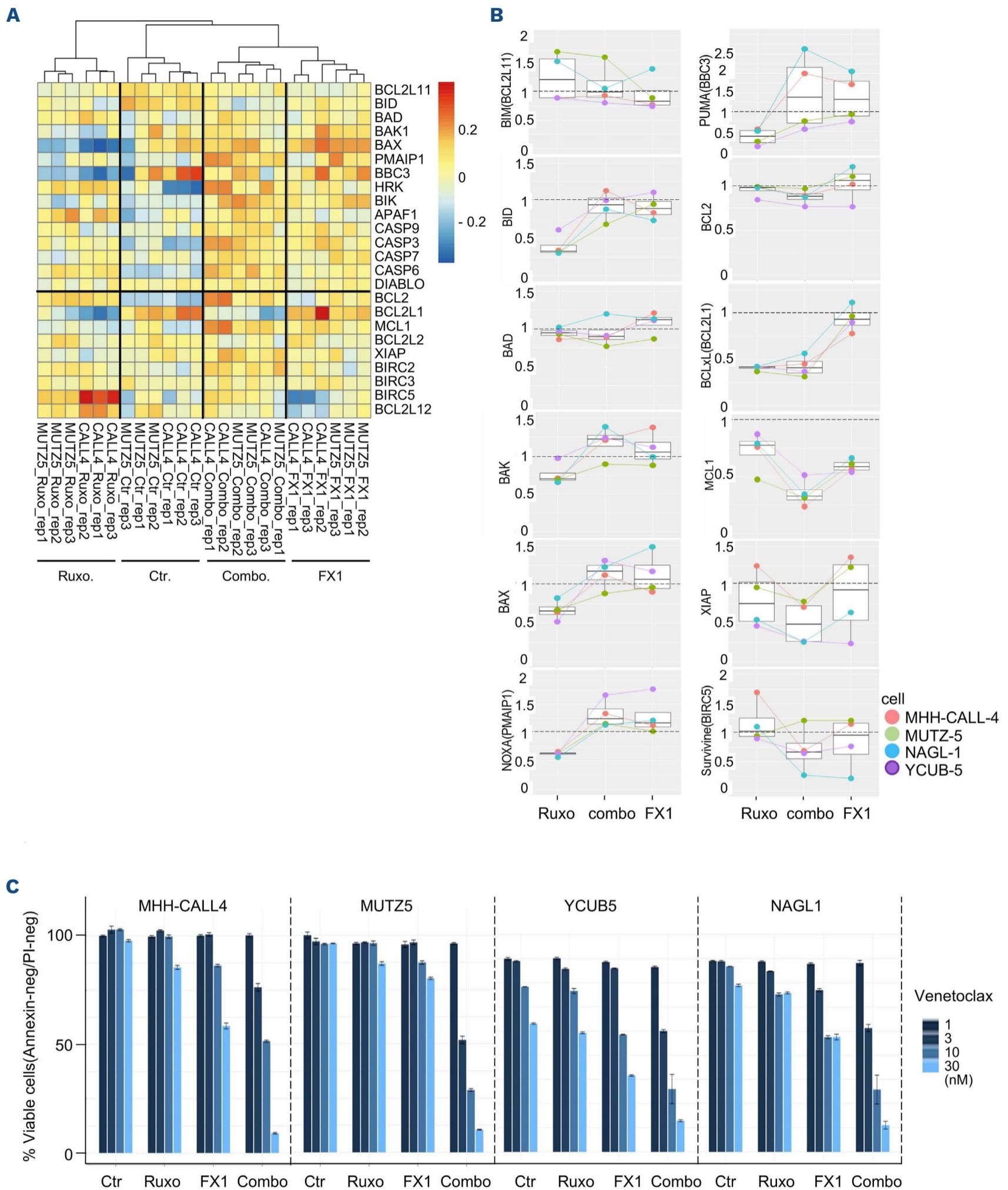
## Discussion

CRLF2-ALL responds inadequately to type I JAK1/2 inhibitors, such as ruxolitinib.<sup>4,14,15</sup> This study identified upregulated BCL6 as a reason for this. Upregulated BCL6 was

accompanied by a decrease in TP53 protein, with blunted gene expression in the TP53 tumor suppressor pathway. Conversely, BCL6 inhibitors restored TP53 levels and its downstream gene expression. In addition, ruxolitinib and BCL6 inhibitors downregulate MYC in concert. Therefore, these two drugs act synergistically in the treatment of CRLF2-ALL. Although BCL6 upregulation is implicated in imatinib resistance in Ph ALL,<sup>42</sup> this study revealed the role of BCL6 in ruxolitinib resistance in CRLF2-ALL.

