Supplementary Materials for

JAK/BCL2 inhibition acts synergistically with LSD1 inhibitors to selectively target ETP-ALL

Aissa Benyoucef, Katharina Haigh, Andrew Cuddihy, Jody J. Haigh

Correspondence to: aissabenyoucef@gmail.com & jody.haigh@umanitoba.ca

This PDF file includes:

Materials
Methods
Figures. S1 to S6
Tables S1 to S2
## Materials

### 1- Western blot Antibodies table

| Antibody Name                        | Catalog No          | Link                          | Dilution |
|--------------------------------------|---------------------|-------------------------------|----------|
| HDAC1 antibody (mAb)                 | 39531               | https://www.activemotif.com   | 1/500    |
| Zeb2 antibody                        | NBP1-82991          | https://www.novusbio.com      | 1/500    |
| LSD1 Antibody (1B2E5)                | NB100-1762          | https://www.novusbio.com      | 1/1000   |
| beta-Actin Antibody (AC-15)          | NB600-501           | https://www.novusbio.com      | 1/2000   |
| STAT5                                | 94205               | https://www.cellsignal.com    | 1/1000   |
| P-STAT5(Tyr694)                      | 9351                | https://www.cellsignal.com    | 1/1000   |
| H3K27me3                             | 07-449              | https://www.emdmillipore.com  | 1/5000   |
| anti-H3K4me3                         | ab8580              | https://www.abcam.com         | 1/5000   |
| anti-pan histone H3                  | 07-690              | https://www.emdmillipore.com  | 1/5000   |
| anti-H3K27Ac                         | ab4729              | https://www.abcam.com         | 1/5000   |
| anti-H3K4m2                          | 39079               | https://www.activemotif.com   | 1/5000   |
| anti-H3K4me1                         | ab8895              | https://www.abcam.com         | 1/5000   |
| **In Vivo MAb anti-mouse IL-7Rα (CD127)** | A7R34               | https://bxcell.com/           | 100ug/ml |

### 2- Flow cytometry Antibodies table

| Antibody Name                        | Catalog No   | Link                          |
|--------------------------------------|--------------|-------------------------------|
| CD45                                 | 557748       | https://wwwbdbiosciences.com   |
| CD7                                  | 564020       | https://wwwbdbiosciences.com   |
| CD3                                  | 555340       | https://wwwbdbiosciences.com   |
| PerCP anti-mouse/human CD11b Antibody| 101229       | https://www.biolegend.com     |
| APC/Cyanine7 anti-mouse Ly-6G Antibody| 127623       | https://www.biolegend.com     |
| PE anti-mouse CD3 Antibody           | 100205       | https://www.biolegend.com     |
| APC Rat Anti-Mouse CD4               | 561091       | https://wwwbdbiosciences.com   |
| PerCP anti-mouse CD8a Antibody       | 100731       | https://www.biolegend.com     |
| APC/Cyanine7 anti-mouse/human CD44 Antibody | 103027 | https://www.biolegend.com |
| PE-Cy™7 Rat Anti-Mouse CD25          | 561780       | https://wwwbdbiosciences.com   |
3- Primer list table (please check the Excel file: Table S2)

Methods

Western blots:

Protein extraction and immunoprecipitation were performed essentially as described (Benyoucef et al 2016). For the Immunoblotting, protein extraction was prepared from 100,000 to 200,000 cells for SDS-PAGE (12%) and proteins were transferred to nitrocellulose membranes and probed with different antibodies, see Abs table. For the immunoprecipitation, briefly, the beads were cross-linked with Abs washed twice with 1M immunoprecipitation buffer (25 mM Tris at pH 7.9, 5 mM MgCl₂, 10% [v/v] glycerol, 0.1 % [v/v] NP40, 1 M KCl, 0.3 mM DTT, protease inhibitors), two washes with 150 mM immunoprecipitation buffer (25 mM Tris at pH 7.9, 5 mM MgCl₂, 10% [v/v] glycerol, 0.1 % [v/v] NP40, 150 mM KCl, 0.3 mM DTT, protease inhibitors) and incubated with Nuclear extraction for ON. Bound proteins were eluted with 5% acetic acid, dried and resolved by SDS-PAGE. Proteins were then identified by Western blot and quantified with ImageJ software.

