

## Immunomodulatory drugs downregulate IKZF1 leading to expansion of hematopoietic progenitors with concomitant block of megakaryocytic maturation

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## **Supplementary Methods**

### **Immunolabeling for fluorescence microscopy**

Cells were cytocentrifuged onto slides and fixed in ice-cold acetone (Sigma Aldrich) for 5 min. Non-specific binding was prevented by 30 min incubation of cells in 1% bovine serum albumin. Anti-GATA1 antibody (N6; Santa Cruz Biotechnology) was subsequently applied to the slides and incubated overnight at 4°C. Slides were then washed 3 times before and after application of the secondary antibody. We performed 4'-6-diamidino-2-phenylindole-2HCl (DAPI) staining in Vectashield (Vector) to visualize cell nuclei.

### **Flow cytometry analyses of cell cycle and apoptotic cells**

Cells were harvested, washed with ice-cold 1×PBS, fixed with 70% ethanol for 1 h on ice, and pretreated with RNase (Worthington) for 30 min, followed by DNA staining with propidium iodide (PI, 20 µg/mL; Sigma Aldrich). The percentage of cells are in G0/G1, S and G2/M phases, as well as the cells in the sub-G1 phase prior to apoptosis be determined and analyzed by flow cytometry and ModFit LT2.0 software (BD Biosciences).

### **Flow cytometry**

All of the antibodies were purchased from either BD Bioscience, Beckman Coulter, Life Technologies or BD Pharmingen. Antibodies included CD42b-FITC (clone AK2), CD41a-PE (clone P2), CD61-PE (clone SZ21), CD38-biotin (clone LS198-4-3), Streptavidin-ECD, CD33-PECy5 (clone D3HL60.251), CD11b-APC (clone 340937), CD41-PC7 (clone P2), CD34-APC-Alexa700 (clone 581), CD45-APCCy7 (clone 2D1). Multi-dimensional flow cytometric acquisition was performed using a 10-color Gallios cytometer (Beckman Coulter). Offline compensation and analyses were performed using VenturiOne software (Applied Cytometry).

## **Western blot analysis**

Cells for Western blotting were prepared as described previously <sup>1</sup>. The following antibodies were used: anti-GATA1 (N6), anti-FOG (M-20) and anti-NFE2 (C-19) from Santa Cruz Biotechnology; anti-cyclin D1 and anti-p16 from Cell Signaling Technology; anti-IKZF1 from Abcam plc; anti-CRBN from Sigma-Aldrich. We used  $\beta$ -actin to normalize the protein quantity.

## **Real-time PCR**

Real-time PCR was performed as described previously <sup>1,2</sup>. The following primer sets were used: GATA1 5'-CAA GCT TCG TGG AAC TCT CC-3' and 5'-ACT GAC AAT CAG GCG CTT CT-3';  $\beta$ -actin 5'-GGA CTT CGA GCA AGA GAT GG-3' and 5'-AGC ACT GTG TTG GCG TAC AG-3'.

## **Chromatin immunoprecipitation assay**

$2 \times 10^7$  CD34<sup>+</sup> cells were treated without or with 1  $\mu$ M POM for 24 hours. Cells were collected and ChIP assays were performed using the Pierce Agarose ChIP kit (Thermo Scientific) according to the manufacturer's instructions. Antibodies used for IP were: IgG and IKZF1 (Abcam plc). EBF1B was used as positive control to qRT-PCR analysis <sup>3</sup>. Specific pairs of primers used for quantitative ChIP are listed as below: GATA1 promoter #5 forward: (5'-CAG TGG GGC GCT AAG TGA GT-3'), reverse: (5'-AAC TAG AGC CTG TGG GAT ACC T-3'); GATA1 promoter #6 forward: (5'-CAC CCC AAG ACA GCC TGT TA-3'), reverse: (5'-CTG GGG CAG CAG ATA AGT CT-3'); GATA1 promoter #7 forward: (5'-CGA GGA ATC ATC CCT GGC TC-3'), reverse: (5'-CCG CAG TAA CAG GCT GTC TT-3'); GATA1 promoter #8 forward: (5'-CAC GTC TGT CTC CTT CTC TA-3'), reverse: (5'-AGG AGT GGG ATA GGG TAG GA-3'); GATA1 promoter #11 forward: (5'-GTG CAT GTG TGT CAG ATG CTT-3'), reverse: (5'-GAA TGT GGC TAG GCC TCC G-3'); EBF1 B promoter forward: (5'-GGG TTA GTG TGC CTG TGT TTA G-3'), reverse: (5'-CCC TGC TGG ATG GAG ATT CTG-3').

