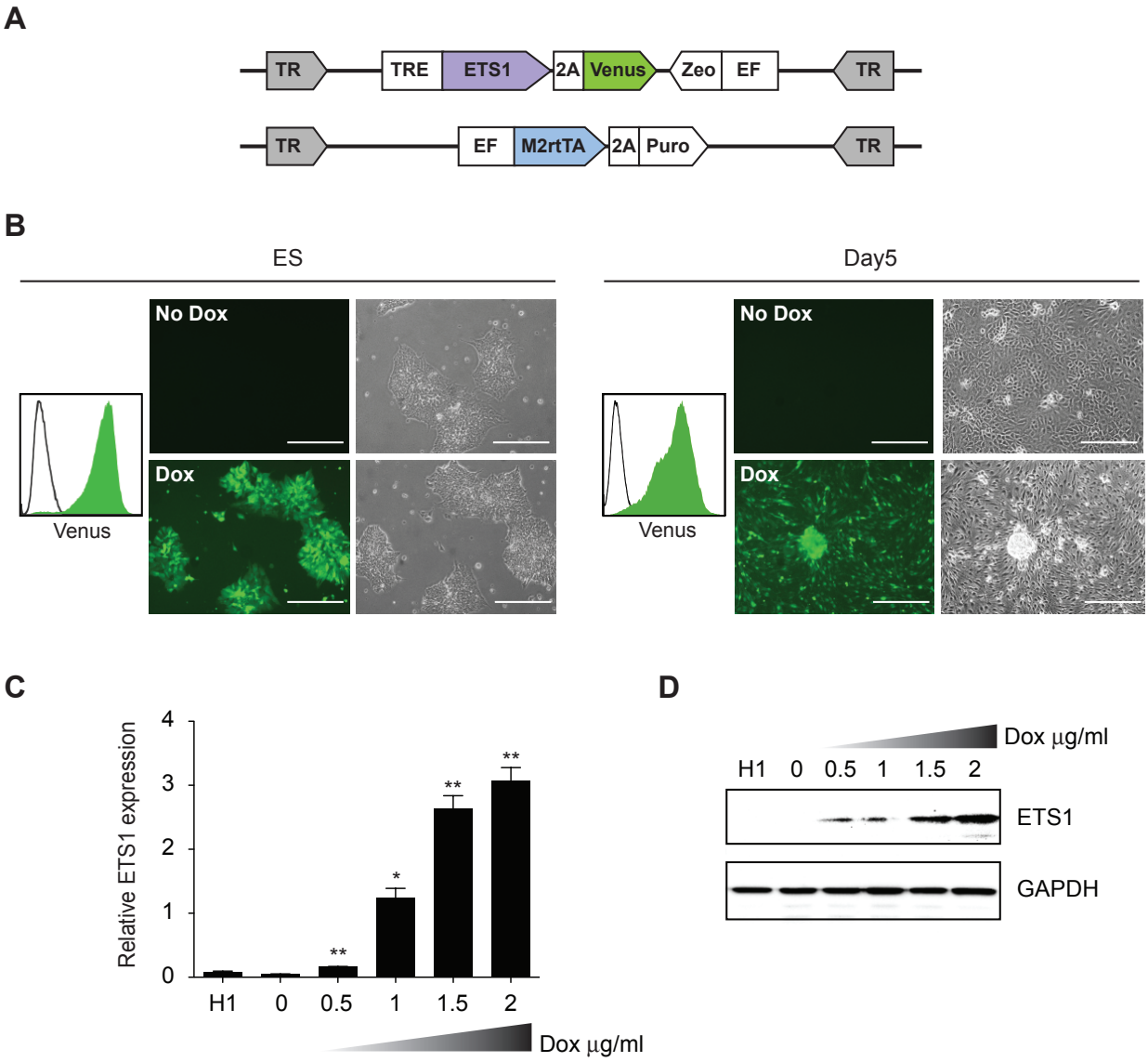
Cell Reports, Volume *23*

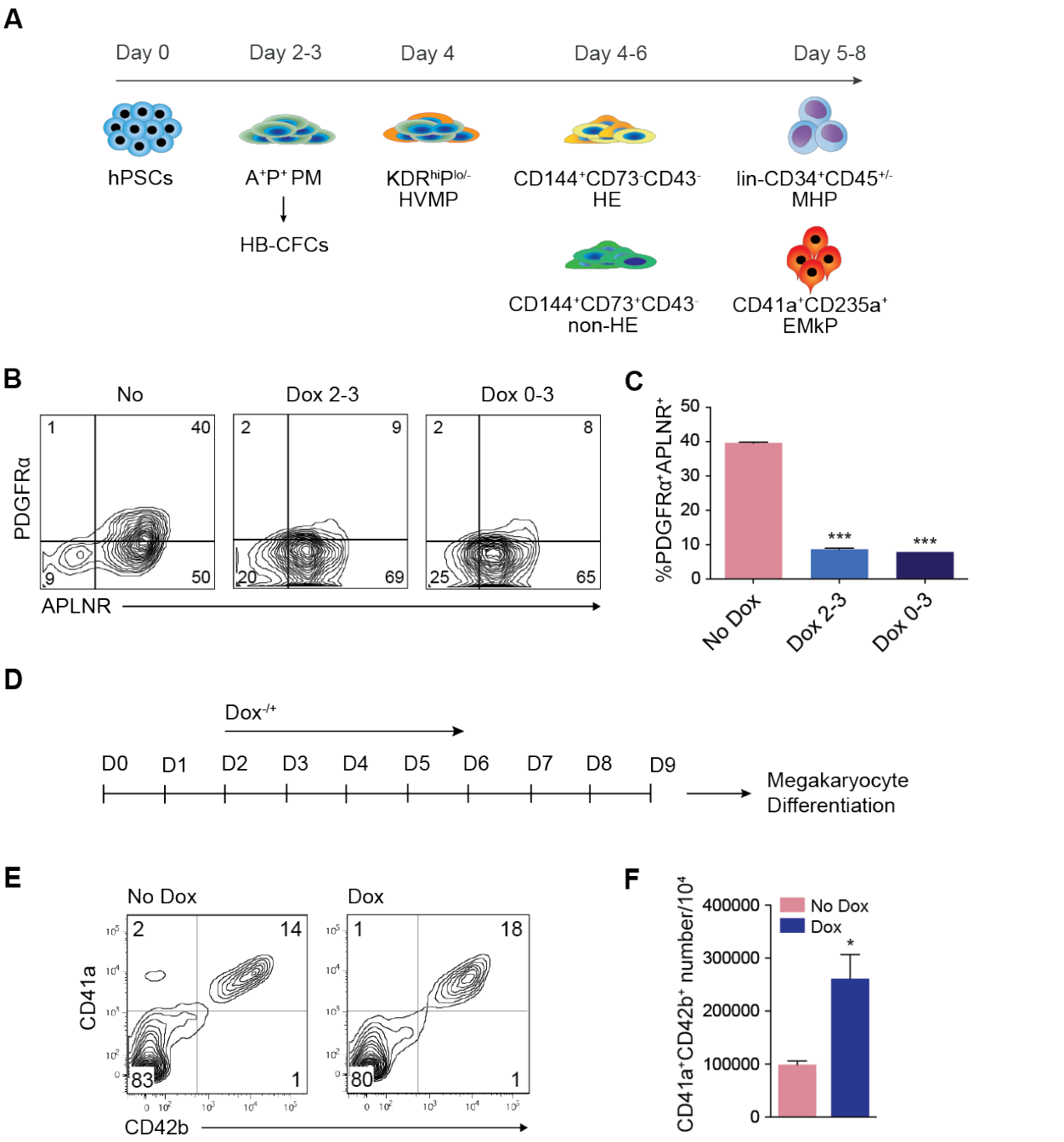
Supplemental Information

Activation of the Arterial Program Drives Development of Definitive