Bioinformatic analysis of ENCODE ChIP-seq data:

The high-throughput sequencing data were obtained from https://www.encodeproject.org STAT5b (ENCFF248XJW) and its control (ENCSR766WPH). KDM1A/LSD1 (ENCFF087TDC) and its control (ENCSR000AKY). ZEB2 (ENCSR004GKA) and its control (ENCSR173USI, ENCSR554FDZ). The Raw data were trimmed to remove low-quality reads and mapped to the NCBI build 37.1 (hg19) human reference genome using BWA (version 0.7.17.4). To detect regions of enrichment, we used model-based analysis of ChIP combined with massively parallel sequencing (MACS2 callpeak) version 2.1.1.20 using default values with a ctrl ChIP as a background and a Minimum FDR (0.05 q-value) cutoff for peak detection. The common and
unique peaks were defined by bedtools Intersect intervals (version 2.29.0). We used the plotHeatmap (Version 3.3.2.0.1) to create a heatmap for score distributions across LSD1, ZEB2 and STAT5 peak genomic regions. Association of common peaks (ZEB2, LSD1 and STAT5) with genes was performed with GREAT (http://great.stanford.edu/public/html/index.php) using the Basal plus extension setting. Check the GO biological process obtained with GREAT. The list of genes related to regulation of intrinsic apoptotic signaling pathway was illustrated by STRING (https://string-db.org).

**Animal experimentation and handling.**

All experiments were performed according to the regulations and guidelines of the Ethics Committee for care and use of laboratory animals at the University of Manitoba. The mouse (Mus musculus) cohorts used in these experiments were sibling littermates and maintained. Both male and female mice were included in the various analyses and ranged in age from 3 to 60 weeks. For the transplantation experiments, primary thymic tumours (Lck-Cre, R26-Zeb2^Tg/Tg, p53^F/F^) were dissected under aseptic conditions. Cell was prepared in sterile PBS and the indicated cell numbers were intravenously injected in 6- to 10-week-old total body irradiated C57BL/6J wild-type recipient mice (Charles River). A similar approach was used for the transplantation of LOUCY cells into NSG mice (Charles River). For drug treatments, after 3-7 days post-injection, the engraftment was measured routinely (5-10%) in peripheral blood, and randomly the mice were treated with SP2509 (Cedarlane lab, CA) prepared in solvent buffer (20% PEG-40, 20% dimethyl sulfoxide, 60% sterile water) by intra-peritoneal injection. ABT-199 (Cedarlane lab, CA) prepared in 2.5%dimethyl sulfoxide, 50% polyethylene glycol 300, 5% Tween 80, and 42.5% of 0.5% methyl cellulose in water, administrated in mice by oral gavage. For Ruxolitinib phosphate (Chemscene. USA) prepared in vehicle (20% Captisol in 58 mM citrate buffer) twice daily by oral gavage. For GSK-LSD1(Chemscene. USA) prepared in vehicle (sterile NaCl 0.9%) administrated in mice by oral gavage.
Patient-derived xenograft models in NSG mice.

NSG (NOD.Cg-Prkdc<scid>Ii2rg<tm1Wjl>SzJ) male/female mice (8–10 wk of age) were purchased from The Jackson Laboratory and maintained under sterile conditions at the University of Manitoba Animal Care Facility. Leukemic blasts from patients (characterized and provided by The Quebec Leukemia Cell Bank) were expanded in NSG mice by transplanting $0.5 \times 10^6$ to $2 \times 10^6$ cells via intravenous injection. Engraftment of human leukemic blasts in the peripheral blood was assessed by FACS staining for human CD45 and human CD7 during a period of 1-6 months. Mice were euthanized when the engraftment of leukemic blasts was 5-30%, and BM and spleen cells were harvested. All the ETP-ALL and mature T-ALL cell patients are characterized and provided by The Quebec Leukemia Cell Bank (BCLQ) with the consent of all subjects involved.

