Supplementary Materials and Methods

Semi-quantitative Reverse Transcription- Polymerase Chain Reaction (RT-PCR)

After cDNA synthesis using the First Strand cDNA synthesis kit (Invitrogen), RT-PCR was performed in a 20-μL reaction mixture consisting of 1× PCR buffer (Invitrogen), 2 mM $MgCl_2$, 0.2 mM of each dNTP (Roche Applied Science, Pleasanton, CA), 0.2 mM of each primer, and 1.5 U Platinum® *Taq*DNA polymerase (Invitrogen). Primers used for the *MASL1* gene were 5′-GCACTACACCGTGCACATTC-3′ and 5′-CAACATTCTTCTTGGAACAGG-3′, which amplified a region located between exon 2 and exon 3. The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was used as an internal control (primers: 5′-AACAGCGACACCCACT-3′ and 5′-GCCAAATTCGTTGTCATAC-3′). PCR was performed using an initial denaturation at 94°C for 2 minutes, followed by 35 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 45 seconds. After 35 cycles, an additional elongation step was performed at 72°C for 7 minutes. Amplified PCR products were run on 1.5% agarose gels containing 0.5 μg/mL ethidium bromide.

qRT-PCR

Total RNA was extracted from cells using the RNeasy Mini kit and digested with RNase-free deoxyribonuclease to eliminate DNA contamination (Qiagen). First-strand cDNA was synthesized using the First Strand cDNA synthesis kit (Invitrogen) with 500 ng of total RNA according to the manufacturer's protocol. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the Mx3000P®QPCR system using Brilliant® II QRT-PCR Master Mix (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Primers and probes were designed using Real Time Design software (Biosearch Technology, Novato, CA) and synthesized by Eurofins MWG Operon (Huntsville, AL). The primers and probe for *MASL1* were 5′-GCCACCCAGCTCCATCAG-3′ (forward), 5′-AAGCAGCAACCGAATGACA-3′ (reverse), and TaqMan probe 5′-TATGTGGAGGGCTTTCTGTTGCA-3′. The results were analyzed using Mx3000P software (Stratagene). Relative expression was calibrated by normalizing to GAPDH RNA

(primers: 5′-AACAGCGACACCCACT-3′ and 5′-GCCAAATTCGTTGTCATAC-3′; probe: 5′-CCTCCACCTTTGACGCTGGG-3′).

Western-blot analysis

Cells were washed once in PBS and lysed using the M-PER protein extraction kit (Thermo Scientific). Lysates were electrophoresed on NUPAGE 4–12% Bis (2-hydroxyethyl)-Tris (tris(hydroxymethyl)aminomethane) gels (Invitrogen) and then transferred to polyvinylidene difluoride membranes (Invitrogen). The membranes were blocked in Tris-buffered saline (TBS) with 0.1% Tween-20 with 5% blotting milk (Bio-Rad, Hercules, CA) for 1 hour and then incubated with primary antibody at 4°C overnight. The primary antibodies used in these studies included: anti-MASL1 (K-19), anti-β-actin (ACTBD11B7), anti-hemoglobin-α (H-80), anti-phospho-Raf1 (Ser-259), anti-phospho-MEK1/2 (Ser-218/222; 7E10), anti-phospho-Erk1/2 (Thr-202/Tyr-204; 12D4), anti-phospho-Elk1 (Ser-383; B4), anti-SOS1 (A9), anti-GPA (NaM10-6G4), anti-CD13 (3D8) and anti-GAPDH (0411), all of which were obtained from Santa Cruz Biotechnology. Anti-Myc (9B11), anti-c-Raf (Raf1), anti-MEK1/2 (D1A5), anti-Erk1/2 (3A7), anti-Jak2 (D2E12), anti-Akt (C67E7), anti-phospho-Akt (Ser473), anti-Stat5 (3H7), and anti-phospho-Stat5 (Tyr694) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Blots were developed using horseradish peroxidase-labeled anti-mouse IgG or anti-rabbit IgG (GE Healthcare BioScience, Piscataway, NJ) and SuperSignal chemiluminescent reagents (Thermo Scientific). Western blot signals were quantitated using Quantity One software (Bio-Rad).

Red blood cell isolation

Human red blood cells were isolated from the peripheral blood of healthy volunteers by Ficoll-Paque (GE Healthcare BioScience) density centrifugation according to the manufacturer's protocol. All human participants provided written informed consent.

Supplementary Figure Legends

Figure S1. *MASL1* knockdown reduces erythroid differentiation and the Raf/MEK/ERK signaling pathway in CD34$^+$ cells at the early stage of erythropoiesis. (A) Western-blot analysis of protein lysates prepared from mock-, control shRNA-, MASL1 siRNA-, and MASL1 shRNA-transfected CD34$^+$ cells at day 3 of EPO-induced differentiation. β-actin was used as an internal control. (B) Flow-cytometry analysis of CD71$^+$ and GPA$^-$ expression in mock-, control shRNA-, MASL1 siRNA-, or MASL1 shRNA-transfected CD34$^+$ cells at day 3 of EPO-induced differentiation. Error bars represent the SD from 3 individual experiments; *****$P < .05$. (C) Upper panels, representative density plots for data presented in B. The percent of CD71$^+$ GPA$^+$ and CD71$^+$ GPA$^-$ cells is labeled on each density plot. Lower panels, morphology of cells corresponding to flow-cytometric analysis obtained by May-Grünwald-Giemsa staining (original magnification ×20). Results are representative of 3 independent experiments.

