SUPPLEMENTARY MATERIALS

1) Supplemental Methods

Preparation of primary mouse bone marrow (BM) cells

C57BL/6 mice (8-12 weeks) were purchased from Charles River Laboratories and kept in the Division of Comparative Medicine animal facility within the faculty of Medicine. The animal protocols were approved by the University of Toronto Faculty Advisory Committee on Animal Services. Bone marrow cells were flushed from the femurs, tibiae and iliac crests using 23-gauge needles into Hank's Balanced Salt Solution (HBSS; Invitrogen) containing 2% fetal bovine serum (collectively called HF). To disperse clumps, cells were gently passed through a 23-gauge needle three times. Cells were then spun down at 500g for 5 minutes, suspended in HF and ammonium chloride solution (Stem Cell Technologies) at a volume:volume (v:v) ratio of 1:1, and incubated for 2 minutes on ice. Cells were washed once in HF before passed through a 40μm strainer to obtain single cell suspension. Nucleated cells were enumerated using a hemocytometer.

Magnetic- and fluorescence-activated cell sorting (MACS and FACS)

Mouse BM cells (after RBC lysis) were collected in HF supplemented with 1mM ethylenediaminetetraacetic acid (EDTA) and subsequently subjected to lineage depletion (including CD5, CD11b, CD19, CD45R/B220, Ly-6G/Gr1, Ter119, 7-4) by EasySep^{TM} magnetic sorting according to manufacturer's instructions (Stem Cell Technologies). To isolate c-Kit^{+}CD71^{high}Ter119^{-} cells, lineage negative (Lin^{-}) cells were stained with phycoerythrin (PE)-

conjugated anti-CD71, PE-cy7-conjugated anti-Ter119, and allophycocyanin (APC)-conjugated anti-c-Kit/CD117 antibodies (BD Biosciences) for 20 minutes on ice. Cells were washed and suspended in HF containing 2μg/mL 7-aminoactinomycin D (7-AAD; Invitrogen) which was used to discriminate dead cells. BD CompBeads (BD Biosciences) were used as compensation controls according to manufacturer's instructions. Fluorescence minus one (FMO) controls were used to set the threshold gate so that 99.9% of the cell population was below the gate for the corresponding color that was missing. 7-AAD^{-}c-Kit^{+}CD71^{high}Ter119^{-} cells were sorted by a fluorescence-activated cell sorter (MoFlo, Beckman Coulter, Miami, FL) at the SickKids-UHN Flow Cytometry Facility.

Flow cytometry and colony-forming cell (CFC) assay

Cultured cells from day 0, 1, 2, 3 and 3.5 were suspended in HF and incubated with rat anti-mouse CD16/32 antibody (Fc Block; BD Biosciences) for 5 minutes on ice. Cells were then stained with antibodies against c-Kit, CD71 and Ter119. Enucleation was assessed based on DRAQ5 staining according to manufacturer's instructions (Cell Signaling Technology). Samples were acquired on a BD FACSCanto flow cytometer and data were analyzed and presented using FlowJo7 software (Tree Star, San Carlos, CA). For CFC assays, cells were plated in duplicate in the semisolid culture media (MethoCult® M3434, Stem Cell Technologies). At day 2, cell colonies were stained with benzidine (Sigma-Aldrich) and counted under the microscope.

Survival and Proliferation Assays

Sorted c-Kit^{+}CD71^{high}Ter119^{-} cells were incubated in Iscove's Modified Dulbecco's Medium (IMDM) + 10%BIT for 30 minutes prior to exposure to SCF, EPO or SCF+EPO. To assess apoptosis, cells were stained with FITC-Annexin V (BD Biosciences) and 7-AAD. In 5-ethynyl-2'-deoxyuridine (EdU) proliferation assays, cells were incubated in the presence of 10μM EdU (Click-iT^{TM} EdU flow cytometry assay kit, Invitrogen) with SCF, EPO or SCF+EPO for 6 hours before stained for EdU incorporation levels according to manufacturer's instructions.

Quantitative real-time polymerase chain reaction (RT-PCR)

Purified c-Kit^{+}CD71^{high}Ter119^{-} cells were incubated in IMDM + 10%BIT for 30 minutes prior to exposure to SCF, EPO or SCF+EPO. At 0, 0.5, 1, 2, 4 and 6 hours, RNA was prepared using the PicoPure^{TM} RNA isolation kit (Arcturus Bioscience, Mountain View, CA). Reverse-transcription reactions were performed using Superscript III (Invitrogen) on Biometra® cycler (Montreal Biotech). Quantitative RT-PCR was carried out with SYBR Green master mix (Roche) on the 7900HT fast real-time PCR system (Applied Biosystems, Carlsbad, California). PCR primer pairs were custom made by Sigma-Aldrich and the sequences are listed in Table S2.

2) Supplemental Tables

Table S1. List of small molecule inhibitors and ligand and the concentrations used for the screen of signaling mediators of EPO-dependent downregulation of *c-Kit*.

Inhibitor/ligand	Stock concentration	Final concentration	Putative targets
1-Naphthyl PP1	50 mM	100 μM	v-Src, c-Fyn, c-Abl
Tyrphostin AG1478	5 mM	0.1 μM	EGFR
Bafetinib	10 mM	0.1, 1, 10 μM	Lyn
BIO	10 mM	0.5 μM	GSK3β
CHIR99021	6 mM	3 μM	GSK3β
Dasatinib	10 mM	0.1, 0.2, 1, 10 μM	Src family kinases
Dkk1	150 μg/mL	150 ng/mL	Wnt
GDC0941	100 mM	1 μM	PI3K
Jak Inhibitor I	10 mM	2 μM	Jak family
LDN193189	1 mM	2.5 μM	BMP type 1 receptor
MG132	3 mM	6 μM	Proteosome
SB203580	5 mM	10 μM	p38 MAPK
SU11274	10 mM	20 μM	c-Met tyrosin kinase
Wortmannin	2 mM	0.5 μM	PI3K and others

Table S2. List of primer sequences for the 17 candidate genes.

Gene	MGI ID	primer sequence
Bim	MGI:1197519	F:gctggtgggacctgtttcta R:ttcagtgagccatcttgacg
Bcl-xL	MGI:88139	F:ccttcaggcctctctctcct R:ccagcagctcctcacacata
Pim1[1]	MGI:97584	F:ttctggactggttcgagagg R:gctcctcgttcggtgataaa
cyclin D2	MGI:88314	F:ctgagtctggttggtgctga R:acacccgagaccacagaaac
cyclin G2	MGI:1095734	F:gcacctgtgtgaaagcagaa R:ccatcaccacacagaattgc
JunD	MGI:96648	F:tgccacacttgggaatatga R:ccagggtagccatcagttgt
CDK2	MGI:104772	F:cattcctcttcccctcatca R:gcagcccagaagaatttcag
GATA-1	MGI:95661	F:gatggaatccagacgaggaa R:gccctgacagtaccacaggt
PU.1	MGI:98282	F:ggcagcaagaaaaagattcg R:tttcttcacctcgcctgtct
GATA2	MGI:95662	F:tgcatgcaagagaagtcacc R:accacccttgatgtccatgt
Hbβmajor	MGI:96021	F:gctggttgtctacccttgga R:acgatcatattgcccaggag
c-Fos	MGI:95574	F:ccagtcaagagcatcagcaa R:taagtagtgcagcccggagt
c-Myc	MGI:97250	F:gcccagtgaggatatctgga R:atcgcagatgaagctctggt
Egr1	MGI:95295	F:gacgagttatcccagccaaa R:ggcagaggaagacgatgaag
c-Jun	MGI:96646	F:aaaaccttgaaagcgcaaaa R:cgcaaccagtcaagttctca
EpoR	MGI:95408	F:cccaagtttgagagcaaagc R:tgcaggctacatgactttcg
c-Kit	MGI:96677	F:ttatcctttaggccgtgtgg R:tgtggccccttaagtacctg
GAPDH	MGI: 95640	F:aggtcggtgtgaacggatttg R:ctgtagaccatgtagttgaggtca

F: Forward; R: Reversed

1. Menon MP, Fang DM, Wojchowski DM. Core erythropoietin receptor signals for late erythroblast development. Blood. 2006;107:2662-2672.