Drug treatment of xenograft Patient-derived cells.

To ensure the high purity of blasts harvested from the BM of engrafted NSG mice, we used the EasySep™ FITC Positive Selection Kit II (StemCell Technologies, 17682) to sort the stained blasts with the anti-human CD7 FITC. For the drug treatment in vitro, from each condition, the sorted blasts were cultured on OP9 stromal cells expressing the NOTCH ligand Delta-like-1 (DL1) as previously described (Benyoucef et al. 2015). Briefly, $1 \times 10^5$ cells from each sample were plated in α minimum essential medium with 10% fetal calf serum (StemCell Technologies, 06450) and 10% serum (Sigma-Aldrich, H4522) supplemented with 50 ng/mL hSCF cytokine, 20 ng/mL Flt3-L cytokine, 20 nM insulin, 10ng/mL IL-7, and 100 U/mL penicillin and 100 mg/mL streptomycin. All human cytokines are provided by peprotech Canada. The cells are treated for 5 days with DMSO (as control condition), Ruxolitinib alone (5uM), GSK-LSD1 alone (100nM), SP-
2905 alone (250nM), ABT-199 alone (25nM), or with drug combination indicted in the Figure 7 and S8. The apoptosis of blasts after treatment was assessed as mentioned previously.

**Human mRNA expression in primary human samples.**

We used the mRNA expression data in human patient ETP-ALL (n=12) vs mature T-ALL (n=40) samples previously published (Zhang J et al, 2012, Gutierrez A et al. 2011), and publicly available at Gene Expression Omnibus-NCBI, GSE28703.

**Figure S1.**

(A) Western blot (WB) analysis of uncrosslinked immunoprecipitation of 3XFLAG tagged ZEB2 performed in T-ALL (P53−/−) and ETP-ALL (ZEB2tg/tg P53−/−) cell line nuclear extracts (NEs) with Abs against endogenous ZEB2, LSD1 and STAT5. (B) ZEB2 knockdown (KD) with 3 independent ShRNAs measured by Western blot. (C) Cell growth measured by the fold change number cells (D10/D1) after the ZEB2 knockdown in T-ALL (P53−/−-WT-1) and ETP-ALL (ZEB2tg/tg P53−/−-TG-50) from 3 independent experiments (D) Representative cytometry plots of the experiments used to measure the cell growth of T-ALL (P53−/−-WT-1) and ETP-ALL (ZEB2tg/tg P53−/−-TG-50) cell line (illustrated in (C)) after the efficient KD of ZEB2 with ShZeb2#2 & ShZeb2#3. Representative flow cytometry blots of ShZeb2#2 & ShZeb2#3 that were used to assess the growth of transduced GFP+ T-ALL (P53−/−-WT-1) and ETP-ALL (ZEB2tg/tg P53−/−-TG-50) cells at day 1 and day 10 of the culture (E) Viability assays in the T-ALL (P53−/−) cell lines and ETP-ALL (ZEB2tg/tg P53−/−) cell lines treated for 48h with (open circles) or without (black squares) IL7 cytokine (10ng/ml) plus increasing concentrations of ORY-1001 or (F) with SP2509. All experiments were performed in triplicate. *p< 0.05; **p< 0.01, ***p< 0.001.
Figure S2.

Exploring the transcriptional role of ZEB2, LSD1 and STAT5a in leukemic context by using publicly available high throughput sequencing data (ENCODE Project) (A) Average profile and heat map for LSD1, ZEB2 and STAT5b ChIP–seq in same leukemic context (K562) data obtained from ENCODE project 3/4. Metagene plot ± 5 kb is shown. \( P < 0.05 \) (B) Venn diagram of overlapping peaks bound by LSD1, ZEB2 and STAT5b protein using significance threshold at \( P < 0.01 \). (C) analysis the genome binding distribution of the common peaks (ZEB2-LSD1-STAT5b). (D) GO analysis of common-bound genes (LSD1/ZEB2/STAT5b) classified by the Binomial \( p \) value. (E) STRING interaction network of the genes involved in regulation of intrinsic apoptosis signaling pathway (highlighted by * in D) and potentially regulated by LSD1, ZEB2 and STAT5b (n=109 genes).