Figure S2. ***MASL1* knockdown does not affect CD34$^+$ cells treated with G-CSF.** (A) Cell counts per mL in culture at day 3, 5, 7, and 14 of G-CSF-induced granulocytic differentiation for mock-, control shRNA-, MASL1 siRNA-, or MASL1 shRNA-transfected CD34$^+$ cells. Error bars represent the SD from 3 individual experiments. (B) Flow-cytometry analysis of CD13$^+$ CD66b$^+$ expression and representative density plots in mock-, control shRNA-, MASL1 siRNA-, or MASL1 shRNA-transfected CD34$^+$ cells at day 3 and 7 of G-CSF-induced differentiation. Error bars represent the SD from 3 individual experiments. The percent of CD13$^+$ and CD66b$^+$ cells is labeled on each density plot. (C) Flow-cytometry analysis of annexin V-negative/PI-negative (viable), annexin V-positive/PI-negative (early apoptotic), and annexin V-positive/PI-positive (late apoptotic) and representative density plots in mock-, control shRNA-, MASL1 siRNA-, or MASL1 shRNA-transfected CD34$^+$ cells at day 3 and 7 of G-CSF-induced differentiation. Error bars represent the SD from 3 individual experiments. The percent of annexin V-positive/PI-negative (early apoptotic) and annexin V-positive/PI-positive (late apoptotic) cells is labeled on each density plot. (D) Differential counting of mock-, control shRNA-, MASL1 siRNA-, or MASL1 shRNA-transfected CD34$^+$ cells at day 3 and 7 of G-CSF-induced differentiation. (E) Western-blot

analysis of protein lysates prepared from mock-, control shRNA-, MASL1 siRNA-, and MASL1 shRNA-transfected $CD34^+$ cells at day 3 of G-CSF-induced differentiation. β-actin was used as an internal control.

Figure S3. *MASL1* knockdown in $CD34^+$ cells reduces cell proliferation and induces apoptosis during erythroid differentiation. (A) Mean proliferation rate of mock-transfected, control shRNA-transfected, and *MASL1*-knockdown $CD34^+$ cells at day 0, 7, 10, and 14 of EPO-induced differentiation using the MTT colorimetric assay. Error bars represent the SD from 3 independent experiments; $*P < .05$. (B) Mock-transfected, control shRNA-transfected, and *MASL1*-knockdown $CD34^+$ cells were costained with FITC-annexin V and propidium iodide (PI) to evaluate apoptotic cells. The data are expressed as the mean percentage of viable (annexin V-negative/PI-negative), early apoptotic (annexin V-positive/PI-negative), late apoptotic (annexin V-positive/PI-positive), and necrotic (annexin V-negative/PI-positive) cells at day 7 of differentiation. Error bars represent the SD from 3 independent experiments; $*P < .05$. (C) Representative density plots for data presented in B and at days 3, 5, 7, and 14 of differentiation. The percent of annexin V-positive/PI-negative (early apoptotic) and annexin V-positive/PI-positive (late apoptotic) cells is labeled on each density plot. Results for unstained cells (Auto) are also presented.

Figure S4. Stat5 and Akt expression is not reduced in *MASL1*-knockdown $CD34^+$ cells during erythroid differentiation. Western-blot analysis of protein lysates prepared from mock-, control shRNA-, MASL1 siRNA-, and MASL1 shRNA-transfected $CD34^+$ cells at day 14 of EPO-induced differentiation. β-actin was used as an internal control.

Figure S5. Erk1/2 phosphorylation in *MASL1*-knockdown $CD34^+$ cells is diminished in the absence of EPO and induced by restimulation with EPO. Western-blot analysis of protein lysates prepared from mock-, control shRNA-, MASL1 siRNA-, and MASL1 shRNA-transfected $CD34^+$ cells. β-actin was used as an internal control. (A) Cells were both transfected and grown in the presence of 4 U/mL EPO. Two days after transfection, the cells were washed in culture medium without serum and EPO, resuspended at 4×10^5 cells/mL, then starved of EPO for 4 hours before being hyperstimulated by 10 U/mL EPO for 10

minutes (Erk1/2 activation) or 30 minutes (Akt and Stat5 activations). Minus (-) and Plus (+) indicate that cells were without or with a 4-hour period of EPO starvation, respectively. (B) Cells were both transfected and grown in EPO-free culture medium. Two days after transfection, the cells were induced with 4 U/mL EPO for 15 or 30 minutes.

Figure S6. Alteration of Ras-like GTPase region in *MASL1* reduces erythroid differentiation in human erythroid progenitor CD34$^+$ cells. (A) Schematic diagram of domain structure and phosphorylation sites of myc-DDK-tagged *MASL1* and mutants. Myc-DDK-tagged MASL1 S450A and MASL1 Δ414-556 represent the mutation from serine (S) to alanine (A) at amino acid position 450 and deletion of amino acid positions 414 to 556 of the Ras-like GTPase region in *MASL1*, respectively. The acronyms designate different domains: LRR, leucine-rich repeats; ROC, Ras of complex protein; COR, C-terminal of ROC. *MASL1* point mutation S450A (MASL1 S450A) and deletion of Ras-like GTPase region residues 414-556 (MASL1 Δ414-556) constructs were obtained by site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). Primers used for generation of the *MASL1* point mutation and deletion constructs were as follows: forward primer 5′-GCTACCCACCGGCACCTCCCCCTGTG-3′, and reverse primer 5′-CACAGGGGGAGGTGCCGGTGGGTAGC-3′ for S450A and 5′-CAGCCCCGGCTCAAGCTGCTCCAGATCGCCCTGCAGGAGAAG-3′ for Δ414-556. Both constructs were verified by DNA sequencing at Eurofins MWG Operon. (B) Visual depiction of cell pellets at day 14 of EPO-induced erythroid differentiation for mock-, control pCMV6-, myc-DDK-tagged MASL1-, MASL1 S450A-, or MASL1 Δ414-556 plasmid DNA-transfected CD34$^+$ cells. Induced erythroid differentiation is evident by the pink-red cell pellets. (C) Flow-cytometry analysis of CD71$^+$ and GPA$^+$ expression in mock-, control pCMV6-, myc-DDK-tagged MASL1-, MASL1 S450A-, or MASL1 Δ414-556 plasmid DNA-transfected CD34$^+$ cells at day 14 of EPO-induced differentiation. Error bars represent the SD from 3 individual experiments; *$P < .05$. (D) Upper panels, representative density plots for data presented in C. CD34$^+$ cells were stained with FITC-conjugated anti-CD71 and PE-conjugated anti-GPA monoclonal antibodies. The percent of CD71$^+$ and GPA$^+$ cells is labeled on each density plot. Lower panels, morphology of cells corresponding to flow-