Hemogenic Endothelium with Lymphoid Potential

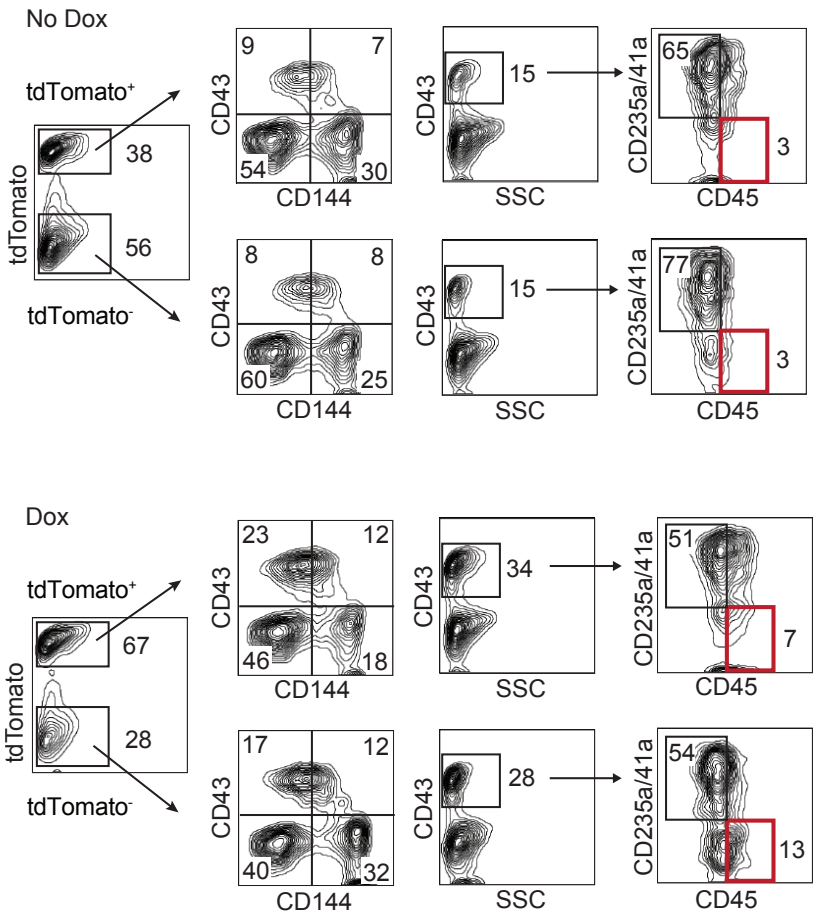
Mi Ae Park, Akhilesh Kumar, Ho Sun Jung, Gene Uenishi, Oleg V. Moskvin, James A. Thomson, and Igor I. Slukvin