3) Supplemental Data

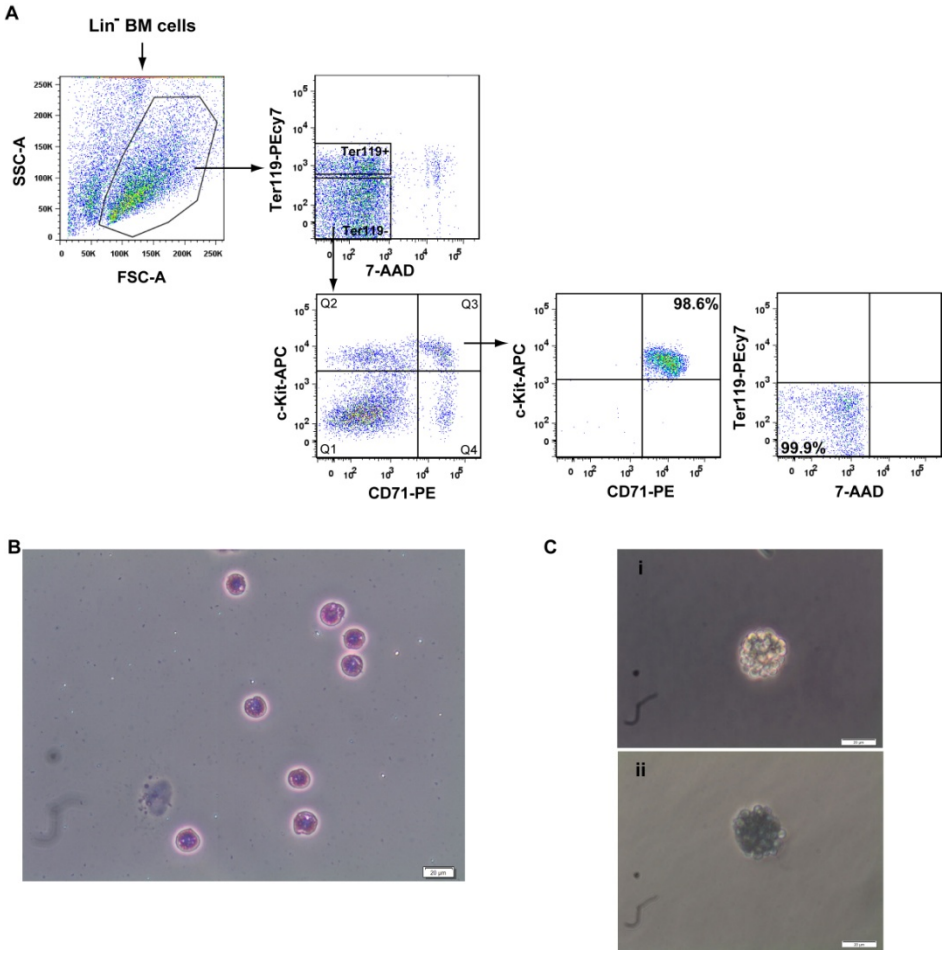


Figure S1. Isolation of CFU-E cells by flow cytometry. (A) C-Kit^{+}CD71^{high}Ter119^{-} cells were purified by lineage depletion of bone marrow cells using EasySep (Stem Cell Technologies) magnetic sorting followed by FACS. (B) C-Kit^{+}CD71^{high}Ter119^{-} cells were spun onto cytospin slides and stained with Giemsa-benzidine. (C) Morphology of day 2 CFU-E colonies derived from a single c-Kit^{+}CD71^{high}Ter119^{-} cell cultured in MethoCult M3434 (Stem Cell Technologies) without (i) and with (ii) benzidine staining. 72% ± 3% (±SD) of c-Kit^{+}CD71^{high}Ter119^{-} cells gave rise to CFU-E colonies (n = 5). No other colonies were detected. Images were visualized using an Olympus CKX41 microscope equipped with an Olympus SC30 camera. CellSense Standard software was used to capture and process images (scale bar: 20μm).

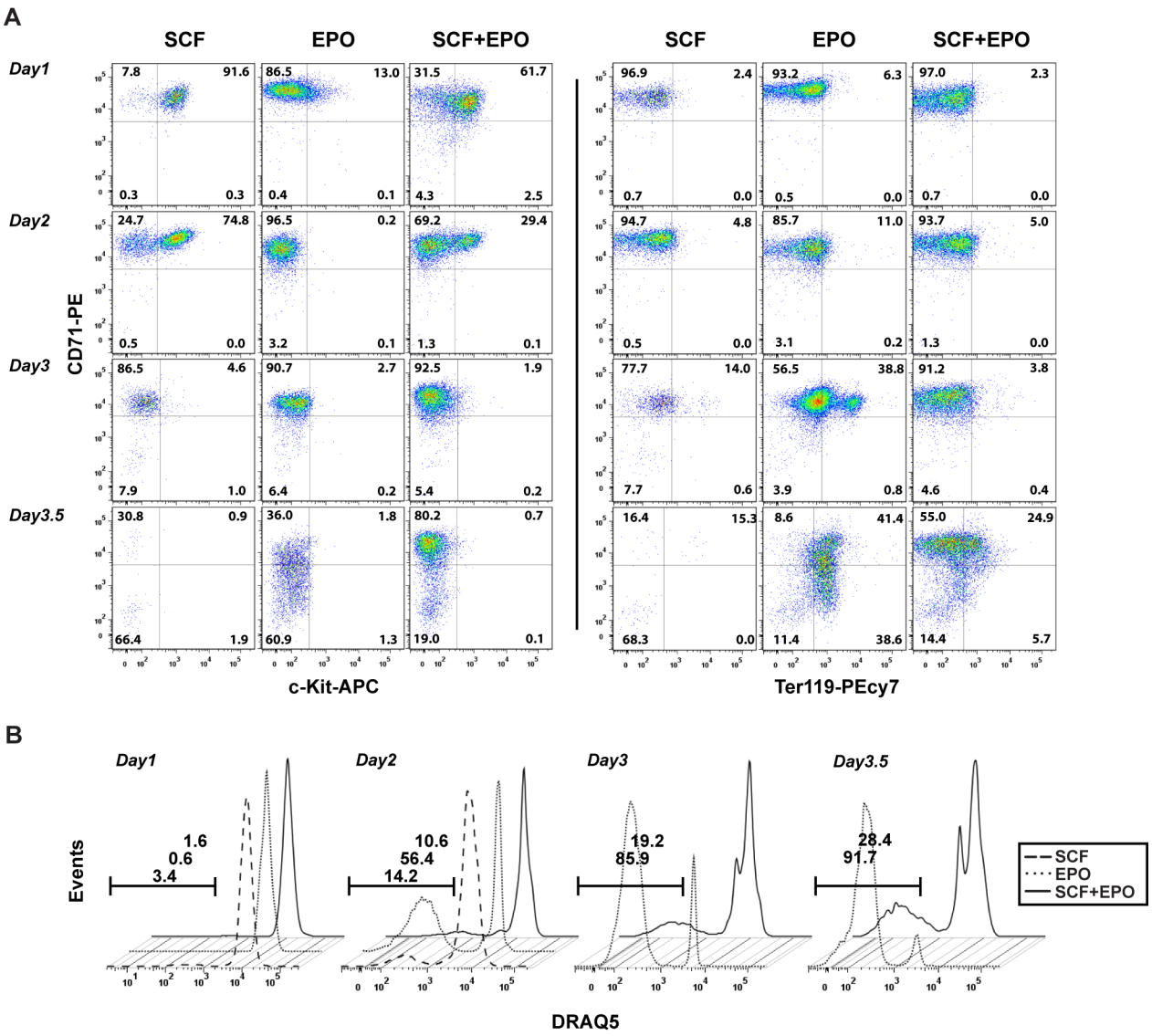


Figure S2. Flow cytometric analysis of c-Kit^{+}CD71^{high}Ter119^{-} cell differentiation in the presence of SCF, EPO or SCF+EPO. Shown are representative (A) density plots of the expression levels of c-Kit, CD71 and Ter119 and (B) profiles of DRAQ5 staining with numbers indicating the frequencies of reticulocytes (DRAQ5^{-}).

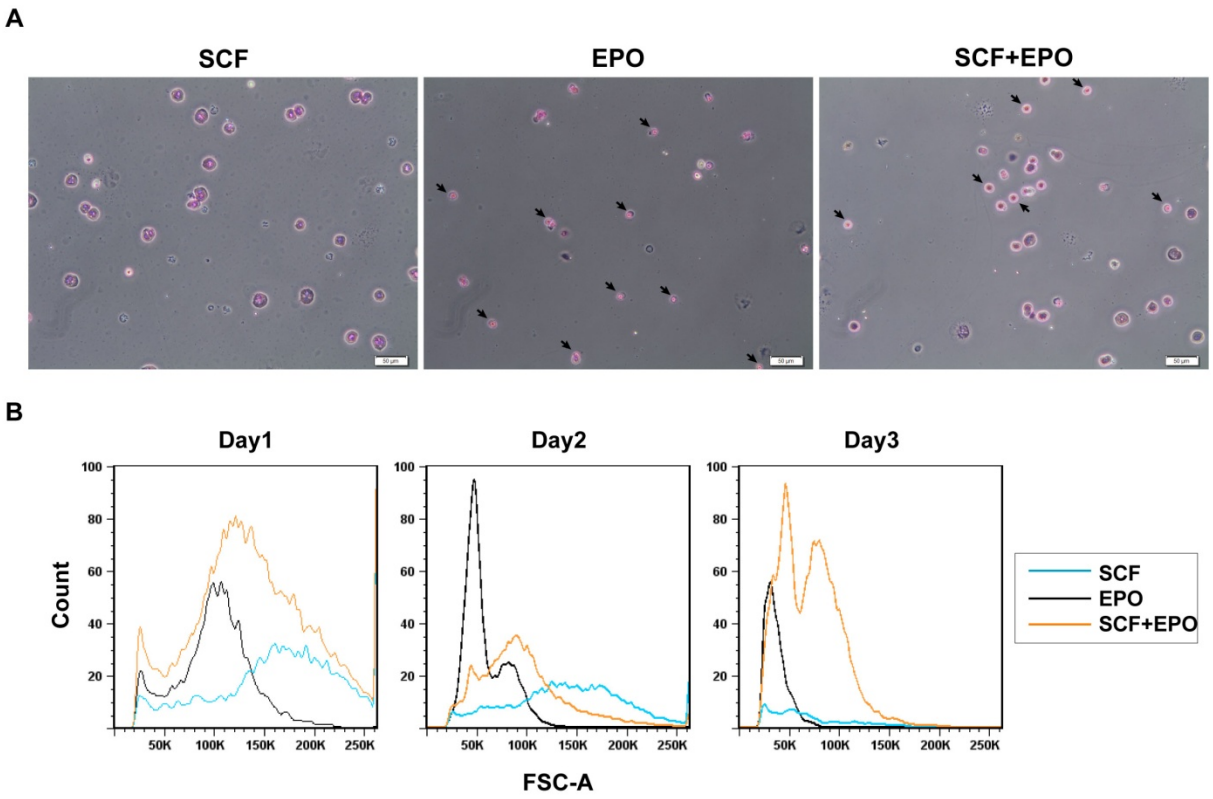


Figure S3. Changes in cell morphologies associated with terminal differentiation. (A) Cytospin preparation of cultured cells with SCF, EPO and SCF+EPO harvested from day 2. Cells cultured with SCF alone maintained the blast-like cell morphology whereas cells cultured with EPO alone underwent significant reduction in cell size, nuclear condensation and were positive for hemoglobin expression, as judged by positive staining with benzidine (brown color; benzidine$^+$ cells are indicated by arrows). Cells cultured with SCF+EPO underwent the same morphological changes as those cultured with EPO, but at a slower pace (scale bar: 50μm). (B) Maturation was also assessed based on changes in forward size scatter (FSC) analyzed by flow cytometry. Shown are representative histogram overlays for each culture condition (SCF, EPO, SCF+EPO) at day 1, 2 and 3.