### **Lentiviral production and gene transduction**

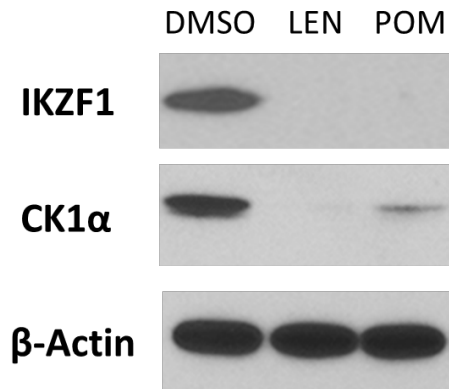
To silence IKZF1 expression, CD34<sup>+</sup> cells were transduced using pGreenpuro (System Biosciences) lentiviral particles containing IKZF1 targeting shRNAs, which express green fluorescent protein (GFP). The shRNA stable transfectants were obtained by cell sorting based on GFP expression. The target sequences were as follows: IKZF1 shRNA #1 (5'-CCG CTT CCA CAT GAG CTA AAG-3'); IKZF1 shRNA #2 (5'- GCA TTT GGA AAC GGG AAT AAA-3'). IKZF1, GATA-1 and CRBN protein levels were determined in control and silenced cells by Western blotting.

The pLenti V5 GATA1 expression vectors and vector alone were kindly provided by Dr. Griffin P. Rodgers. Lentivirus encoding GATA1 or vector alone were packaged into 293TN lentiviral producer cells (System Biosciences (SBI)) and supernatants containing viral particles were collected 48 h after transfection according to the ViraPower Lentiviral Expression Systems instructions (Invitrogen). The viral product was concentrated 100 times by PEG-it™ Virus Precipitation Solution (SBI) and titers were determined. Gene transduction of CD34<sup>+</sup> stem cells, was carried out according to Zhu J et al. with minor modifications<sup>4</sup>. Briefly,  $4 \times 10^6$  viral particles were preloaded onto RetroNectin-coated plates (Takara Shuzo) and centrifuged for 2 h at 32°C. Just before infection, the viral supernatant was discarded.  $4 \times 10^5$  purified CD34<sup>+</sup> cells grown in serum-free HPGM supplemented with 10 ng/mL rhIL-3, 10 ng/mL rhIL-6, and 50 ng/mL rhSCF for 2 days were added to the viral-preloaded plate and cultured for 3 days. After that, gene-transduced CD34<sup>+</sup> cells were reseeded into HPGM containing 10 ng/mL rhTPO, 10 ng/mL rhIL-3, 10 ng/mL rhIL-6, 50 ng/mL rhSCF, and 3 µg/mL blasticidin (Invitrogen). IMiDs or vehicle was added daily as mentioned before. Cells were harvested for colony assay, Western blot and FISH after 3 days, 6 days and 10 days of culturing respectively.

## References

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Figure S1



Purified CD34<sup>+</sup> cells were cultured in serum-free HPGM hematopoietic growth medium containing 10 ng/mL recombinant human thrombopoietin (rhTPO), 10 ng/mL recombinant human interleukin-3 (rhIL-3), 10 ng/mL recombinant human interleukin-6 (rhIL-6), and 50 ng/mL recombinant human stem cell factor (rhSCF) with 10 μM LEN, POM or 0.1% DMSO as vehicle control for 24 h. The expression of the indicated proteins was analyzed by Western blotting. β-actin was used as the loading control .