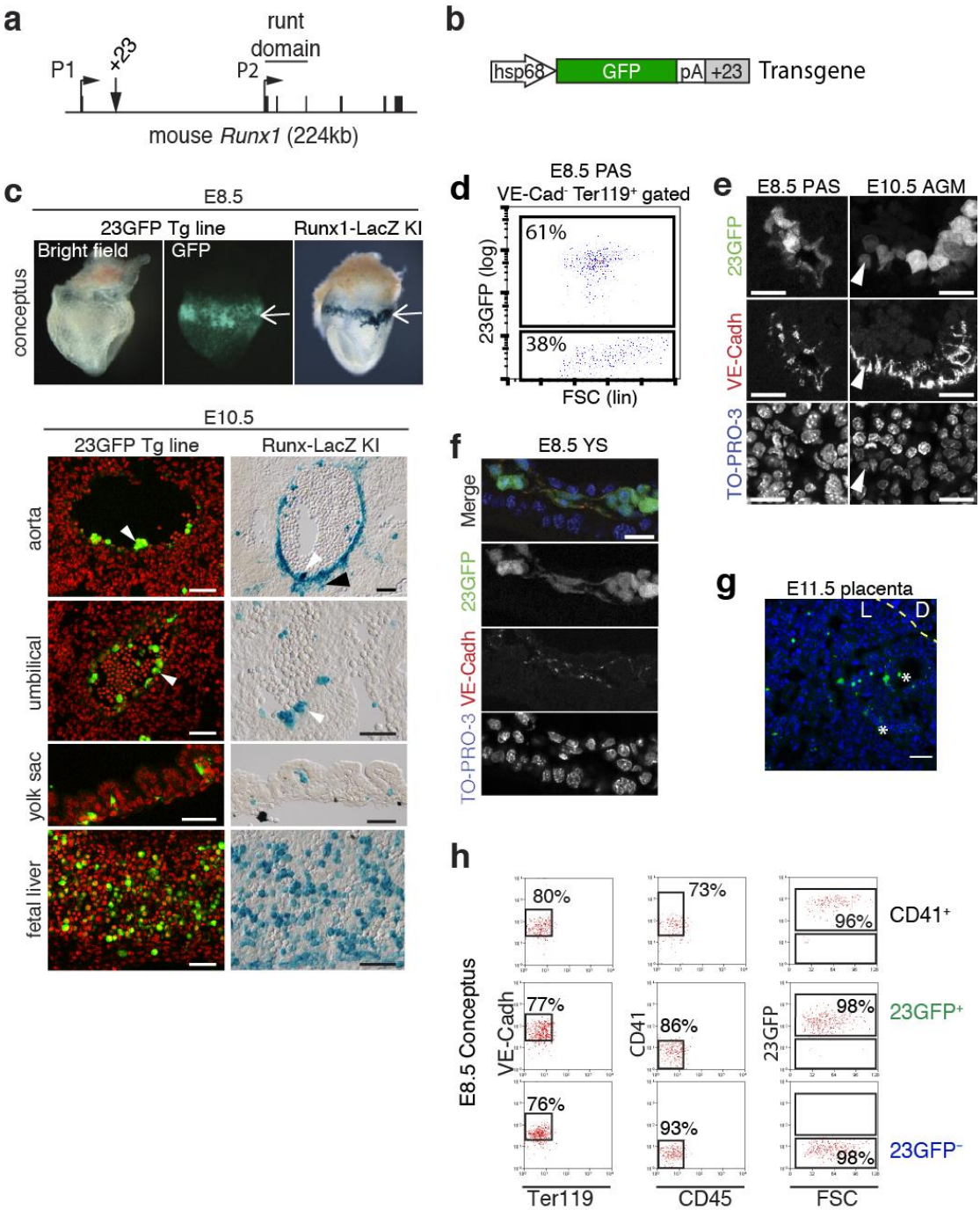
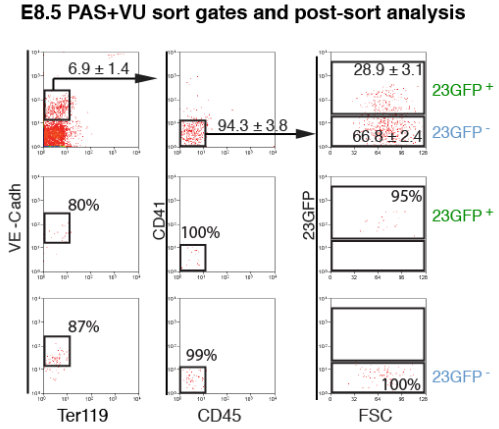