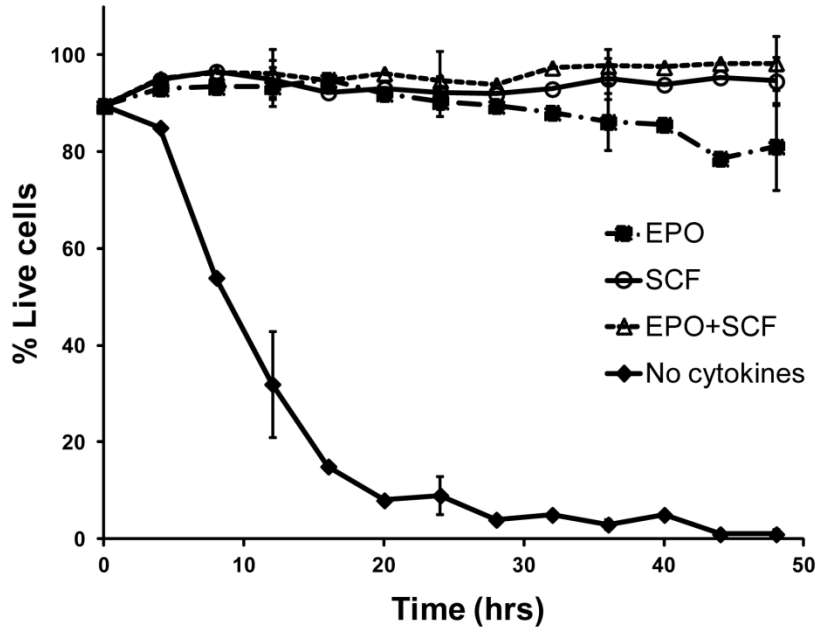


Figure S4. Culture of mouse fetal liver c-Kit^{+}CD71^{high}Ter119^{-} cells in serum-free condition supplemented with SCF (100 ng/mL) for 48 hours resulted in minimal cell death.

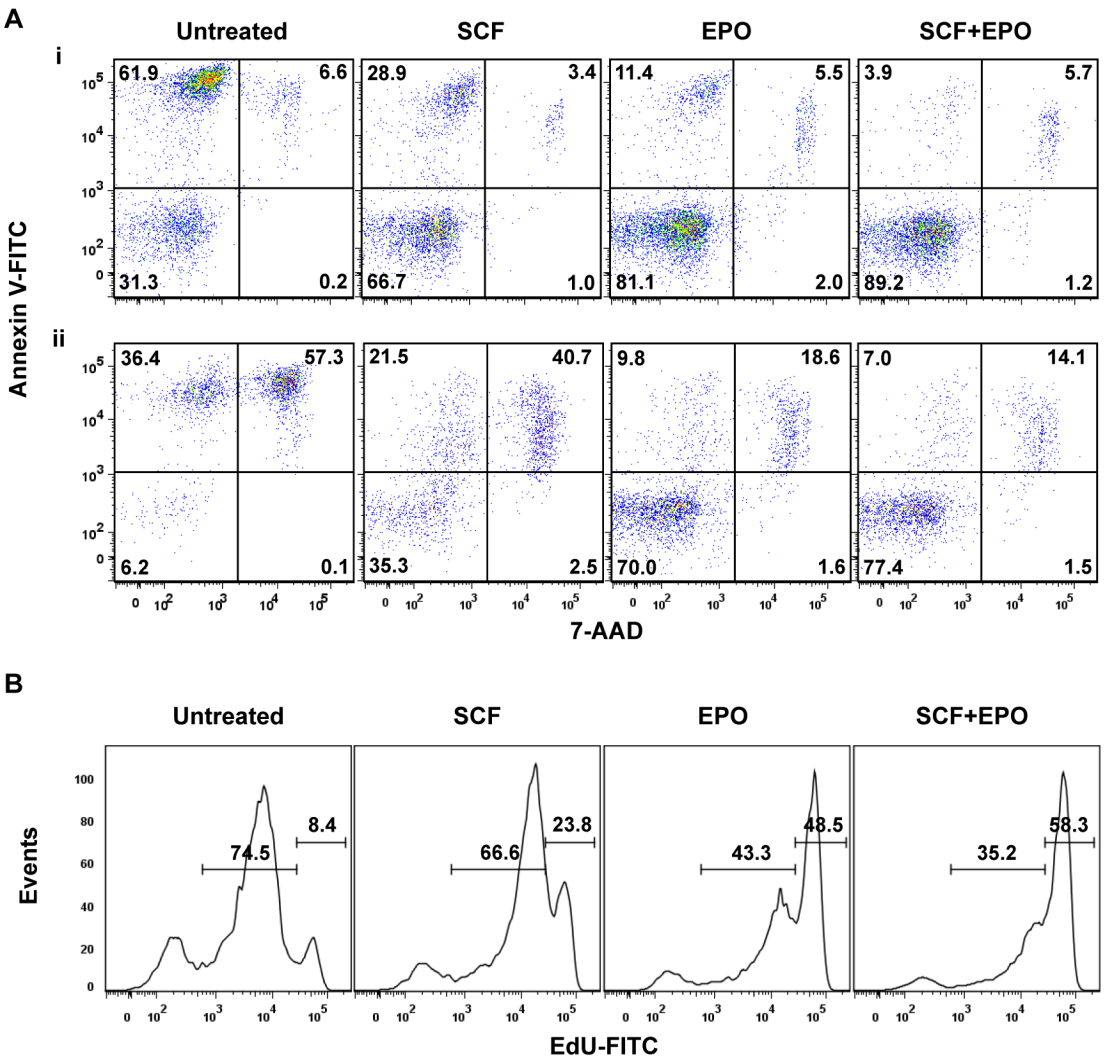
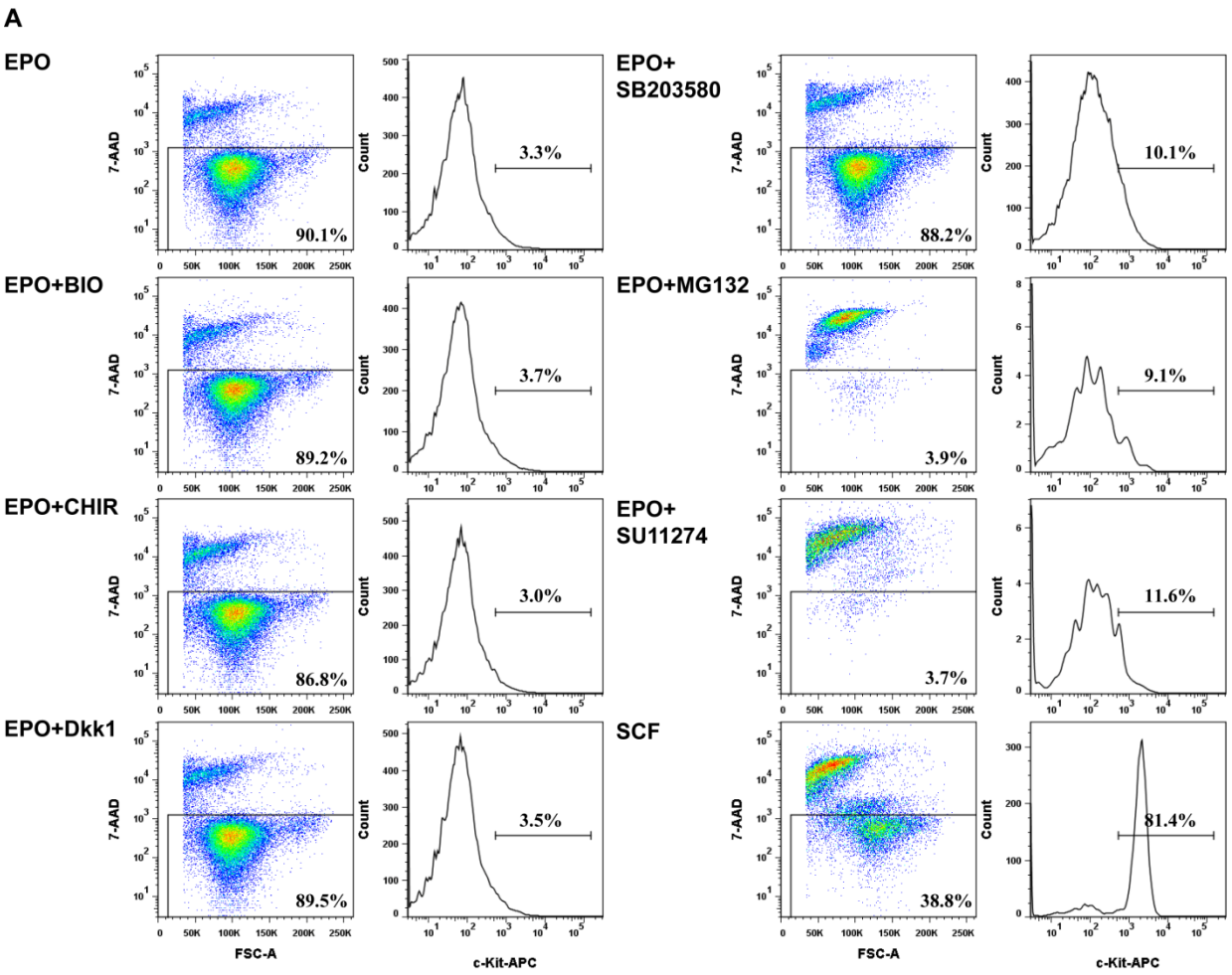
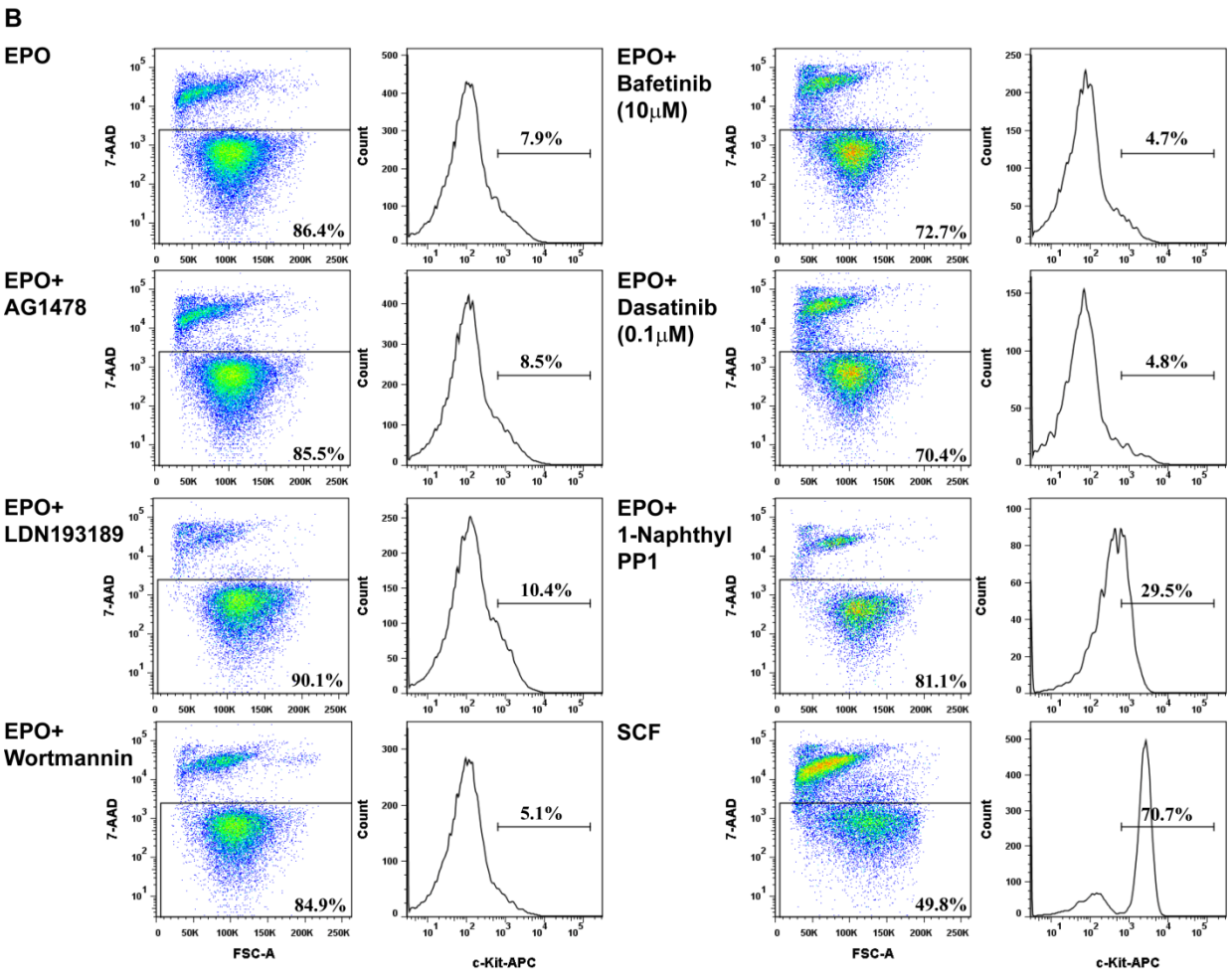