cytometric analysis obtained by May-Grünwald-Giemsa staining (original magnification ×20). Results are representative of 3 independent experiments. (E) Western-blot analysis of protein lysates prepared from mock-, control pCMV6-, myc-DDK-tagged MASL1-, MASL1 S450A-, or MASL1 Δ414-556 plasmid DNA-transfected $CD34^+$ cells at day 14 of EPO-induced differentiation. β-actin was used as an internal control.

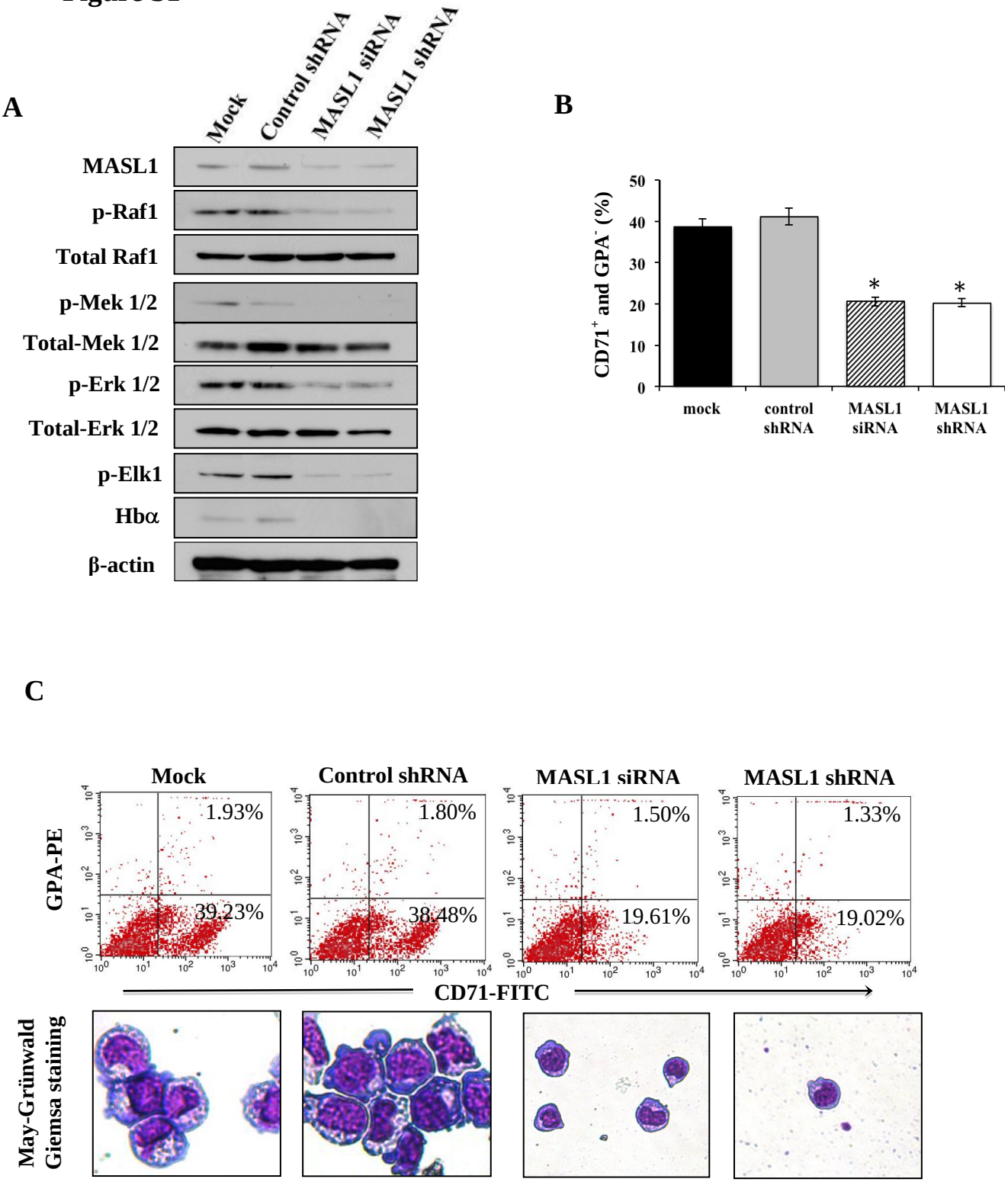
Figure S7. Inhibition of SOS1 activity reduces *MASL1* expression and diminishes erythroid differentiation. For inhibitor assays, 50 μM Jak2 Inhibitor V (EMD4Biosciences, Gibbstown, NJ),[1] 25 μM LY294002 (PI3K inhibitor) (Sigma-Aldrich),[2] or 100 nM Son of Sevenless (SOS) SH3 domain inhibitor (Santa Cruz Biotechnology)[3] was added to $CD34^+$ cell cultures on day 0 of EPO-induced differentiation after a 6-day period of expansion in SFEM medium with 1× CC100 cytokine mix and 2% penicillin/streptomycin. The inhibitors were added every 48 hours. Cells were harvested at day 7 of differentiation for further analysis. (A) Semi-quantitative RT-PCR analysis of *SOS1*, *MASL1*, *Jak2*, and *PI3K* gene expression in $CD34^+$ cells treated without EPO (no EPO), with 4 U/mL of EPO (EPO), with EPO + 50 μM Jak2 inhibitor (EPO+Jak2), with EPO + 25 μM PI3K inhibitor (EPO+PI3K), or with EPO + 100 nM SOS SH3 domain inhibitor (EPO+SOS). Cells were analyzed at day 7 of EPO-induced differentiation; all of inhibitors were added starting at day 0 of differentiation and replaced every 48 hours. Primers used for the *SOS1* gene were 5′-GGAACTGGCATTTGATCCAT-3′ and 5′-CTAACTGCTTCAAAAGTTCA-3′; primers used for the *Jak2* gene were 5′-CTGGAAATTGAACTTAGCTC-3′ and 5′-CTCGCTCGACAGCAAAAGTC-3′; and primers used for the *PI3K* gene were 5′-CCAGGTGGAATGAATGGC-3′ and 5′-CTCTAAGCATGGAGTTTCT-3′. GAPDH was used as an internal control. (B) Mean relative *MASL1* gene expression levels in $CD34^+$ cells treated as described in A, shown as fold induction compared with levels in $CD34^+$ cells grown without EPO by qRT-PCR. Values were normalized to the expression level of the housekeeping gene *GAPDH*. Error bars represent the SD from 3 individual experiments; $*P < .05$. (C) Western-blot analysis of protein lysates in $CD34^+$ cells treated as described in A. β-actin was used as an internal control. (D) Flow-cytometry analysis of cells at S-phase of the cell cycle based on propidium iodide staining at day 7. The percentage of G2/M phase is