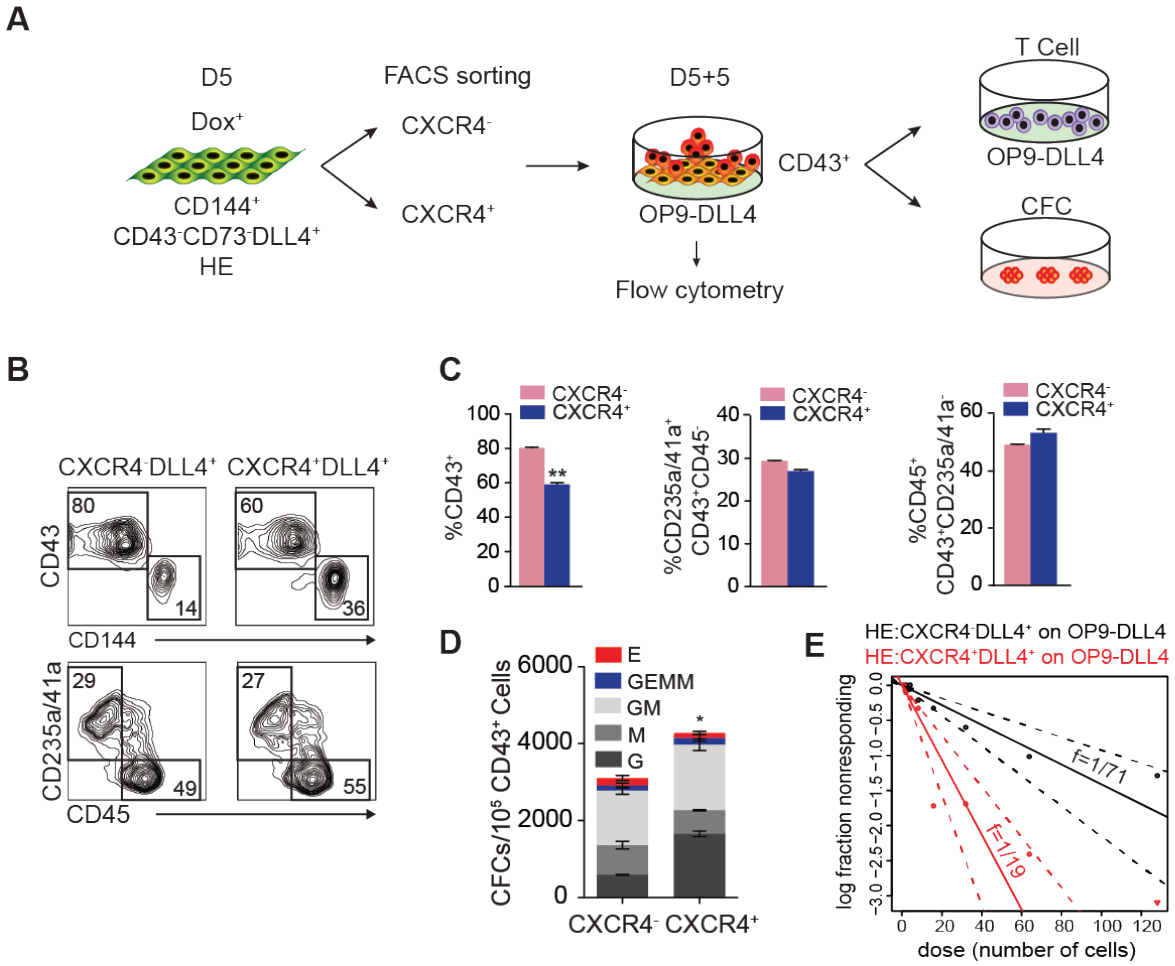
Supplementary Figure S1. Generation of conditional H1 hESC cell line (iETS1-hESC), Related to Figure 1. (A) Schematic diagram of PiggyBac system used to generate iETS1. (B) Expression of Venus reported in undifferentiated and day 5 differentiated iETS1 cells. Image scale bar is 300 μm. Dose-dependent effect of DOX on ETS1 expression in undifferentiated iETS1-hESCs as determined by RT-qPCR (C) and Western Blot (D).