Figure S5. SCF+EPO supported maximal cell survival and proliferation. (A) Shown are representative FACS plots of cells stained with FITC-Annexin V and 7-AAD after (i) 2 and (ii) 6 hours. Numbers indicate the percentages of live (Annexin V^- 7-AAD^-), early apoptotic (Annexin V^+ 7-AAD^-), and dead (Annexin V^+ 7-AAD^+) cells. (B) Representative FACS histograms of EdU incorporation profiles at 6 hour. Gates were set for low versus high EdU incorporation populations.





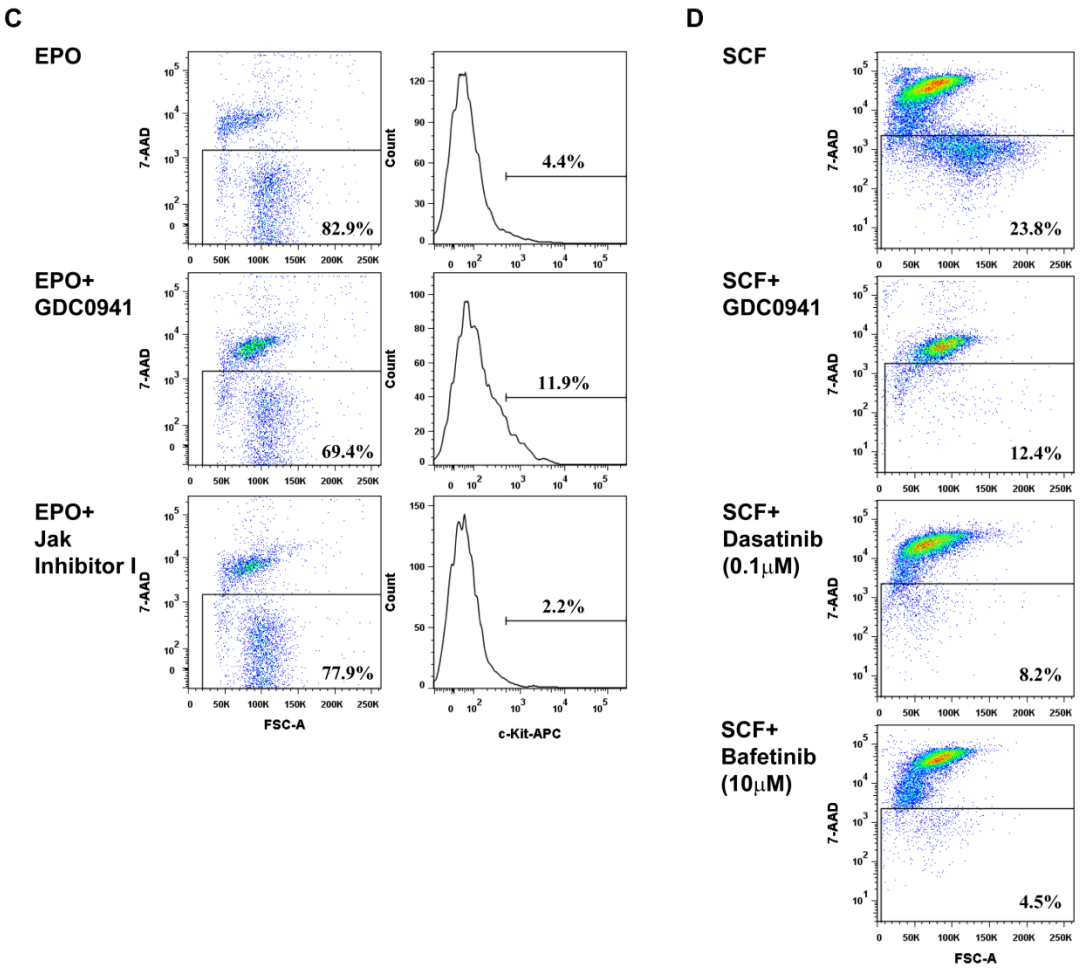
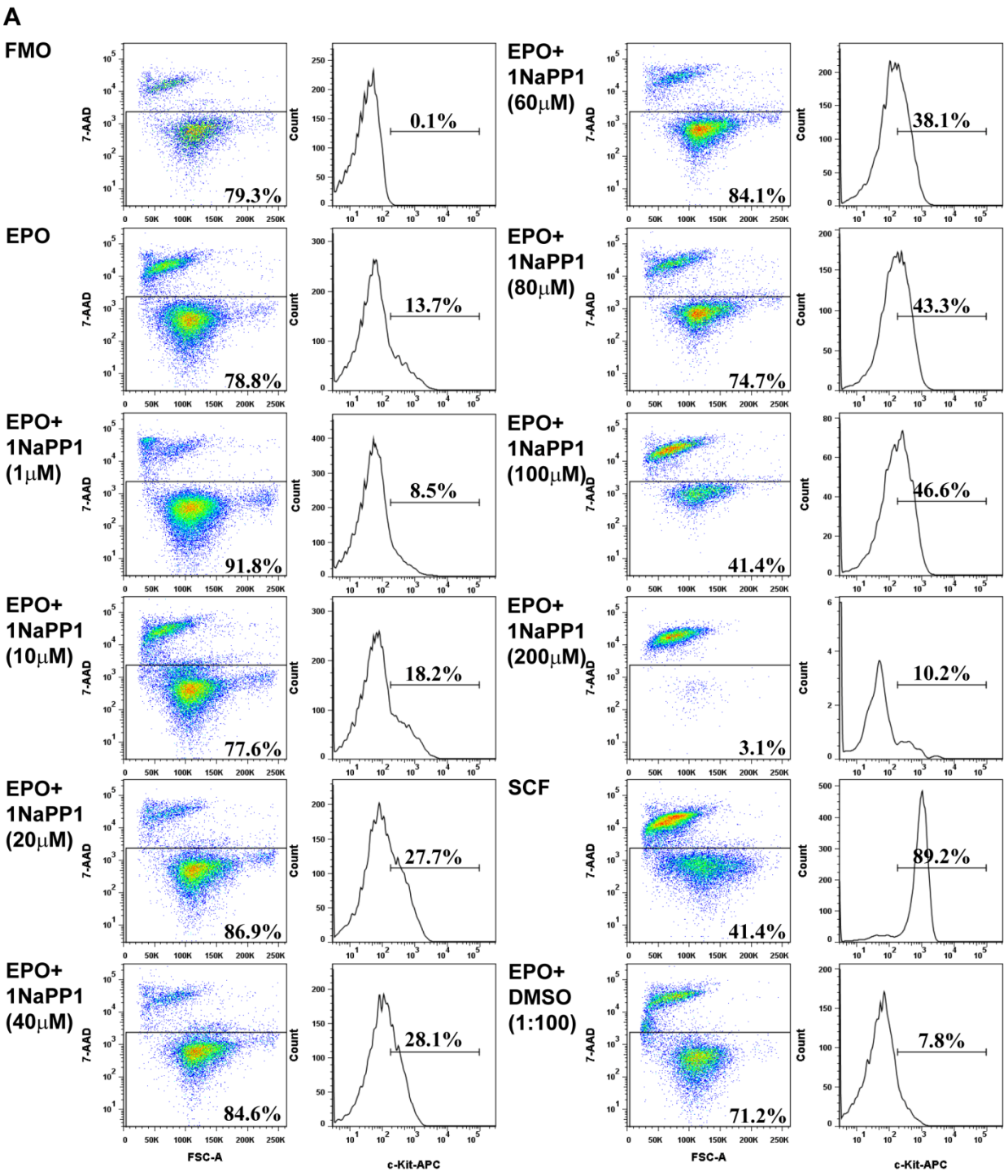