Supplementary Figure S1. Characterisation of the 23GFP reporter line. (a) Schematic of mouse *Runx1* locus with the distal P1 and proximal P2 promoters and location of the +23 enhancer highlighted. (b) Schematic of the 23GFP reporter construct. pA = SV40 polyadenylation site. (c) Comparison of the 23GFP reporter expression with Runx1-lacZ knockin mice, showing expression in

hematopoietic territories as described in Nottingham et al.[22]. At E8.5 23GFP expression is detected in the yolk sac blood island (arrow) in a pattern similar to that of the Runx1-LacZ KI. At E10.5 23GFP expression is expressed in hematopoietic sites only, in a pattern similar to the Runx1-LacZ KI mice (white arrowhead, example of hematopoietic cell). The 23GFP enhancers specificity for the hematopoietic lineage is also seen from the absence of widespread 23GFP expression in the subaortic mesenchyme (black arrowhead, marked in the Runx1-LacZ KI), which are mostly smooth muscle cells[56]. Scale bar = 50 μm (d) Flow cytometric analysis of E8.5 PAS region for Ter119, a marker of primitive erythrocytes. (e) Single channel images for the images shown in Fig. 1a: 23GFP (top), VE-Cadh (middle) and nuclear (TO-PRO-3; bottom) expression at E8.5 and E10.5. Arrowhead: example of 23GFP expression in VE-Cadh^{+} endothelial cells, scale bar = 20 μm. (f) 23GFP expression is also detected in endothelial (and haematopoietic) cells of the E8.5 YS. Representative image of VE-Cadh immunostaining (red) on a 10 μm section through 23GFP transgenic (green) yolk sac. Nuclear TO-PRO-3 in blue, scale bar = 20 μm. (g) 23 GFP expression is detected in the placenta. L = labyrinth, D = decidua. Asterisk = vessel. Scale bar = 50 μm. (h) Representative sort re-analysis (with sort gates shown) for E8.5 conceptus. Percentages reveal the proportion of events within the sort gate when re-analysed.

a



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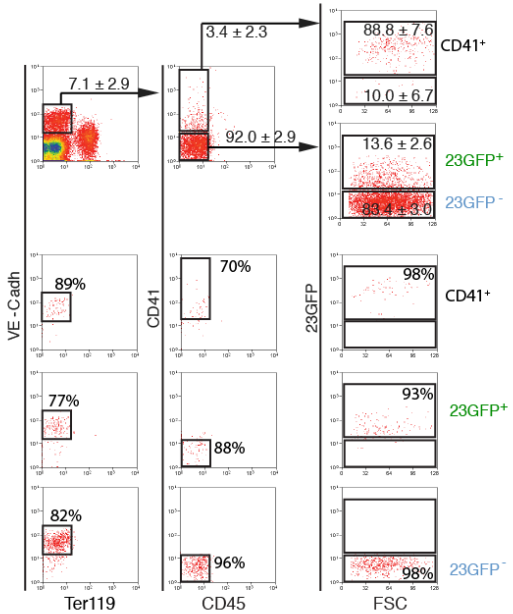

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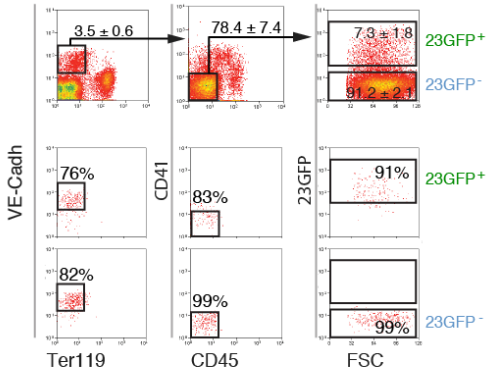
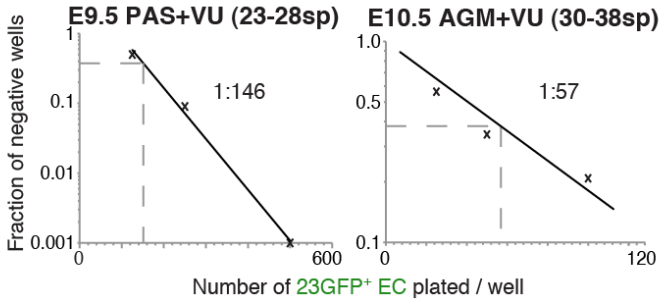