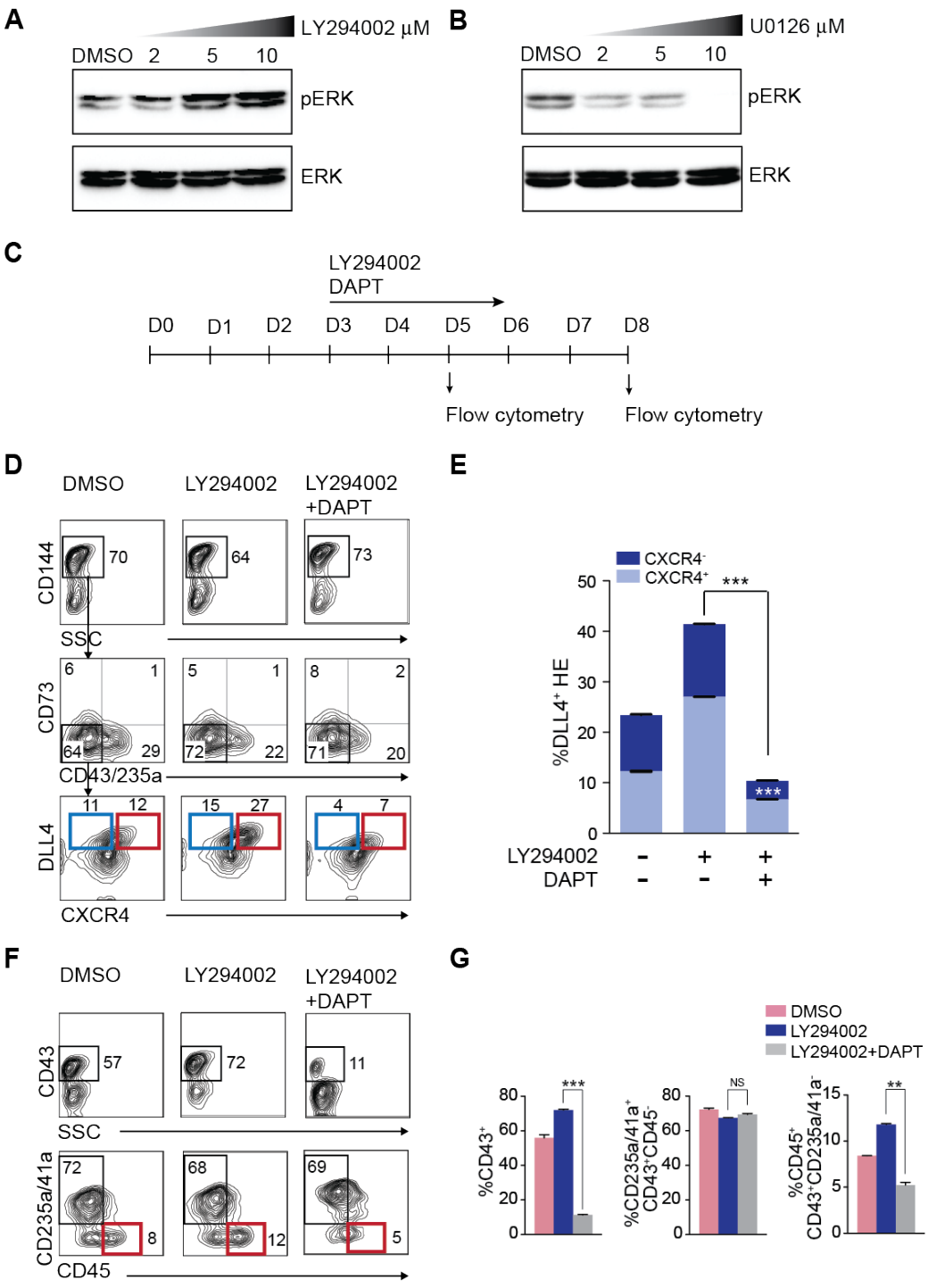
Supplementary Figure S2, Related to Figure 2 and 3. Effect of ETS1 overexpression on development of primitive posterior mesoderm and megakaryocytes. (A) Schematic diagram depicts the major stages of hematopoietic development from hESCs. A^+P^+ PM is $APLNR^+PDGFR\alpha^+$ primitive posterior mesoderm; HB-CFC is hemangioblast CFCs; $KDR^{hi}P^{lo/-}$ HVMPs is $KDR^{high}PDGFR\alpha^{low/-}$ hematovascular mesodermal progenitors; HE, hemogenic endothelium; MHPs, multipotent hematopoietic progenitors; EMkPs, erythromegakaryocytic progenitors. (B) Representative contour plots show the DOX effect on A^+P^+ PM. (C) Percentages of A^+P^+ PM cells in DOX-treated and untreated cultures. (D) Experimental scheme to assess the effect of ETS1 on megakaryocyte production. (E) Representative flow cytometry contour plots show megakaryocytic cells obtained from hematopoietic progenitors in DOX+ and DOX- conditions. (F) Bar graph shows the total number of MK cells generated from 10^4 $CD43^+$ cells obtained from iETS1-hESCs in DOX-treated and untreated conditions. Bar graphs in (C) and (F) are mean ± SD of three independent experiments; *p <0.05, ***p<0.001.