Figure S6. Inhibitor screen to identify the signaling mediators responsible for EPO-induced downregulation of c-Kit. Sorted c-Kit^{+}CD71^{high}Ter119^{-} cells were incubated with the inhibitors for 1 hour prior to the addition of EPO or SCF. After 24 hours of culture in the serum-free condition, cells were harvested, stained and analyzed for the cell surface expression of c-Kit by flow cytometry. Due to limited numbers of c-Kit^{+}CD71^{high}Ter119^{-} cells, the initial screening was carried out in 3 separate experiments (shown in A, B and C). 1-Naphthyl PP1 was identified to delay the downregulation of c-Kit by EPO. (D) GDC0941, Dasatinib and Bafetinib significantly affected the survival of SCF-cultured cells.



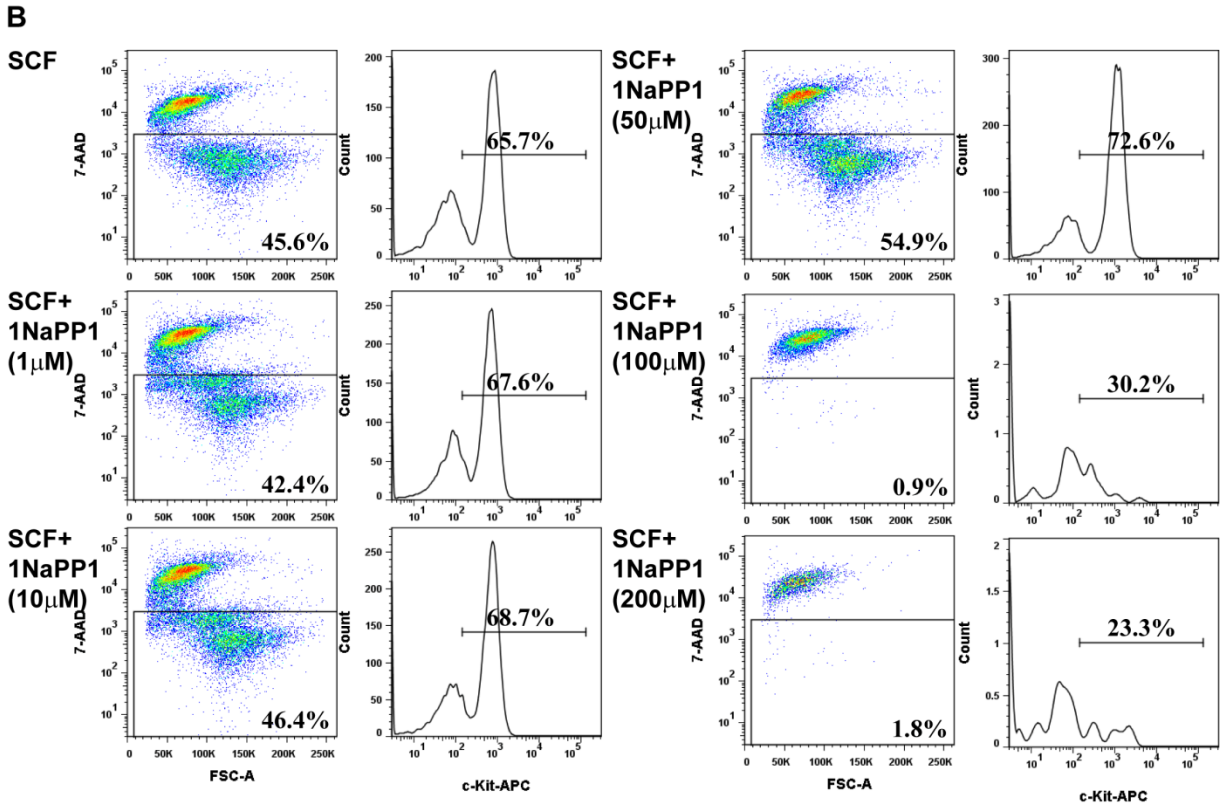
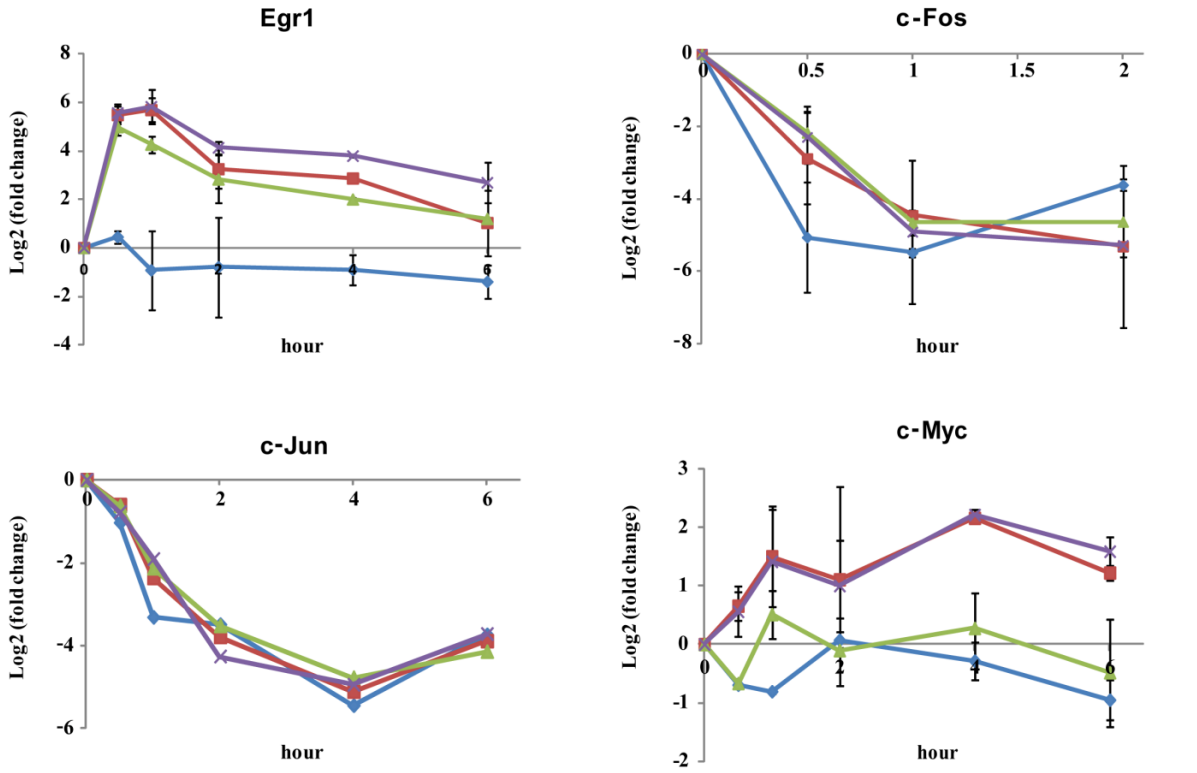
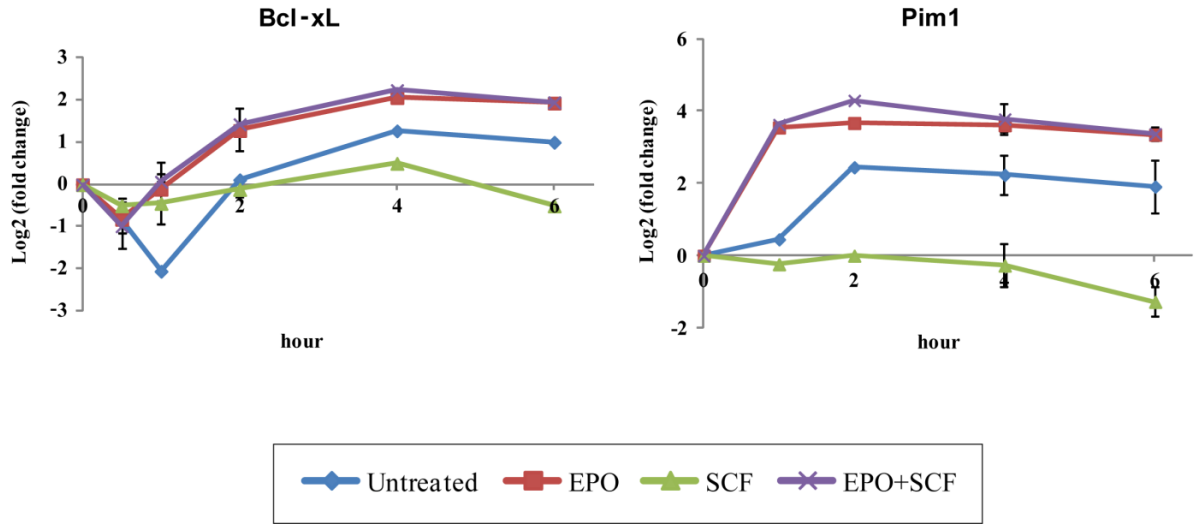