d

Cell population plated on OP9	Cells / well	Wells with haematop. progeny / Total wells	
		E8.5	E10.5
23GFP⁻ EC	1000	0/10	0/7
23GFP⁺ EC	1000	7/9	4/4
CD41⁺ HPC	500	4/4	2/2

e



f

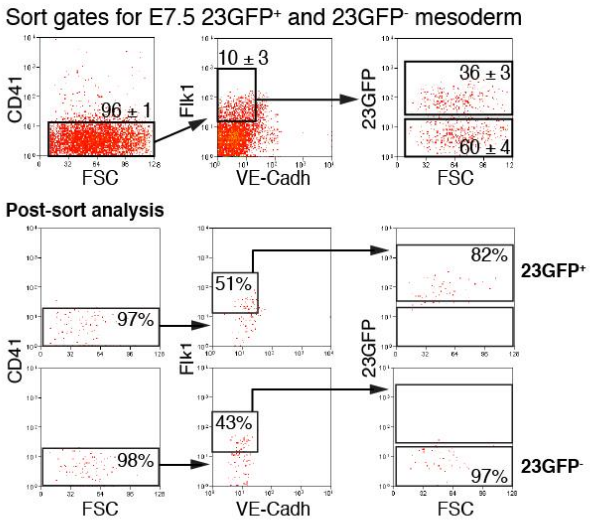
Cell population plated on OP9 (E10.5 YS)	Cells / well	Wells with haematop. progeny / total wells
23GFP⁻ EC	3000	0/3
23GFP⁺ EC	2800	2/2

g

Stage	CFU-C frequency in 23GFP⁺ EC
E8.5 (4-10 sp)	1:3156
E10.5 (32-37 sp)	1:183

Supplementary Figure S2. Representative sort gates and reanalysis for (a) E8.5 PAS+VU, (b) E9.25 PAS+VU, (c) and E10.5 AGM+VU. Numbers within the sort gates are indicated and are the mean percentage ± SD of at least 3 independent experiments. Representative re-analysis percentages as shown. (d) Summary of haemogenic OP9 co-cultures at E8.5 and E10.5. At least 3 independent sorts were performed on pooled tissues dissected from staged embryos (except for E10.5 VE-Cadh^{+}Ter119^{-}CD45^{-}CD41^{+} HPCs where n=2). (e) Limiting dilution analysis of haemogenic output of 23GFP in OP9 co-cultures; data are the mean of 2 (E9.5) and 3 (E10.5) independent experiments. (f) Summary of haemogenic progeny generated during OP9 cultures of E10.5 (34-38sp) YS ECs. Data are from one independent experiment. (g) Frequency of CFU-C formation from 23GFP expressing ECs (VE-Cadh^{+}Ter119^{-}CD45^{-}CD41^{-}). Data are from at least 3 independent sorts performed on pooled tissues dissected from stage-matched embryos.

a



b

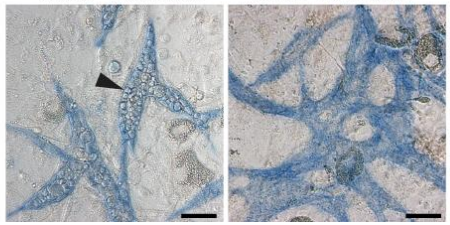
Clonal analysis of E7.5 Flk1^{+} VE-Cadh$^{-/lo}$ CD41^{-} cells on OP9

Flk1^{+} VE-Cadh$^{-/lo}$ CD41^{-} subset (1 cell / well)	Haematopoietic	Endothelial	Haematopoietic and endothelial
23GFP $^{+}$	1:33 (24 / 798)[a]	1:15 (54 / 798)[b]	1:80 (10 / 798)[c]
23GFP $^{-}$	<1:323 (0 / 323)	1:9 (36 / 323)	<1:323 (0/323)