Figure S3.

A) RNA expression of the anti-apoptotic proteins (BCL2, BCL2L1, MCL1) and anti-apoptotic protein BIM (BCL2L11) in T-ALL (n=40) and ETP-ALL (n=12) patients. B) Time course of IL7 stimulation, after 6h without stimulation, the mouse T-ALL (WT-1) and ETP-ALL (TG-50) was stimulated with 10ng/ml of IL7 and the RNA expression of Bcl2, Bcl2l1, Mcl1 and Bcl2l11 (BIM) genes was measured at different time point (0, 30, 60 and 120 min). \( n = 3 \). (*) \( P < 0.05 \); (**) \( P < 0.01 \); (***) \( P < 0.001 \). C) Assessing the role of IL7R in the regulation of the anti-apoptotic proteins (BCL2, BCL2L1, MCL1) in T-ALL (WT-1) and ETP-ALL (TG-50) using the Ruxolitinib (50ng/ml) or the anti-IL-7Rα (100ug/ml).

Figure S4.

(A) Measuring the cell growth of mouse T-ALL (WT-1) and ETP-ALL (TG-50) after treatment with increasing concentration of specific inhibitor of MCL1 (AZD5991), inhibitor of BCL2L1 (WEHI-
539), or inhibitor of BCL2 (ABT-199) in presence or absence of GSK-LSD1 inhibitor (100nM) \( n = 3 \). (*) \( P < 0.05 \); (**) \( P < 0.01 \). B&C apoptosis measurement after 48h of treatment with MCL1 inhibitor (AZD5991, 10nM), BCL2L1 inhibitor (WEHI-539, 10nM) or BCL2 inhibitor (ABT-199, 10nM) in combination or not of GSK-LSD1 inhibitor (100nM). \( n = 3 \). (*) \( P < 0.05 \); (*** \( P < 0.001 \).

**Figure S5.**

UCSC genome browser tracks showing ChIP–seq of LSD1, ZEB2 and STAT5 in representative Bcl2, Bcl2l11 and CD8a loci in leukemic context (K562) using the ENCODE project data (See Sup Figure 2A). The sonication optimization (~90 cycles our optimal condition) (B) and the validation by western blot of epigenetic marks antibodies used in ChIPqPCR experiments (C) in T-ALL (P53\(^{+/−}\)-WT-1) and ETP-ALL (ZEB2\(^{tg/tg}\) P53\(^{+/−}\)-Tg-50) cell lines. (D) Quantification of western results for global methylation marks in T-ALL and ETP-ALL cells.

**Figure S6.**

Assessing the toxicity and the tolerability of combination therapy for GSK-LSD1 and Ruxolitinib or ABT-199 drug in vivo. (A) Experimental strategy. Healthy C57BL/6J mice were treated by gavage with vehicle control, GSK-LSD1 (1.5mg/kg/day) and/or Ruxolitinib (30mg/kg/ twice a day) for 2 consecutive weeks. (B) the impact of treatment with single or combined drugs on different hematopoietic cells using the cell surface markers(Ter119 (Erythroid), Ly6G (Neutrophils), Thy1.2 (T cells), CD11b (Mono/eosinophils), CD41 (Megakaryocytes) and B220 (B cells) (C) and assessed in different tissue by flow cytometry assays that revealed slight increases in mono/eosinophils in spleen tissue but overall, no sign of toxicity or intolerability was observed during and after the 2 weeks of treatment. \( n = 4 \) mice/group *p< 0.05.