indicated on each histogram plot (E) Flow-cytometry analysis of $CD71^+$ and GPA^+ expression at day 7 in $CD34^+$ cells treated as described in A. Error bars represent the SD from 3 individual experiments; $*P < .05$. (F) Upper panels, representative density plots for data presented in E. $CD34^+$ cells were stained with FITC-conjugated anti-CD71 and PE-conjugated anti-GPA monoclonal antibodies. The percent of $CD71^+$ and GPA^+ cells is labeled on each density plot. Lower panels, morphology of cells corresponding to flow-cytometric analysis obtained by May-Grünwald-Giemsa staining (original magnification ×20). Results are representative of 3 independent experiments.

Supplementary References

1. Sayyah J, Magis A, Ostrov DA, Allan RW, Braylan RC, Sayeski PP. Z3, a novel Jak2 tyrosine kinase small-molecule inhibitor that suppresses Jak2-mediated pathologic cell growth. *Mol Cancer Ther*. 2008;7(8):2308-2318.
2. Vlahos CJ, Matter WF, Hui KY, Brown RF. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem*. 1994;269(7):5241-5248.
3. Cussac D, Vidal M, Leprince C, et al. A Sos-derived peptidimer blocks the Ras signaling pathway by binding both Grb2 SH3 domains and displays antiproliferative activity. *Faseb J*. 1999;13(1):31-38.

Figure S1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MASL1 shRNA
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Total Raf1
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Total-Mek 1/2
p-Erk 1/2
Total-Erk 1/2
p-Elk1
Hbα
β-actin
B
CD71+ and GPA- (%)
50
40
30
20
10
0
*
*
mock
control shRNA
MASL1 siRNA
MASL1 shRNA
C
Mock
Control shRNA
MASL1 siRNA
MASL1 shRNA
GPA-PE
1.93%
39.23%
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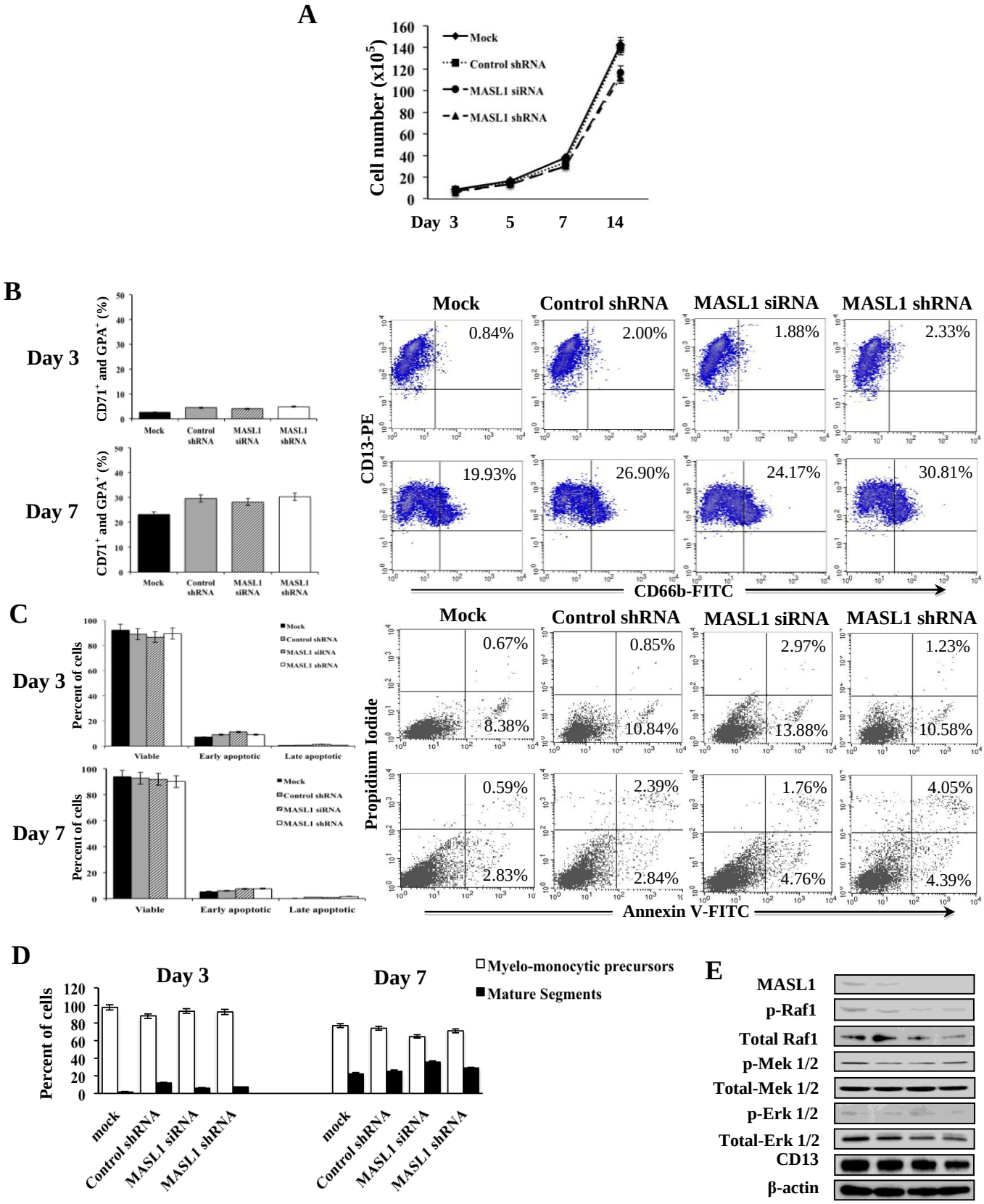
19.61%
1.33%
19.02%
CD71-FITC
May-Grünwald Giemsa staining