Figure S7. 1-Naphthyl PP1 inhibited the EPO-dependent downregulation of c-Kit expression in a dose-dependent manner. (A) C-Kit^{+}CD71^{high}Ter119^{-} cells were pre-treated with different concentrations of 1-Naphthyl PP1 for 1 hour before the addition of EPO or SCF. After 24 hours, the expression of c-Kit was analyzed by flow cytometry. 1% (v:v) DSMO (the volume added is equivalent to 500μM of the inhibitor) had no effect on the percentage of live cells. 1-NaPP1: 1-Naphthyl PP1; FMO: fluorescence minus one control (cells stained with 7-AAD only) used to set the threshold gate for c-Kit^{+} population. (B) 1-Naphthyl PP1 had no effect on the c-Kit expression in SCF-cultured cells. Shown are FACS plots from a representative experiment.

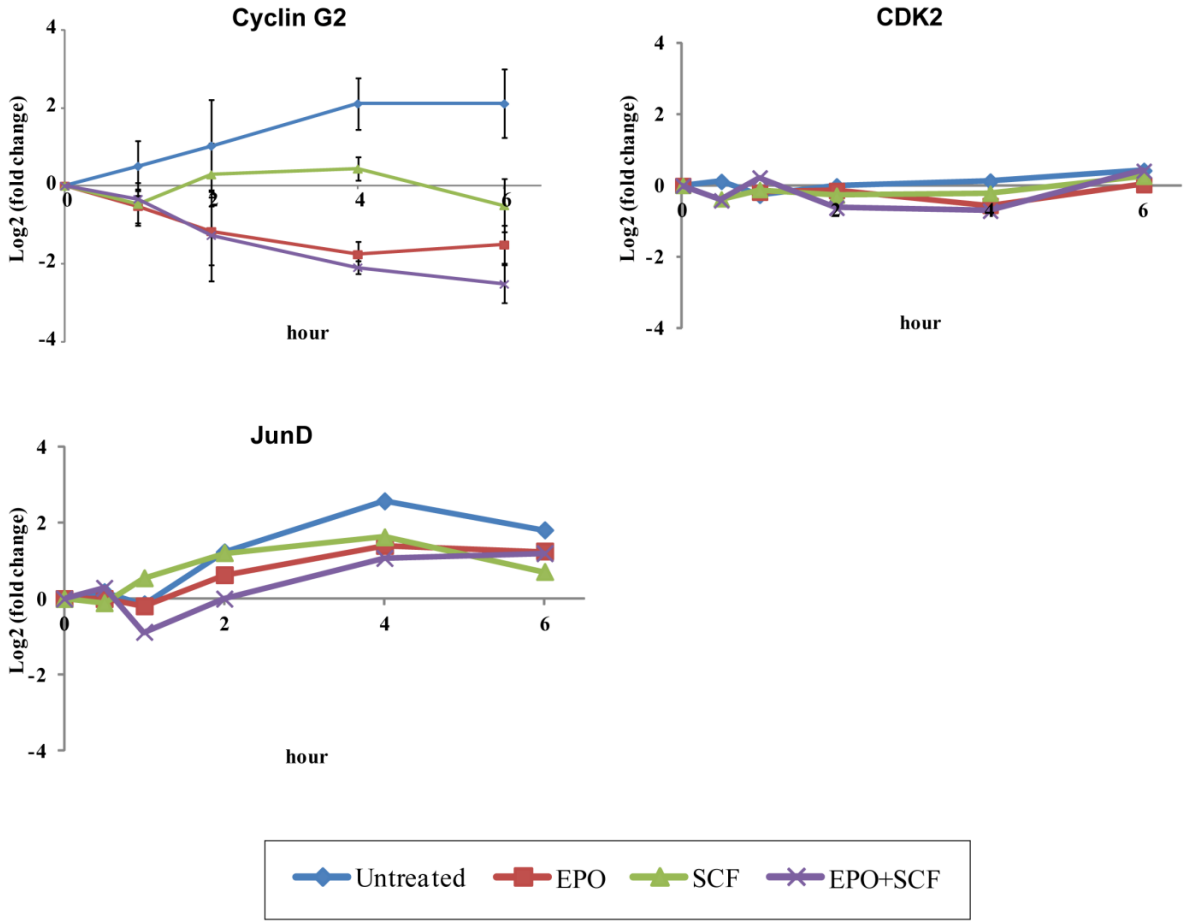
(A) Immediate early genes (IEGs)



(B) "Survival" genes



(C) “Proliferation” genes



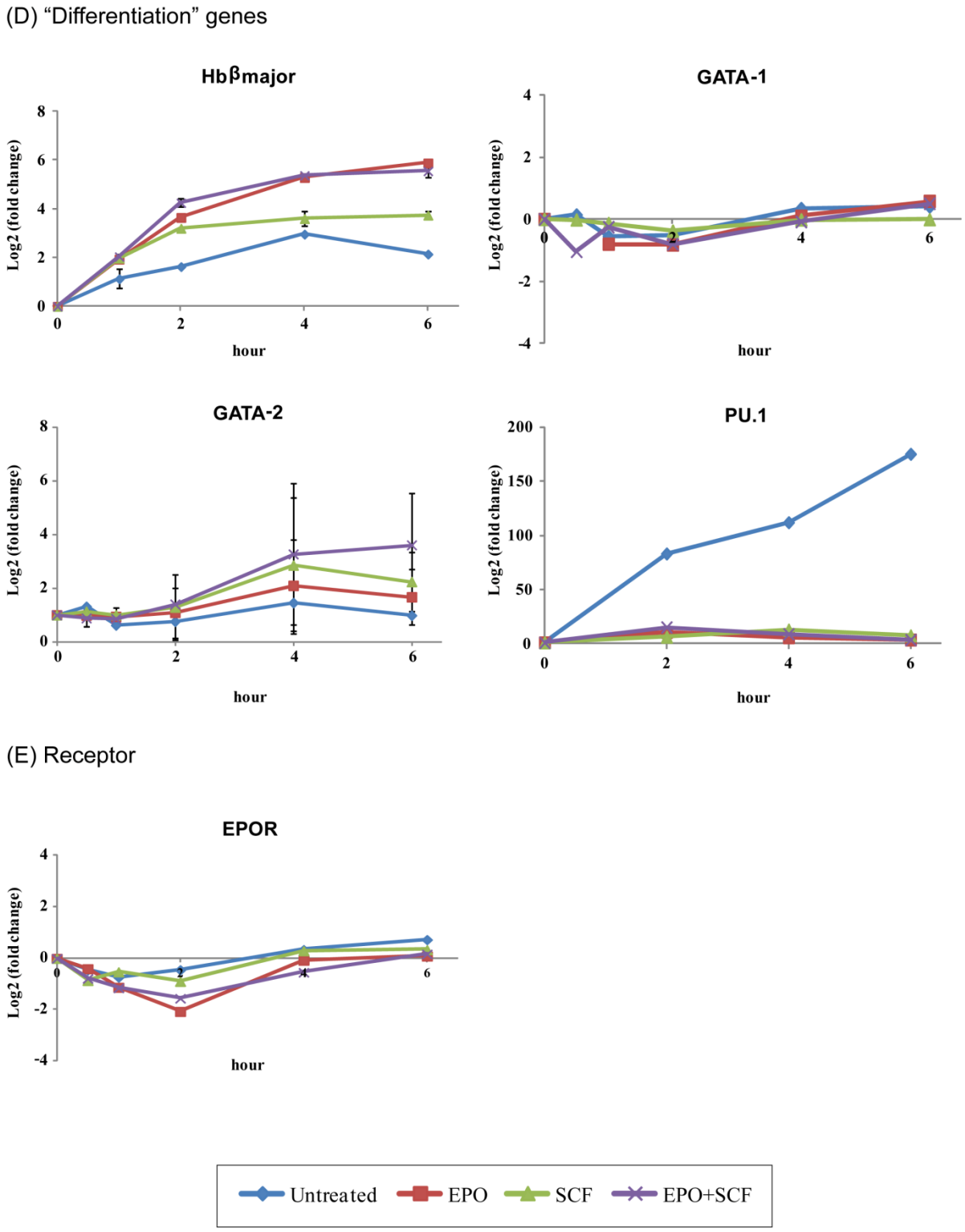


Figure S8. Time-course of the expression profiles of the genes examined (error bars represent SD).