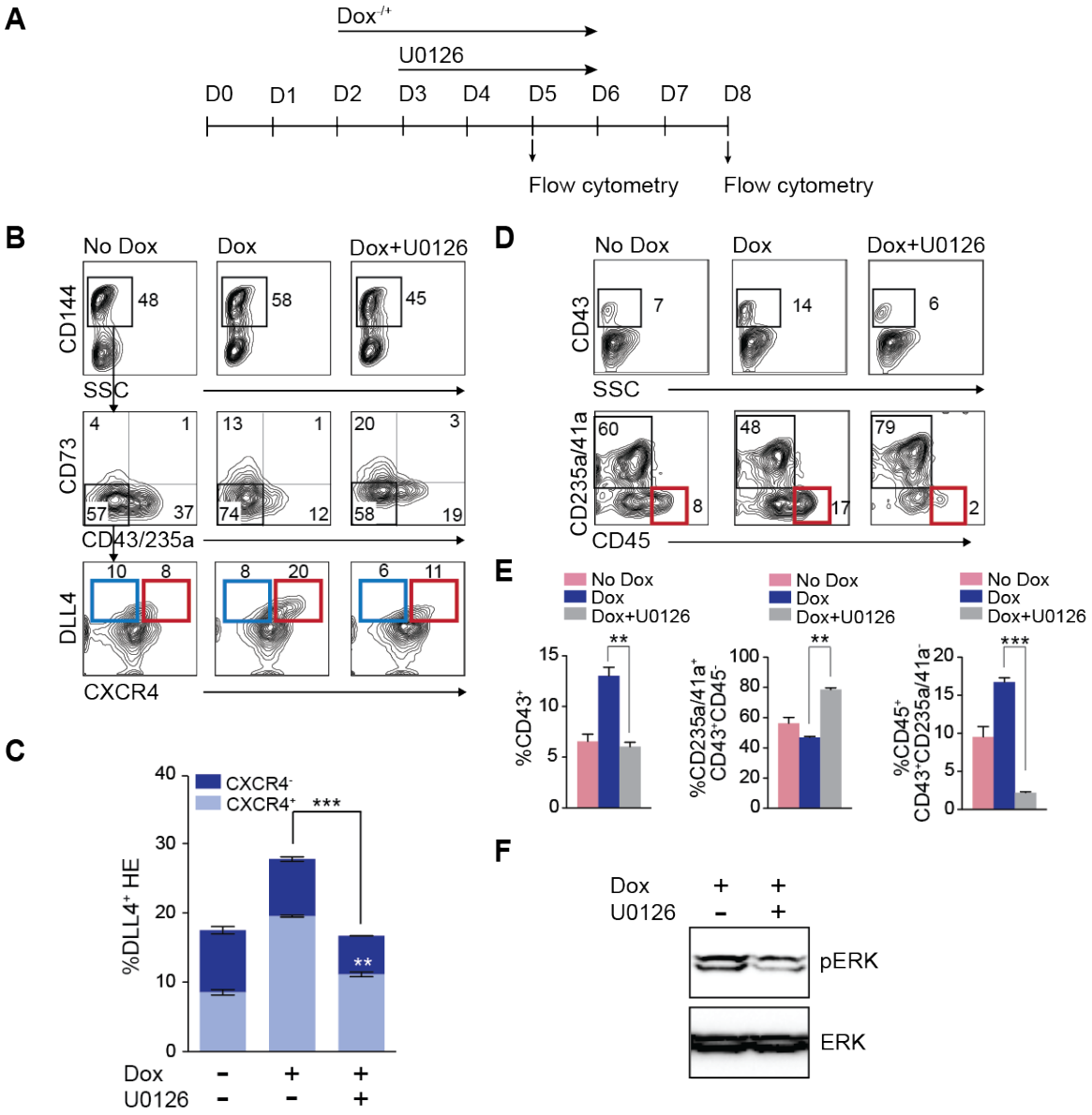
Supplementary Figure S3, Related to Figure 4. Evaluation of ETS1 effect using chimeric (wild type tdTomato$^+$ and iETS1 tdTomato$^-$) H1 hESCs. Representative contour plots show flow cytometric analysis of hematopoietic development on day 8 of differentiation following gating tdTomato$^+$ or tdTomato$^-$ (iETS1) cells in DOX-treated and untreated cultures.



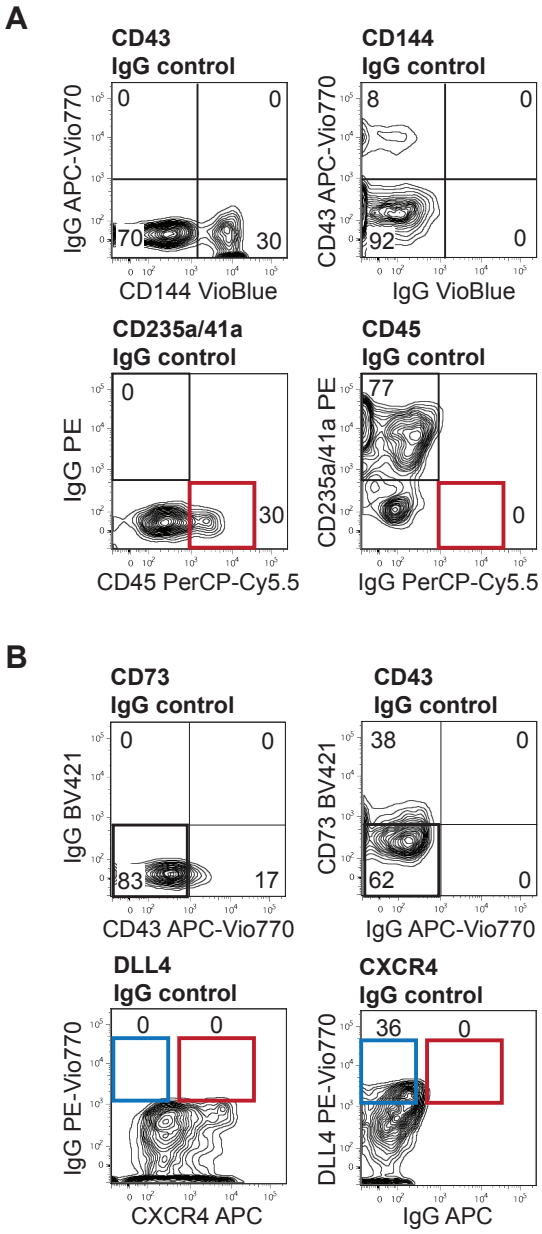
Supplementary Figure S4, Related to Figure 5. Hematopoietic potential of CXCR4$^+$ and CXCR4$^-$ DLL4$^+$ arterial HE. (A) Schematic diagram of experiments. (B) and (C) flow cytometric analysis of hematopoiesis from CXCR4$^+$ and CXCR4$^-$DLL4$^+$ HE. (D) CFC potential of CD43$^+$ cells generated from CXCR4$^+$ and CXCR4$^-$DLL4$^+$ HE. (E) Limiting dilution assay to determine the frequency of T cell progenitors from CXCR4$^+$ and CXCR4$^-$ DLL4$^+$ HE. Bars in (C) and (D) are mean ± SD of three independent experiments; *p<0.05 and **p <0.01.