Figure S2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Cell number (x10^5)
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Control shRNA
MASL1 siRNA
MASL1 shRNA
Day 3 5 7 14
B
Day 3
Day 7
CD71+ and GPA+ (%)
Mock
Control shRNA
MASL1 siRNA
MASL1 shRNA
CD13-PE
CD66b-FITC
0.84%
2.00%
1.88%
2.33%
19.93%
26.90%
24.17%
30.81%
C
Percent of cells
Viable
Early apoptotic
Late apoptotic
Propidium Iodide
Annexin V-FITC
0.67%
0.85%
2.97%
1.23%
8.38%
10.84%
13.88%
10.58%
0.59%
2.39%
1.76%
4.05%
2.83%
2.84%
4.76%
4.39%
D
Day 3
Day 7
Myelo-monocytic precursors
Mature Segments
mock
Control shRNA
MASL1 siRNA
MASL1 shRNA
E
MASL1
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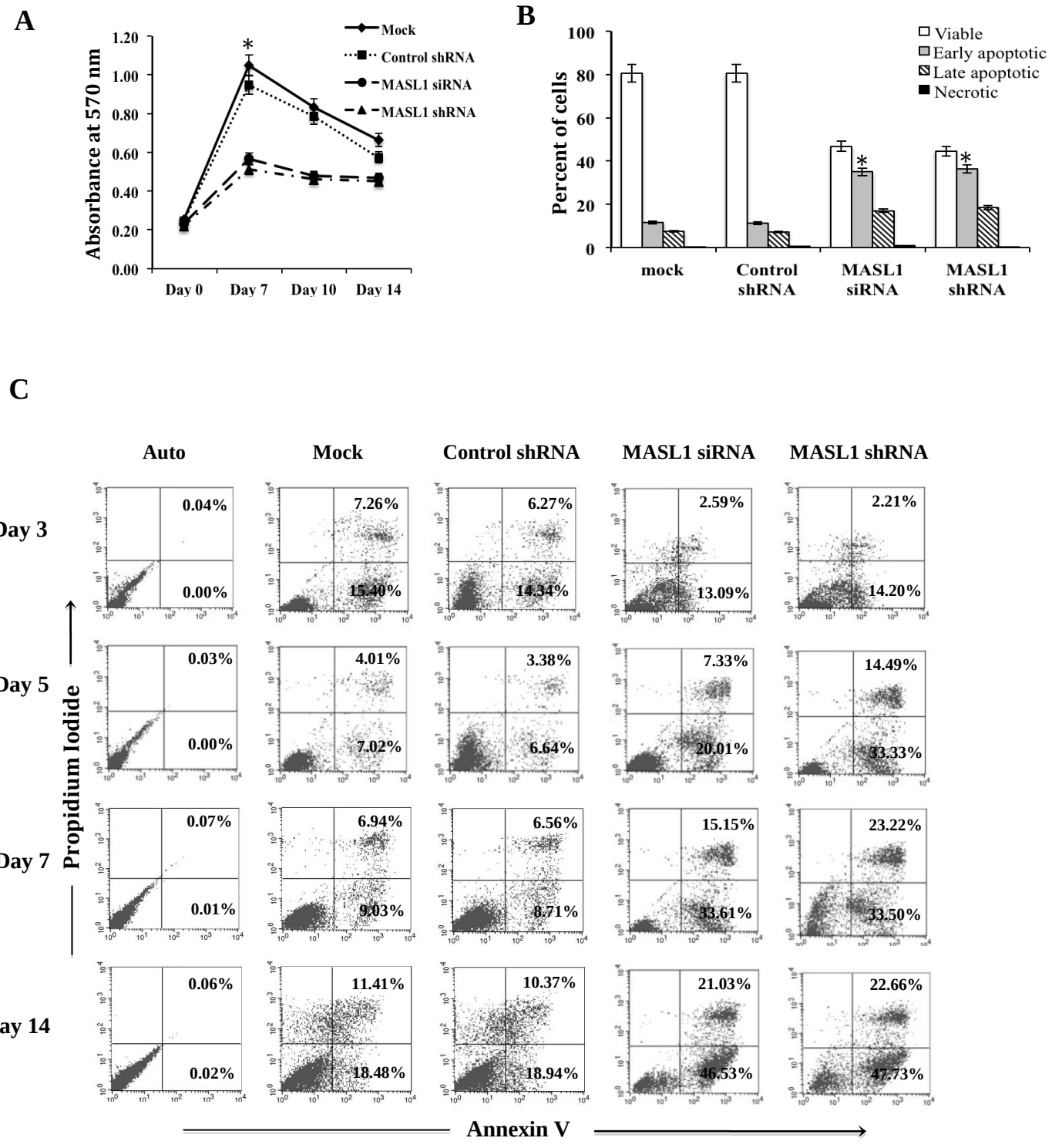
Total-Erk 1/2
CD13
β-actin