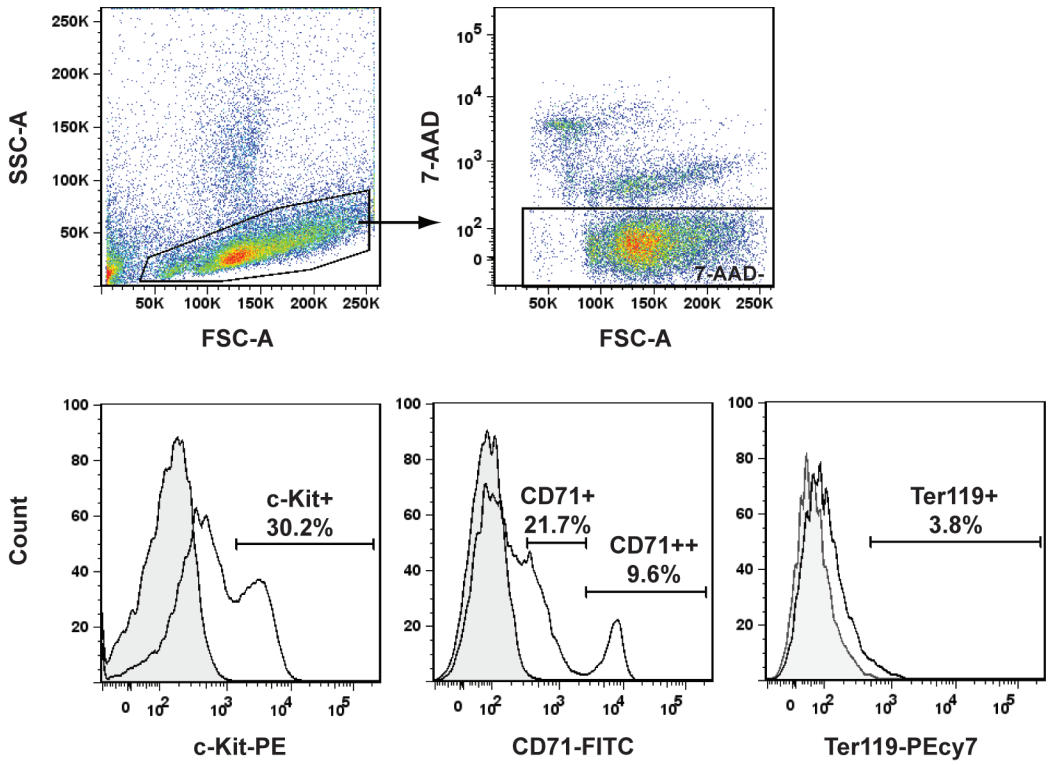


Figure S9. The gating strategy to define cell subsets in BM Lin$^-$ cells. Illustrated are representative FACS plots of the staining profiles of c-Kit, CD71 and Ter119 in fixed and permeablized Lin$^-$ cells. Shaded histogram in each plot represents the background staining level of the corresponding FMO (fluorescence minus one) control.

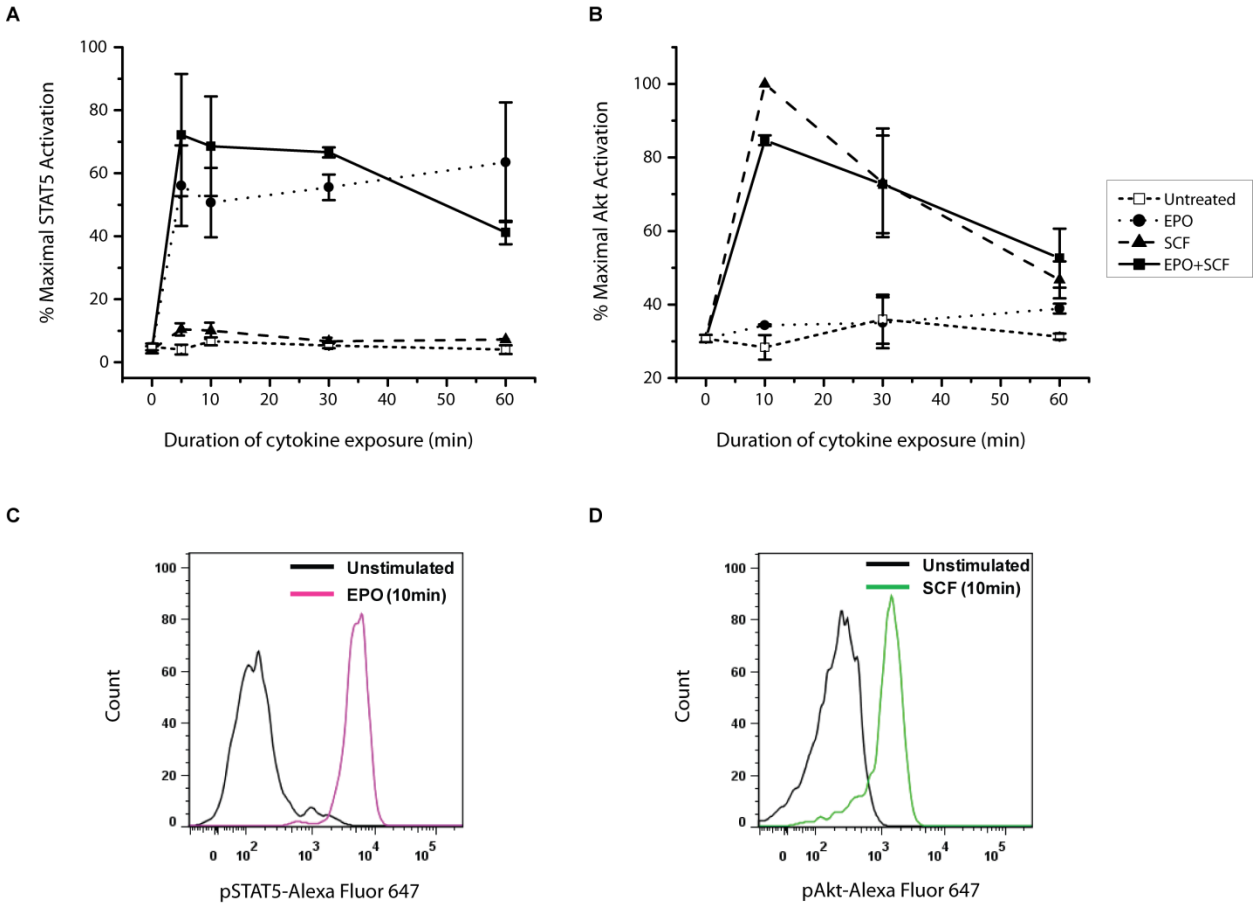


Figure S10. Time-course of STAT5 and Akt activation in c-Kit^{+}CD71^{high}Ter119^{-} cell subset. Lin^{-} cells were exposed to SCF, EPO or both after starvation in IMDM + 10%BIT for 30min. Alexa Fluor®647-conjugated anti-phospho-STAT5 (BD Biosciences) or anti-phospho-Akt (Cell Signaling Technology) antibody was used to detect the (A) pSTAT5 and (B) pAkt level in cell subpopulations defined by the expression levels of c-Kit, CD71 and Ter119. Data were normalized against the maximal fold-change in each experiment. Values are mean ± SEM from 3 independent experiments. Also shown are histogram overlays of (C) pSTAT5 and (D) pAkt staining profiles in unstimulated *vs.* stimulated cells with EPO or SCF respectively at 10 minutes from a representative experiment.

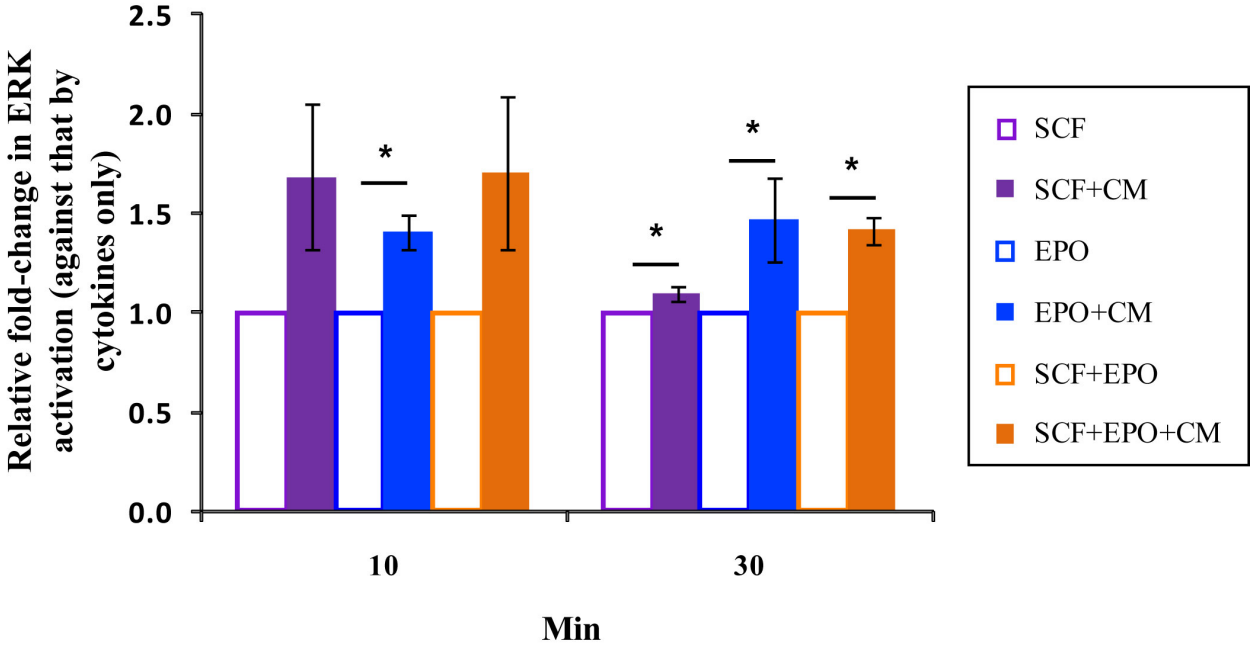


Figure S11. CM enhanced SCF-, EPO- and SCF+EPO-induced ERK activation. Lin- BM cells were exposed to SCF, EPO or SCF+EPO with or without CM. After 10 or 30 minutes, cells were fixed, permeabilized and stained for pERK. For each experiment, the fold-change in ERK activation induced by SCF+CM, EPO+CM or SCF+EPO+CM was normalized against that induced by SCF, EPO or SCF+EPO respectively. Values are mean ± SEM from 3 independent experiments. * denotes statistical significance using one-tail Student's T-test ($p < .05$).