Supplementary Figure S5, Related to Figure 6. MAPK/ERK signaling promotes arterial HE formation and hematopoiesis through upregulation of NOTCH signaling. (A) and (B) Western blot analysis of pERK expression. Day 3 differentiation cultures were treated for 1 hour with LY294002 (A) or 24 hours with U0126 (B). After treatment total cultures were collected for Western blot analysis. Total ERK is included as a loading control. (C) Schematic diagram of experiment to investigate whether ERK effect is mediated through NOTCH signaling. (D) and (E) The effect of DAPT treatment arterial HE specification in cultures treated with LY294002. (F) and (G) The effect of DAPT treatment blood production in cultures treated with LY294002. Bar graphs in (E) and (G) are mean ± SD of three independent experiments; **p <0.01; ***p<0.001.



Supplementary Figure S6, Related to Figure 6. ERK inhibition suppress ETS1 effect on arterial HE specification and blood production. (A) Schematic diagram of experiment to assess the role of MAPK/ERK signaling in ETS1-mediated effect on hematopoiesis. (B) and (C) The effect of U0126 treatment on arterial HE specification. (D)-(E) The effect of U0126 treatment on blood production. (F) Western blot shows pERK expression in cultures treated with DOX and U0126. Bar graphs in (C) and (E) are mean ± SD of three independent experiments; **p <0.01; ***p<0.001.



Supplementary Figure S7, Related to Experimental procedures. Ig isotype controls for flow cytometric analysis of arterial HE and blood production. (A) Representative flow cytometry contour plots of hematopoietic development on day 8. Ig isotypes control for CD43, CD144, CD235a/41a and CD45 were used to determine the background. (B) flow analysis of arterial HE specification on day 5. Ig isotypes control for CD73, CD43, DLL4 and CXCR4 were used to determine the background.

Table S1. List of antibodies used for FACS, Related to Experimental Procedure.

Antigen	Label	Company	Cat. Number
CD4	APC	BD Biosciences	555349
CD5	PE	BD Biosciences	555353
CD7	FITC	BD Biosciences	555360
CD8	PE	BD Biosciences	555635
CD10	PE	BD Biosciences	555375
CD19	APC	Miltenyi Biotec	130-091-248
CD34	PE	BD Biosciences	555822
CD41a	PE	BD Biosciences	555467
CD41a	PE	Miltenyi Biotec	130-105-560
CD41a	APC	BD Biosciences	559777
CD42b	PE	BD Biosciences	555473
CD43	PE	BD Biosciences	560199
CD43	APC	BD Biosciences	560198
CD43	APC-Vio770	Miltenyi Biotec	130-101-174
CD43	BV421	BD Biosciences	562916
CD45	PerCP-Cy5.5	BD Biosciences	564105
CD45	APC	BD Biosciences	555485
CD45	PE-Vio770	Miltenyi Biotec	130-096-616
CD45	BV421	BD Biosciences	563879
CD73	PE	BD Biosciences	550257
CD73	BV421	BD Biosciences	562430
CD93	PE	BioLegend	336107
CD144	PE	Miltenyi Biotec	130-100-714
CD144	PE-Vio770	Miltenyi Biotec	130-100-722
CD144	PerCP-Cy5.5	BD Biosciences	561566
CD144	PerCP-Vio700	Miltenyi Biotec	130-100-718
CD144	Alexa Fluor 647	BD Biosciences	561567
CD144	VioBlue	Miltenyi Biotec	130-100-724
CD235a	PE	BD Biosciences	555570
CD235a	APC	BD Biosciences	551336
KDR (CD309)	PE	BD Biosciences	560494
KDR (CD309)	Alexa Fluor 647	BD Biosciences	560495
DLL4	PE	Miltenyi Biotec	130-096-567
DLL4	PE-Vio770	Miltenyi Biotec	130-101-587
CXCR4	APC	eBioscience	17-9999-42
PDGFα (CD140a)	PE	BD Biosciences	556002
APLNR	APC	R&D Systems	FAB856A
TRA-1-85	APC	R&D Systems	FAB3195A

Table S2. List of primers used for RT-qPCR, Related to Experimental Procedure.