Figure S3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Day 0
Day 7
Day 10
Day 14
Mock
Control shRNA
MASL1 siRNA
MASL1 shRNA
*
B
Percent of cells
100
80
60
40
20
0
mock
Control shRNA
MASL1 siRNA
MASL1 shRNA
Viable
Early apoptotic
Late apoptotic
Necrotic
*
*
C
Auto
Mock
Control shRNA
MASL1 siRNA
MASL1 shRNA
Day 3
Day 5
Day 7
Day 14
Propidium Iodide
Annexin V
0.04%
0.00%
7.26%
15.40%
6.27%
14.34%
2.59%
13.09%
2.21%
14.20%
0.03%
0.00%
4.01%
7.02%
3.38%
6.64%
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20.01%
14.49%
33.33%
0.07%
0.01%
6.94%
9.03%
6.56%
8.71%
15.15%
33.61%
23.22%
33.50%
0.06%
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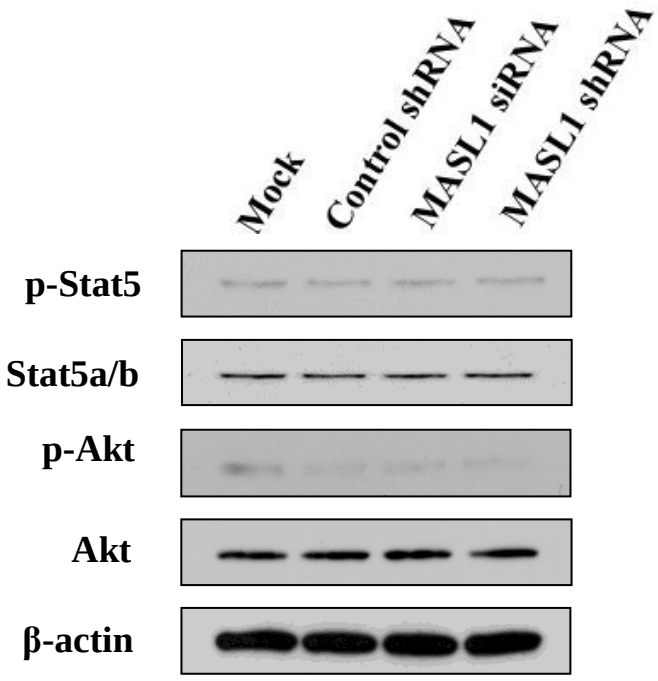
Figure S4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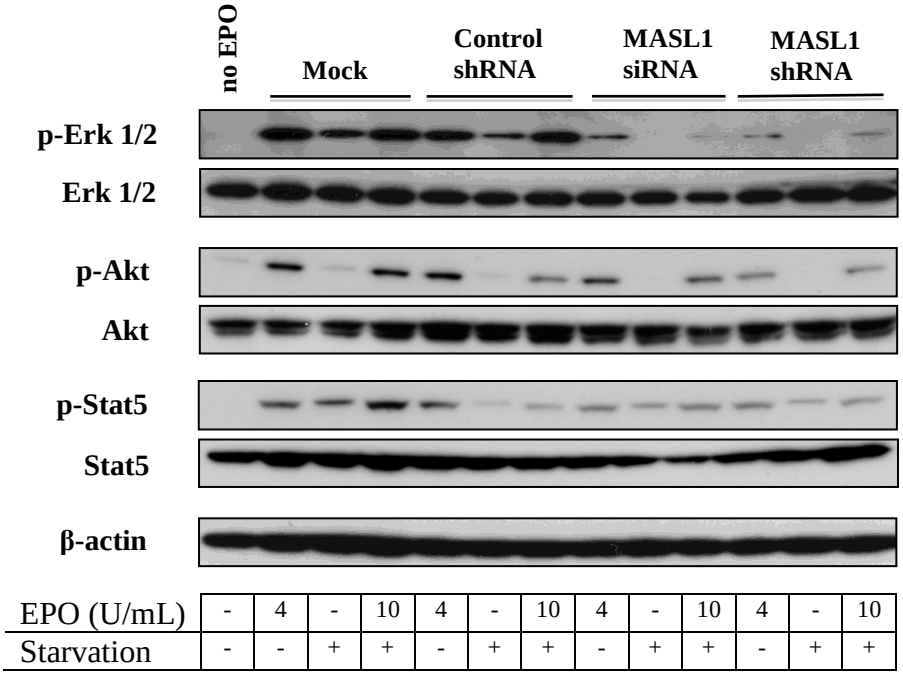
p-Akt
Akt
β-actin