**Figure S7.**
(A) RNA expression of the known oncogenes (LYL1, LMO2, HHEX) and ZEB2 in T-ALL (n=40) and ETP-ALL (n=12) patients. (B) ZEB2 protein measurement with western blot in human ETP-ALL cell line (LOUCY) transduced with shCtrl or 3 independent shRNA against ZEB2. (C) Flow cytometry analysis of GFP+ cells over time of transduction human ETP-ALL LOUCY cell line with ShCtrl or 3 independent shRNA_ZEB2 and (D) measuring the % of GFP+ cells at Day 1 and Day 7 of the culture n = 3. (*) P < 0.05; (**) P < 0.01. (E) Assessing the growth of GFP+ LOUCY cells using fluorescent microscopy at Day 1 and Day 12 (400× magnification [0.4 mm]).

**Figure S8.**

Drug synergy analyzed by using Combbenefit software revealed the absence of synergy between the methylase LSD1 inhibitors (A) GSK-LSD1 (high-dose) or (B) ORY-1001) with Ruxolitinib. (C) the high synergy of scaffolding LSD1 inhibitor (SP2509) with BCL2i (ABT-199) to compromise cell growth of human ETP-ALL LOUCY cell line but not JURKAT cells. (D) Measurement of epigenetic marks from 3 independent experiments performed in human mature T-ALL JURKAT and ETP-ALL LOUCY cell lines cultured with Ruxolitinib (5uM) with/without SP2509 (250nM) assayed by western blot analysis and protein quantification compared to internal loading controls. n = 3 *p< 0.05; **p< 0.01. (E) Using the AnnexinV/PI assay to measure the apoptosis of human mature T-ALL JURKAT and MOLT3 cell lines and unique ETP-ALL LOUCY cell line, and (F) Normalized measurement of AnnexinV/PI+ cells in each cell line treated with ABT-199 (10nM) with/without SP2509 (250nM). n =average of 3 experiments **p < 0.01. (G) Representative AnnexinV/PI flow cytometry results of primary T-ALL (15h025) and ETP-ALL (15h069) for control (DMSO), single drug (Ruxo, GSK-LSD1, SP2509) and combination drug exposure (GSK-LSD1/Ruxo, SP2509/Ruxo). (H) Normalized AnnexinV/PI+ measurement for each primary T-ALL (N=3) and ETP-ALL (N=2) patient sample treated with ABT-199 (25nM)
with/without GSK-LSD1 (100nM) and SP2509 (250nM). Experiments were performed in triplicate. **p<0.01, ***p< 0.001.

Figure S9.
Uncropped western blot analysis of murine T-ALL (WT-1) and ETP-ALL (TG-50) cell lines for effects of GSK-LSD1, ruxolitinib and combination treatment on H3 epigenetic marks.

Figure S10.
Uncropped western blot analysis of human T-ALL (JURKAT) and ETP-ALL (LOUCY) cell lines for effects of SP2509, ruxolitinib and combination treatment on H3 epigenetic marks.

Table S1.
Supplementary table1 excel file. It contains the common ChIP peak locations. List of genes associated with common peaks. The results of the PANTHER, GREAT analysis, STRING intrinsic apoptosis genes of the common peaks.

Table S2.
The list of primers used in this study.
Figure S2

A) LSD1 narrow Peaks  
B) Peaks = 71044  
C) ZEB2 narrow Peaks  
D) STAT5b narrow Peaks  
E) Peaks = 58232  
F) Peaks = 253

B) Common peaks ZEB2/LSD1/STAT5b

C)  

D) GO Biological Process

- log10(Binomial p value)

E) STRING interaction network: regulation intrinsic apoptosis signaling pathway (109 genes)
Figure S3

A

Bcl2 qPCR

Bcl2 IqPCR

Mcl1 qPCR

Bcl2l11 (BIM) qPCR

B

C

T-ALL (P53-)

ETP-ALL (P53-ZEB2 wt)

- + + + - + + +

Ruxolitinib (50ng/ml)

anti-IL-7Ra (100ug/ml)

IL7 (10ng/ml)

150 kDa

100 kDa

50 kDa

37 kDa

ZEB2

pSTAT5

STAT5 (total)