Gene	Direction	Sequences (5'-3')
ETS1	F	CATCCACAAGACAGCGGGG
	R	CTCGTCGGCATCTGGCTTG
EFNB2	F	CTCCTCAACTGTGCCAAACCA
	R	GGTTATCCAGGCCCTCCAAA
SOX17	F	AGAATCCAGACCTGCACAAC
	R	GCCGGTACTTGTAGTTGGG
NOTCH1	F	CAATGTGGATGCCGCAGTTGTG
	R	CAGCACCTTGGCGGTCTCGTA
β-globin	F	GGCACCTTTGCCACACTG
	R	CACTGGTGGGGTGAATTCTT
ε-globin	F	GCCTGTGGAGCAAGATGAAT
	R	GCGGGCTTGAGGTTGT
γ-globin	F	CTTCAAGCTCCTGGGAAATGT
	R	GCAGAATAAAGCCTATCCTTGAAAG
BCL11a	F	AACCCCAGCACTTAAGCAAA
	R	GGAGGTCATGATCCCCTTCT
RPL13a	F	CCTGGAGGAGAAGAGGAAAGAGA
	R	TTGAGGACCTCTGTGTATTTGTCAA

Supplementary Experimental Procedures

Construction of vectors and generation of iETS1 and tdTomato H1 hESC lines
Human ETS1 cDNA was cloned into PiggyBac transposon vector (Transposagen) downstream of TREtight promoter of pTRE-P2A-Venus-EF1α-Zeo plasmid, and then co-electroporated with pEF1α-M2rtTA-T2A-Puro and transposase plasmid into H1 hESCs using human stem cell nucleofector kit 2 (Lonza) (Supplementary Figure S1). The colonies were selected in Zeocin (0.5-1 μg/ml, invitrogen) and Puromycin (0.5-1 μg/ml, Sigma) for 10-15 days and the resistant clones were screened by Venus expression under a fluorescence microscope with DOX treatment. The tdTomato cDNA was cloned downstream of EF1α promoter of a pRMCE-EF1α-Zeo plasmid and into H1 hESCs. 3 days after electroporation, cells were treated with Zeocin (0.5-1 μg/ml, Invitrogen). After 10-15 days, tdTomato positive surviving colonies were picked and expanded in each well of a 12 well plate.

Hemangioblast (HB)-CFC and Hematopoietic CFC assay
HB-CFC were detected as described previously (Vodyanik et al., 2010). HB-CFCs were detected using the semisolid colony-forming serum-free medium (CF-SFM) containing 40% ES-Cult M3120 methylcellulose (2.5% solution in IMDM, Stem Cell Technologies), 25% StemSpan serum-free expansion medium (SFEM, Stem Cell Technologies), 25% human endothelial serum-free medium (ESFM, Invitrogen), 10% BIT 9500 supplement (Stem Cell Technologies), GlutaMAX (1/100 dilution, Invitrogen), Ex-Cyte (1/1000 dilution, Millipore), 100 μM MTG, 50 μg/ml ascorbic acid and 20 ng/ml FGF (Peprotech). Hematopoietic CFCs were detected using serum containing H4435 MethoCult with SCF, IL-3, IL-6 and EPO (Stem Cell Technologies).

RBC differentiation
Floating $CD43^+$ cells at day 9 of differentiation were collected and cultured in RBC differentiated medium consisting of SFEM (serum free expansion medium, Stem Cell Technologies) supplemented with 0.3% Ex-Cyte (Millipore), 1 mg/ml Holo-Transferrin (Sigma), 10 μM dexamethasone (Sigma), 20 ng/ml insulin (Sigma), 2 U/ml EPO (R&D Systems), 50 ng/ml SCF (Peprotech), 50 ng/ml TPO (Peprotech), 5 ng/ml IL-3 (PeproTech) and 10 ng/ml IL-6 (PeproTech) on ultra-low attachment 6 well plate (Corning). After 2 days, cells were cultured on OP9 cells using the same medium without TPO, IL-3 and IL-6 for 20 days with weekly passage. Media changes were performed every 2 days as described previously (Dias et al., 2011).

MK differentiation
Floating $CD43^+$ cells at day 9 of differentiation were collected and cultured in MK differentiation medium consisting of StemSpan SFEM supplemented with 20 ng/ml SCF, 20 ng/ml TPO, 20 ng/ml IL-11 (Peprotech) on ultra-low attachment 6 well plates.

Immunofluorescence
Day 4 $CD144^+$ cells were fixed for 10 minutes with 3.7% formaldehyde (Sigma) and permeabilized with a solution of 0.2% Triton-X-100 (Sigma) for 5 minutes. Cells were blocked with 3% chicken egg albumin (Sigma) blocking buffer and incubated with anti-human COUP-TF II/NR2F2

(Perseus Proteomics Inc, PP-H7147-00, 1:500) antibody. Cells were incubated with anti-mouse secondary antibody, Alexa Fluor 555 conjugated (Invitrogen, A31570, 1:500) and DAPI (2 μg/ml, Sigma) for 10 minutes. The immunolabeled cells were examined using the Nikon Eclipse Ti-E confocal system (Nikon Instruments Inc).

Western Blot

For ETS1 detection, cells were suspended in whole cell lysis buffer containing 1% SDS, 60 mM Tris pH 6.8 and a protease inhibitor cocktail tablet (Roche, Switzerland). ETS1 was detected with mouse anti-ETS1 (Santa Cruz Biotechnology, sc-55581) and horseradish peroxidase (HRP) conjugated anti-mouse IgG-HRP secondary antibody (Santa Cruz Biotechnology, sc-2005). GAPDH was detected with anti-GAPDH (Santa Cruz Biotechnology, sc-25778) and anti-rabbit IgG-HRP secondary antibody (Santa Cruz Biotechnology, sc-2004). For pERK detection, cells were suspended in Pierce IP Lysis Buffer (Thermo Fisher Scientific, 87787) containing a Phosphatase inhibitor cocktail 2 (Sigma Aldrich, P5726) and 3 (Sigma Aldrich, P0044). Phosphorylated pERK and total ERK were detected with anti-pERK1/2 (phospho-p44/42 MAPK, Thr202/Tyr204, Cell Signaling, 4370) or anti-ERK1/2 (p44/42 MAPK, Cell Signaling, 4695) antibodies and anti-rabbit IgG-HRP secondary antibody (sc-2004). The proteins were visualized by ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) using ChemiDoc XRS+ with Image Lab Software Version 5.2.1 (Bio-Rad).