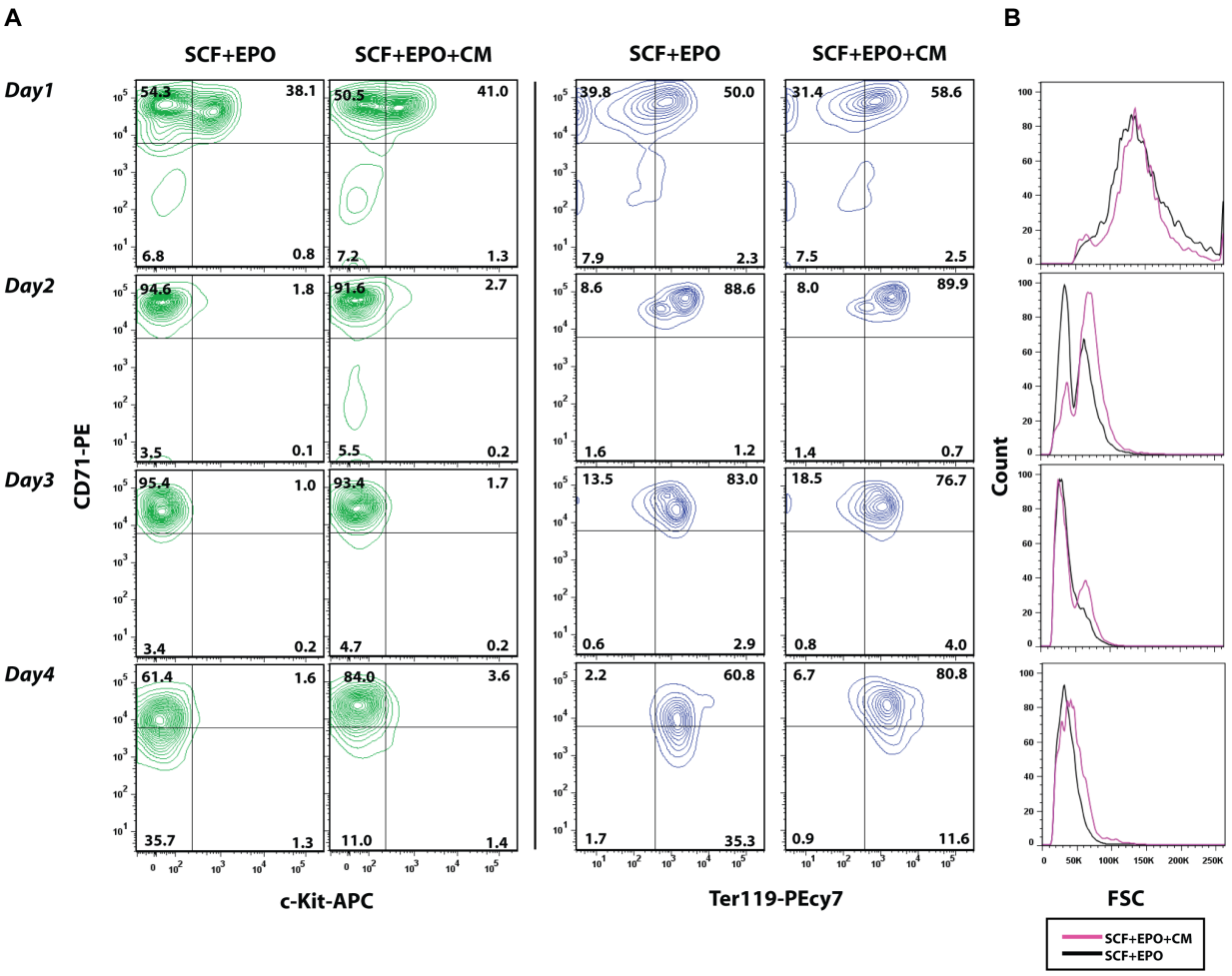


Figure S12. CM moderately delayed the differentiation of c-Kit^{+}CD71^{high}Ter119^{-} cells in the presence of SCF+EPO. BM c-Kit^{+}CD71^{high}Ter119^{-} cells were cultured with SCF+EPO or SCF+EPO+CM in a serum-free condition (IMDM+1%BSA+10%BIT) for 4 days. (A) Shown are expression profiles of c-Kit, CD71 and Ter119 analyzed by flow cytometry from a representative experiment. Numbers indicate the percentage of cells in each quadrant. (B) Histogram overlay of forward scatter distribution of cells cultured with SCF+EPO or SCF+EPO+CM (n =2).

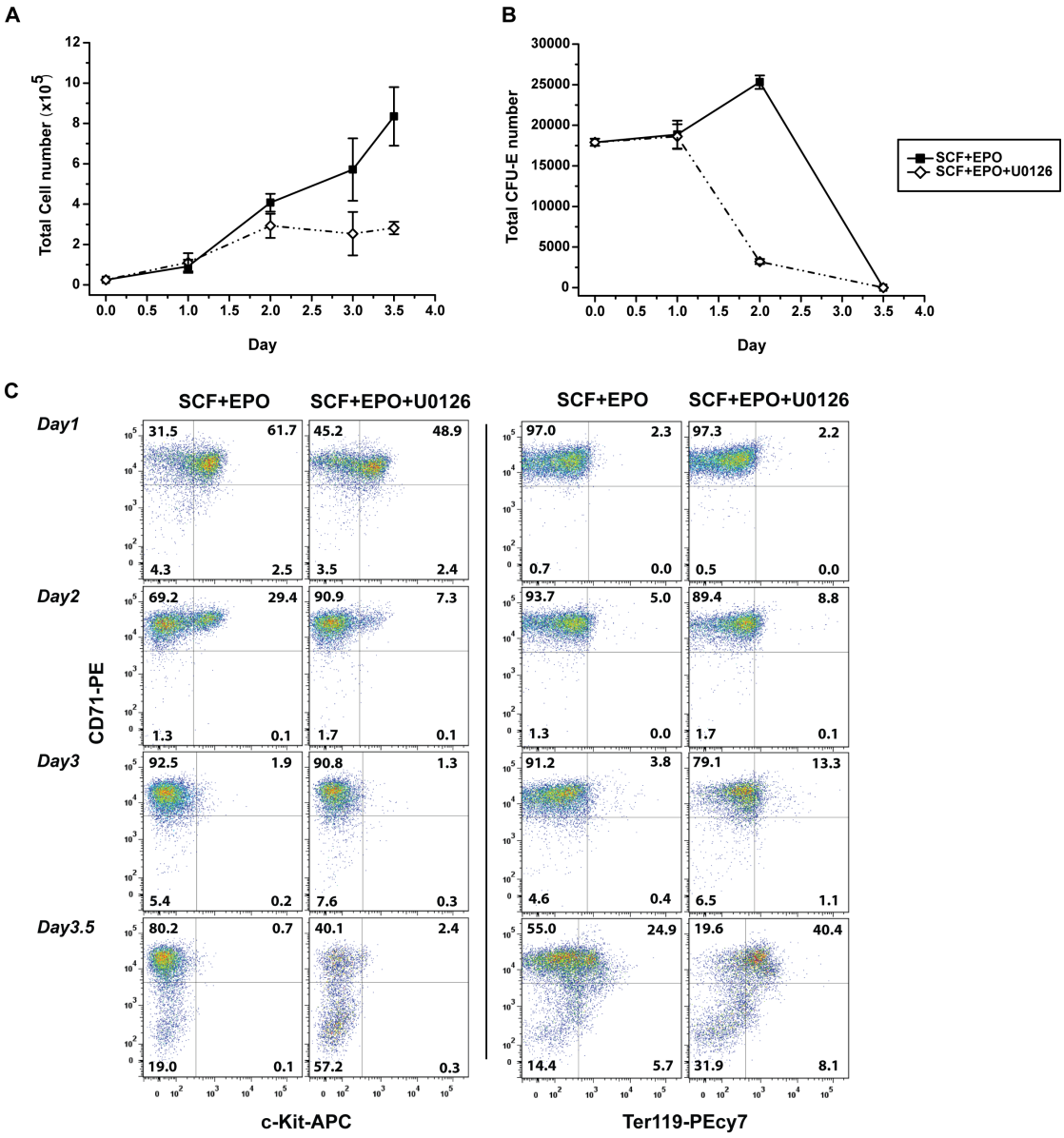


Figure S13. U0126 impaired CFU-E expansion and accelerated the terminal differentiation. C-Kit^{+}CD71^{high}Ter119^{-} cells were cultured in the presence of U0126 in addition to SCF+EPO. Shown are growth curves of (A) total cells and (B) CFU-Es over the course of a 3.5-day serum-free culture. Values are mean ± SEM from 2 or 3 independent experiments. (C) Erythroid cell differentiation was assessed by flow cytometric analysis of cell surface marker expression profiles.