Figure S5

A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Control shRNA
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p-Akt
Akt
p-Stat5
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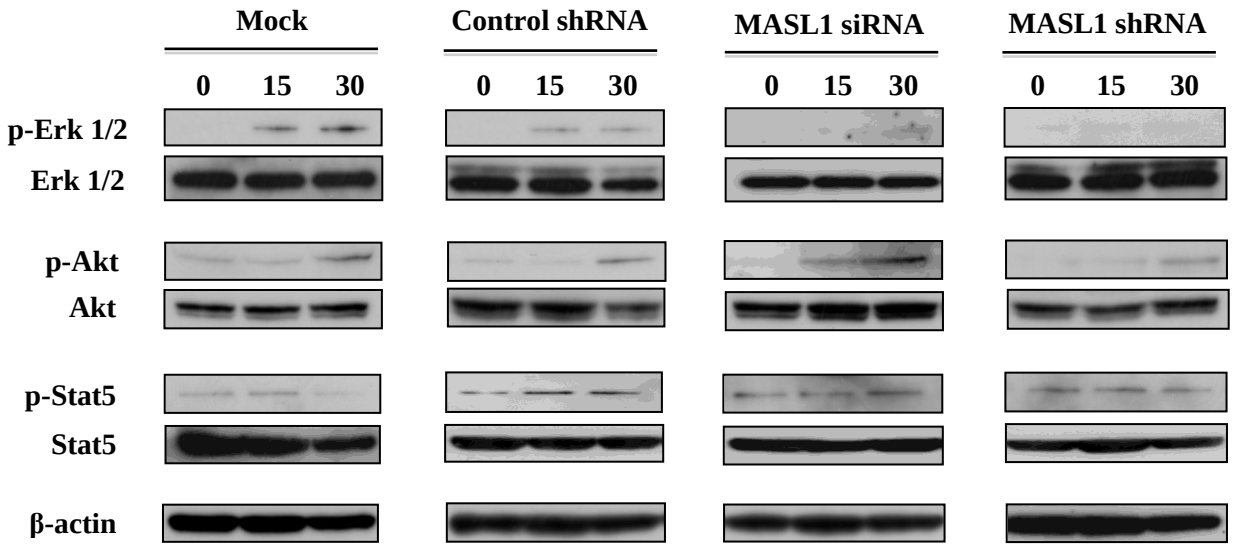
β-actin
EPO (U/mL) - 4 - 10 4 - 10 4 - 10 4 - 10
Starvation - - + + - + + - + + - + +

B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Control shRNA
MASL1 siRNA
MASL1 shRNA
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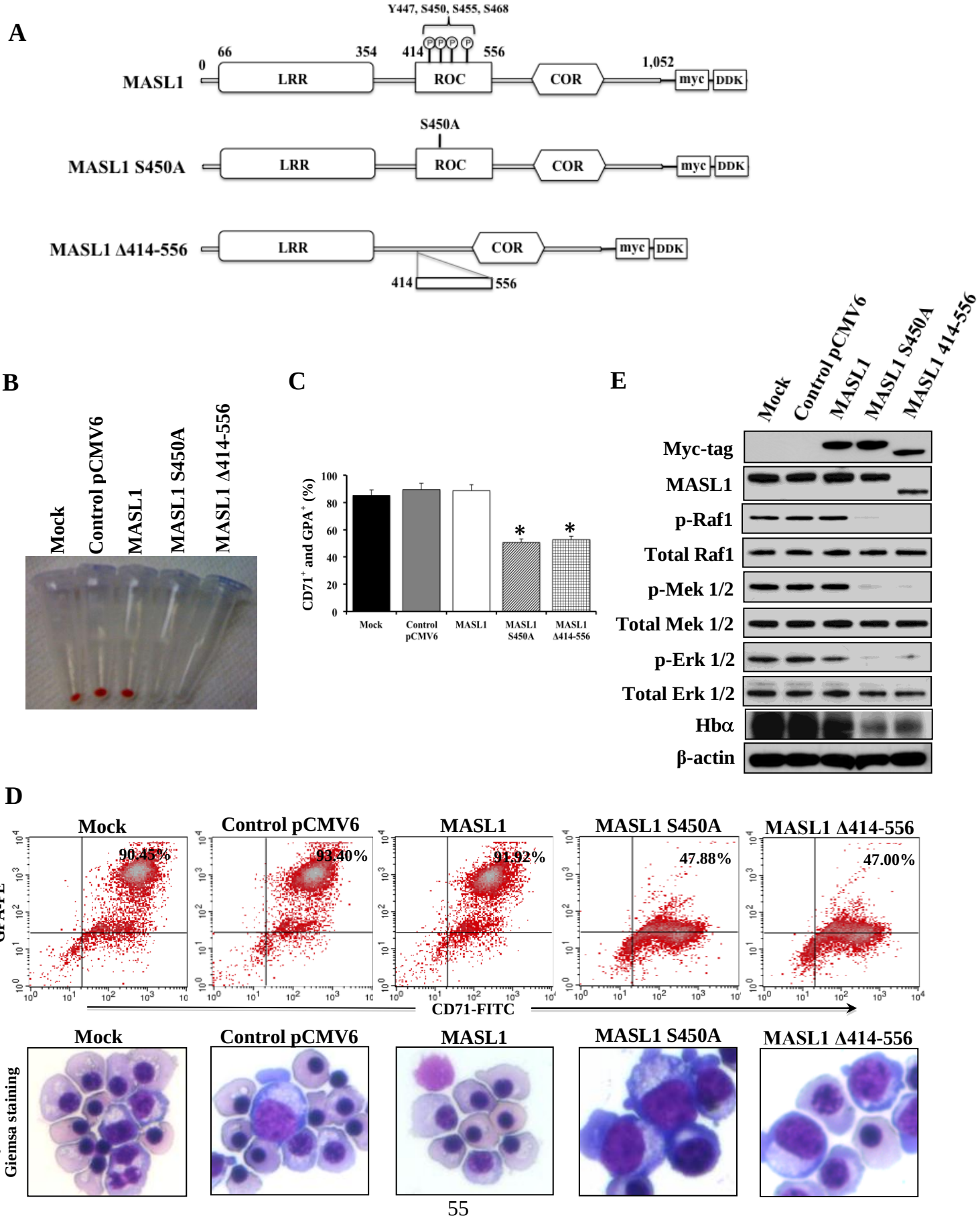
Akt
p-Stat5
Stat5
β-actin