Low level RNAseq data processing.

Day 4 $KDR^{+}CD144^{+}$ or day 5 $DLL4^{+}$ and $DLL4^{-}$ HE cells were isolated from DOX treated and untreated cultures as described above. Total RNA was prepared with RNeasy Plus Micro Kit (Qiagen). RNA purity and integrity were evaluated by capillary electrophoresis on the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Samples were then prepared for sequencing using the Ligation Mediated Sequencing (LM-Seq) protocol, according to the published guidelines (Hou et al., 2015). Final sample libraries were quantitated with the Life Technologies Qubit fluorometer and sequenced on the Illumina HiSeq 3000 (SY-401-3001). Base-calling and demultiplexing were completed with the Illumina bcl2fastq2 utility, v2.17.1.14. Following quality assessment and filtering for adapter molecules and other sequencing artifacts, the remaining sequence reads were aligned to transcript sequences corresponding to hg19 human genome annotation. Bowtie v 1.1.2 was used, allowing two mismatches in a 25 bp seed, and excluding reads with more than 200 alignments (Langmead et al., 2009). RSEM v 1.3.0 was used to estimate isoform or gene relative expression levels in units of ‘‘transcripts per million’’ (tpm), as well as posterior mean estimate of the “expected counts” (the non-normalized absolute number of reads assigned by RSEM to each isoform/gene) (Li and Dewey, 2011; Li et al., 2010). R statistical environment (R core team, 2014) was used throughout all of the stages of downstream data analysis.

Downstream RNAseq bioinformatics analysis:

Testing for differential expression

R statistical environment (R core team, 2014) was used for all stages of downstream data analysis. The entire set of libraries were pre-normalized as a pool to equilibrate 65th percentile of the counts distribution, using the quantile scaling routine from EBSeq package (Leng et al., 2013). For each

gene, maximal counts across all the samples were plotted and the genes representing the lower mode of the distribution were filtered out (only genes that have at least 40 counts in at least 1 sample were retained), restricting the set of genomic features to 12,898. Additional median scaling was applied to the pre-filtered set of genes. Differential expression was called using EBSeq with 10 iterations. The EBSeq's default procedure of filtering low-expressed genes was suppressed by setting the QtrmCut parameter to zero. Genes with an assigned value of Posterior Probability of Differential Expression above 0.95 were preliminary selected. Genes that passed two additional filters were selected for downstream analysis: 1) fold change cutoff of 1.5 and 2) expression level should exceed 20th percentile of genome-wide distribution of expression values in libraries representing the condition with a larger mean expression of that gene.

Visualization of transcriptional network

Using the known transcription-target relationships obtained by combining largely complementary data from HTRIdb (Bovolenta et al., 2012) and CellNet (Cahan et al., 2014), we generated combined sets of targets for 950 transcriptional regulators that involve 130,855 individual transcription factor (TF)-target interactions, for regulon analysis. To visualize the cascades of transcriptional regulation that involve influence of active TFs on TF-encoding genes, we restricted the overall regulatory network to TF-target relationships that involve TF-encoding target genes. The resulting "transcriptional backbone" network has 837 regulators and reduced by over an order of magnitude (12,372) individual TF-target relationships. To isolate the relevant part of this network, we selected 8 transcription factors that demonstrated their activation in response to Dox according to our regulon analysis, and restricted the network to edges that have either outgoing or incoming connections related to the 8 selected factors. The resulting subnetwork had 59 nodes and 175 edges.

Supplementary References

Bovolenta, L.A., Acencio, M.L., and Lemke, N. (2012). HTRIdb: an open-access database for experimentally verified human transcriptional regulation interactions. BMC Genomics *13*, 405.

Cahan, P., Li, H., Morris, S.A., Lummertz da Rocha, E., Daley, G.Q., and Collins, J.J. (2014). CellNet: network biology applied to stem cell engineering. Cell *158*, 903-915.

Dias, J., Gumenyuk, M., Kang, H., Vodyanik, M., Yu, J., Thomson, J.A., and Slukvin, II (2011). Generation of red blood cells from human induced pluripotent stem cells. Stem Cells Dev *20*, 1639-1647.

Hou, Z., Jiang, P., Swanson, S.A., Elwell, A.L., Nguyen, B.K., Bolin, J.M., Stewart, R., and Thomson, J.A. (2015). A cost-effective RNA sequencing protocol for large-scale gene expression studies. Sci Rep *5*, 9570.

Leng, N., Dawson, J.A., Thomson, J.A., Ruotti, V., Rissman, A.I., Smits, B.M., Haag, J.D., Gould, M.N., Stewart, R.M., and Kendziorski, C. (2013). EBSeq: an empirical Bayes hierarchical model for inference in RNA-seq experiments. Bioinformatics *29*, 1035-1043.

Vodyanik, M.A., Yu, J., Zhang, X., Tian, S., Stewart, R., Thomson, J.A., and Slukvin, II (2010). A mesoderm-derived precursor for mesenchymal stem and endothelial cells. Cell Stem Cell *7*, 718-729.