The following findings support the mechanistic link that JAK1/2 inhibition upregulates BCL6, leading to TP53 downregulation: (i) STAT5 activity, estimated by p-STAT5, is inversely correlated to the levels of BCL6 expression (Figure 5A); (ii) manipulation of STAT5 activity suggests that STAT5 harnesses BCL6 expression (Figure 1E; *Online Supplementary Figure S1F*);<sup>24,42,43</sup> (iii) gene expression analysis coupled with publicly available BCL6 chromatin immunoprecipitation sequencing data suggests that BCL6 may downregulate molecules contributing to TP53 protein stabilization (*Online Supplementary Figure S5F, G*); and (iv) pharmacological degradation, inhibition, and knockdown of BCL6 upregulated TP53 (Figure 5, *Online Supplementary Figure S2C*). However, the detailed molecular mechanisms underlying these observations await further investigation.

Six out of seven CRLF2-ALL cells used in this study had no deletion/mutation of the TP53 gene (*Online Supplementary Table S2*); this fact, coupled with the reports that TP53 mutation/deletion in clinical CRLF2-ALL cells are rare (<5%),<sup>10,44,45</sup> suggests that upregulated TP53 protein in CRLF2-ALL by BCL6 inhibitors is mostly functional. This study suggests that the synergy of BCL6 inhibitors with ruxolitinib largely relies on TP53 activation (*Online Supplementary Figures S4D, S5A-D, S6, S7, and S8B, E-G*) but does not exclude the possibility of additional mechanisms. Because BCL6 deletion provokes systemic inflammation in mice,<sup>46</sup> this study primarily used the small-molecule inhibitors FX1 and BI3812 as probes to infer the effects of BCL6 inhibition; these inhibitors diminish the transcriptional suppressor functions of BCL6 by interfering with the binding of BCL6 to co-repressors. This interference does not provoke inflammation.<sup>32</sup> Experiments using these probes suggested that the mere expression of BCL6 does not predict response to BCL6 inhibitors across subtypes of ALL (Figure 1D, F), as in diffuse large B-cell lymphoma.<sup>47</sup> This study implied that CRLF2-ALL is BCL6-dependent, particularly when JAK1/2 inhibitors are used, and is therefore amenable to BCL6 inhibition. However, a small fraction of CRLF2-ALL, particularly in children with National Cancer Institute standard-risk disease, does not show the kinase-activated signature (and is thus not considered Ph-like ALL),<sup>3,7</sup> raising the possibility that this non-Ph-like subset does not show BCL6 dependency. It will, therefore, be important to identify CRLF2-ALL patients who may benefit from the use of BCL6 inhibitors.



**Figure 8. Alterations of intrinsic apoptosis pathway gene expression and their relation to the sensitivity to venetoclax-induced cell death.** (A) Heatmap representation of changes in the expression of genes involved in the intrinsic apoptosis pathway. Cells were treated as in Figure 4A. (B) Box-plot representations of changes in the expression of selected proteins shown in *Online Supplementary Figure S8A*. The level of protein in untreated cells is set as 1, and the relative protein expression levels upon treatment with ruxolitinib, FX1, and their combination are presented. GAPDH protein was used as a loading control. (C) Sensitivity of CRLF4-ALL cells to venetoclax-induced cell death in the presence of ruxolitinib, FX1, and their combination. Cells treated with dimethylsulfoxide served as a control. The color-coded legend indicates venetoclax concentrations. Cells were treated with the indicated drugs for 24 h before treatment with graded concentrations of venetoclax for 12 h. The number of live cells following treatment with vehicle alone is set as 1, and the relative values are shown as the mean  $\pm$  standard deviation. Ruxo: ruxolitinib; Ctrl; control; Combo: ruxolitinib + FX1; PI: propidium iodide.

Previous work suggested the use of a combination of a JAK inhibitor and a BCL2/BCLxL dual inhibitor to treat CRLF-ALL.<sup>18</sup> However, BCLxL inhibition could cause life-threatening thrombocytopenia in a clinical setting, whereas a BCL2 inhibitor (venetoclax) does not.<sup>41</sup> This study revealed that ruxolitinib downregulated BCLxL, probably due to decreased STAT5 binding to the *BCLXL* promoter.<sup>48</sup> FX1 downregulated MCL1 (Figure 8B), likely through TP53-mediated mechanisms (Online Supplementary Figure S5A).<sup>49</sup> In addition, ruxolitinib downregulated, and FX1 restored the expression of the pro-apoptotic proteins PUMA, NOXA, BID, BAX, and BAK (TP53 targets).<sup>49,50</sup> However, ruxolitinib and FX1 did not appreciably affect BCL2 expression (Figure 8A, B). Therefore, the ruxolitinib and FX1 combination primes CRLF2-ALL cells to venetoclax hypersensitivity (Figure 8C, Online Supplementary Figure S8D).

Although the number of patients' samples tested was small and *in vivo* treatment schedules/dosing have not been optimized, this study illuminates a potential clinical utility of a BCL6 inhibitor in conjunction with JAK inhibition as a strategy to treat CRLF2-ALL.

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### Contributions

ST designed the study, performed experiments, and wrote the manuscript. TY performed experiments and contributed to writing the manuscript. HG, KA, and TI provided cell lines. NM, HY, HK, and FH provided patients' cells. SK, AO, TH, HK, and YH provided technical help. FH contributed to writing the manuscript.

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### Data-sharing statement

The RNA-sequencing data have been deposited in ArrayExpress under accession number E-MTAB-10755.

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