Figure S6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Y447, S450, S455, S468
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66
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414
556
1,052
LRR
ROC
COR
myc
DDK
S450A
MASL1 S450A
MASL1 Δ414-556
B
Mock
Control pCMV6
MASL1
MASL1 S450A
MASL1 Δ414-556
C
CD71+ and GPA+ (%)
100
80
60
40
20
0
Mock
Control pCMV6
MASL1
MASL1 S450A
MASL1 Δ414-556
E
Mock
Control pCMV6
MASL1
MASL1 S450A
MASL1 414-556
Myc-tag
MASL1
p-Raf1
Total Raf1
p-Mek 1/2
Total Mek 1/2
p-Erk 1/2
Total Erk 1/2
Hbα
β-actin
D
Mock
Control pCMV6
MASL1
MASL1 S450A
MASL1 Δ414-556
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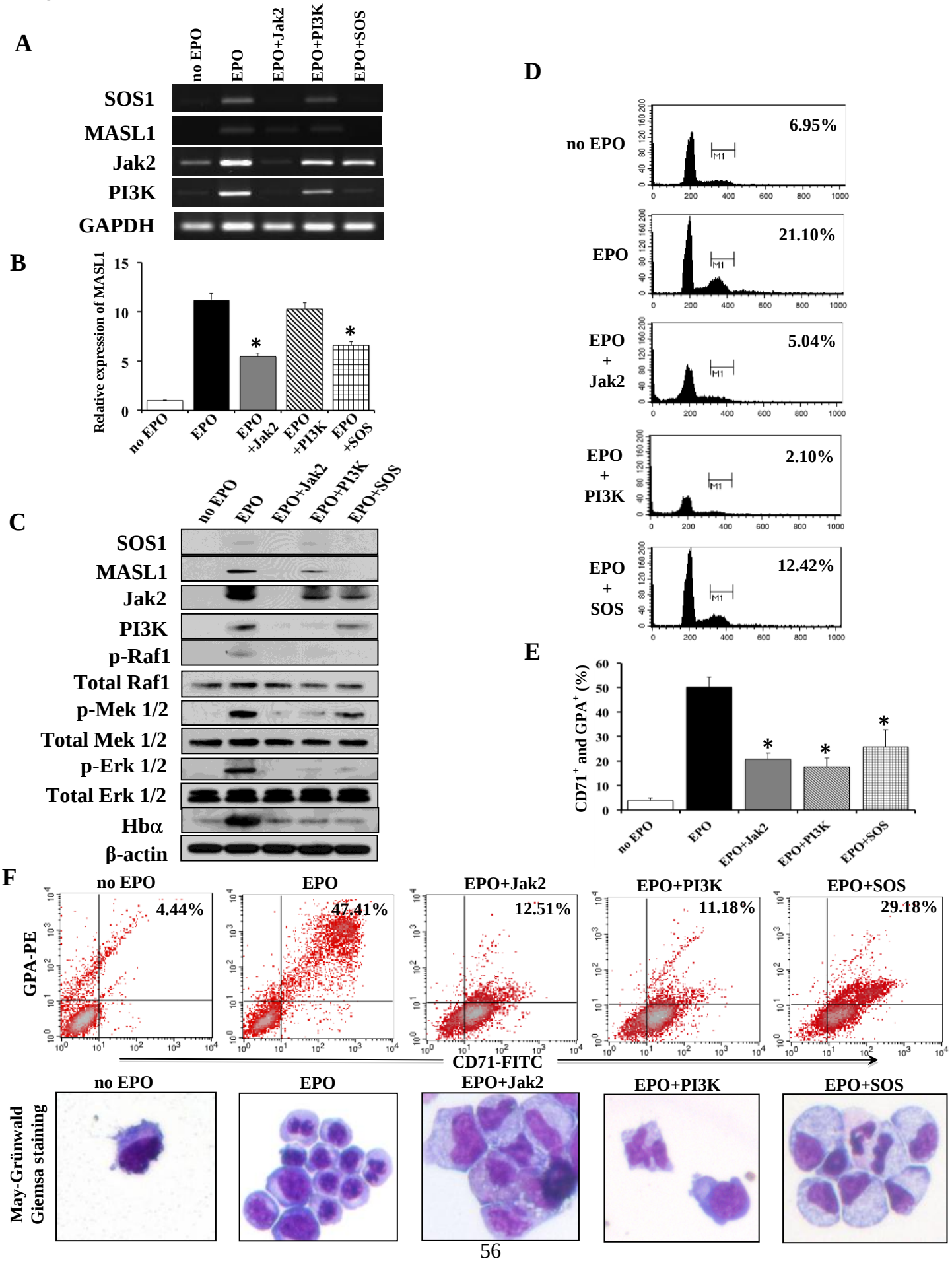
47.00%
GPA-PE
CD71-FITC
May-Grünwald Giemsa staining

Figure S7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EPO+Jak2
EPO+PI3K
EPO+SOS
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Jak2
PI3K
GAPDH
B
Relative expression of MASL1
15
10
5
0
no EPO
EPO
EPO +Jak2
EPO +PI3K
EPO +SOS
C
SOS1
MASL1
Jak2
PI3K
p-Raf1
Total Raf1
p-Mek 1/2
Total Mek 1/2
p-Erk 1/2
Total Erk 1/2
Hbα
β-actin
D
no EPO
6.95%
EPO
21.10%
EPO + Jak2
5.04%
EPO + PI3K
2.10%
EPO + SOS
12.42%
M1
E
CD71+ and GPA+ (%)
60
50
40
30
20
10
0
F
no EPO
4.44%
EPO
47.41%
EPO+Jak2
12.51%
EPO+PI3K
11.18%
EPO+SOS
29.18%
GPA-PE
CD71-FITC
May-Grünwald Giemsa staining