BCL2

BCL2L1

MCL1

ACTIN
**Figure S4**

A. T-ALL (WT-1) (P53<sup>-/-</sup>)

- T-ALL (WT-1) (P53<sup>-/-</sup>)
  - AZD5991 w/o GSK-LSD1
  - AZD5991 with GSK-LSD1

B. T-ALL (WT-1) (P53<sup>-/-</sup>)

- +DMSO +AZD5991 +WEHI-539 +ABT-199
  - GSK-LSD1

C. ETP-ALL (TG-50) (P53<sup>-/-</sup> ZEB2<sup>-/-</sup>)

- +DMSO +AZD5991 +WEHI-539 +ABT-199
  - GSK-LSD1
Figure S6

A

Healthy mice

Drug toxicity and tolerability test

Control
Ruxolitinib
GSK-LSD1
Ruxo+ABT-199
Ruxo+GSK-LSD1

Control
Ruxolitinib
GSK-LSD1
Ruxo+ABT-199
Ruxo+GSK-LSD1

5 days treatment

2 days Off

5 days treatment

B

blood

Spleen

Bone marrow (BM)

Thymus

C

blood

Spleen

Bone marrow (BM)

Thymus
Figure S7

A

Box plots showing transformed count value for ETP-ALL, T-ALL, LMO2, HHEX, and ZEB2.

B

Western blot images for shCtrl and shZEB2#1, #2, #3 with molecular weights of 150 kDa and 50 kDa.

C

Heat maps for Day 1 and Day 7 showing FITC/GFP for shCtrl, shZEB2#1, #2, and #3.

D

Bar graphs showing % GFP Cells for different conditions.

E

Images showing cell culture for Day 1 and Day 12 for shCtrl, shZEB2#1, #2, and #3.
Figure S8

A. ETP-ALL (LOUCY cell line) GSK-LSD1
B. ETP-ALL (LOUCY cell line) GSK-LSD1
C. Mature T-ALL (JURKAT cell line) ABT-199
D. H3k4me1, H3k4me2, H3k4me3, H3k27ac, H3k27me3

E. ETP-ALL (LOUCY cell line) AnnexinV

F. Field change AnnexinV

G. ETP-ALL (LOUCY cell line) AnnexinV

H. Field change AnnexinV
Figure S9

|                | EXP1 | EXP2 | EXP3 |
|----------------|------|------|------|
| T-ALL (WT-1)  | -    | -    | -    |
| (P53^3/4)     | -    | -    | -    |
| ETP-ALL (TG-50)| -    | -    | -    |
| (P53^3/4; ZEB2^tg/tg) | -    | -    | -    |
| T-ALL (WT-1)  | -    | -    | -    |
| (P53^3/4)     | -    | -    | -    |
| ETP-ALL (TG-50)| -    | -    | -    |
| (P53^3/4; ZEB2^tg/tg) | -    | -    | -    |
| T-ALL (WT-1)  | -    | -    | -    |
| (P53^3/4)     | -    | -    | -    |
| ETP-ALL (TG-50)| -    | -    | -    |
| (P53^3/4; ZEB2^tg/tg) | -    | -    | -    |

Ruxolitinib
GSK-LSD1
Figure S10

EXP1

| Mature T-ALL (JURKAT cell line) | ETP-ALL (LOUCY cell line) |
|--------------------------------|---------------------------|
| -                             | -                         |
| +                             | +                         |
| +                             | +                         |
| +                             | +                         |

EXP2

| Mature T-ALL (JURKAT cell line) | ETP-ALL (LOUCY cell line) |
|--------------------------------|---------------------------|
| -                             | -                         |
| +                             | +                         |
| +                             | +                         |
| +                             | +                         |

EXP3

| Mature T-ALL (JURKAT cell line) | ETP-ALL (LOUCY cell line) |
|--------------------------------|---------------------------|
| -                             | -                         |
| +                             | +                         |
| +                             | +                         |
| +                             | +                         |

SP2509 Ruxolitinib

Pan-H3