[a] Number of wells with haematopoietic progeny over the total number of wells seeded. [b] Number of wells with endothelial tubules and/or sheets over the total number of wells seeded. [c] Number of wells with endothelial and haematopoietic progeny over the total number of wells seeded

c

Haematopoietic / endothelial progeny from single cells



d

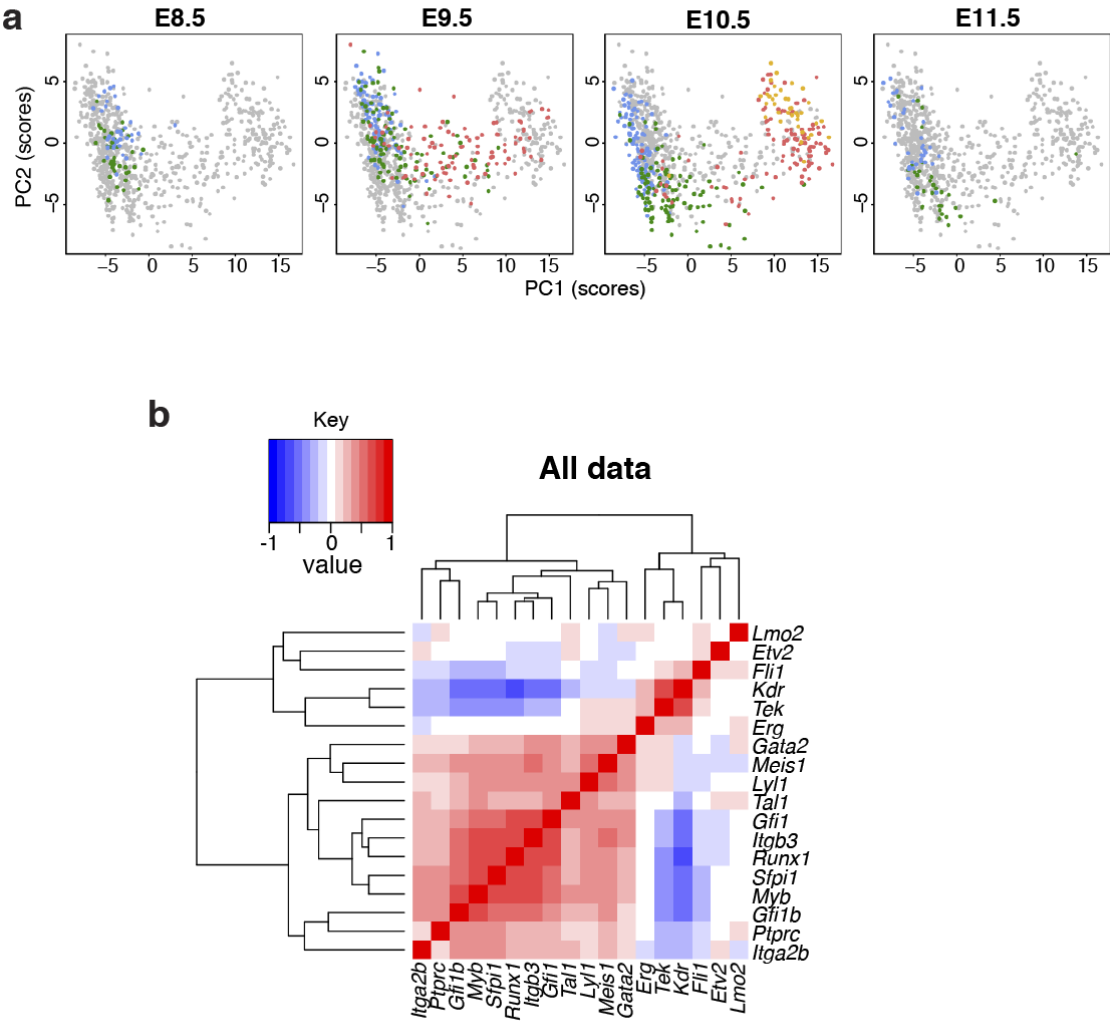
Time lapse imaging of E7.5 Flk1^{+} VE-Cadh$^{-/lo}$ CD41^{-} cells

Subset	No. events plated	Hematopoietic colonies
23GFP^{+}	3000	55 (1:54)
23GFP^{-}	6000	4* (1:1500)

Tracking of individual cells was done up to day 4 of the 7-day cultures. *Two colonies expressed the 23GFP transgene.

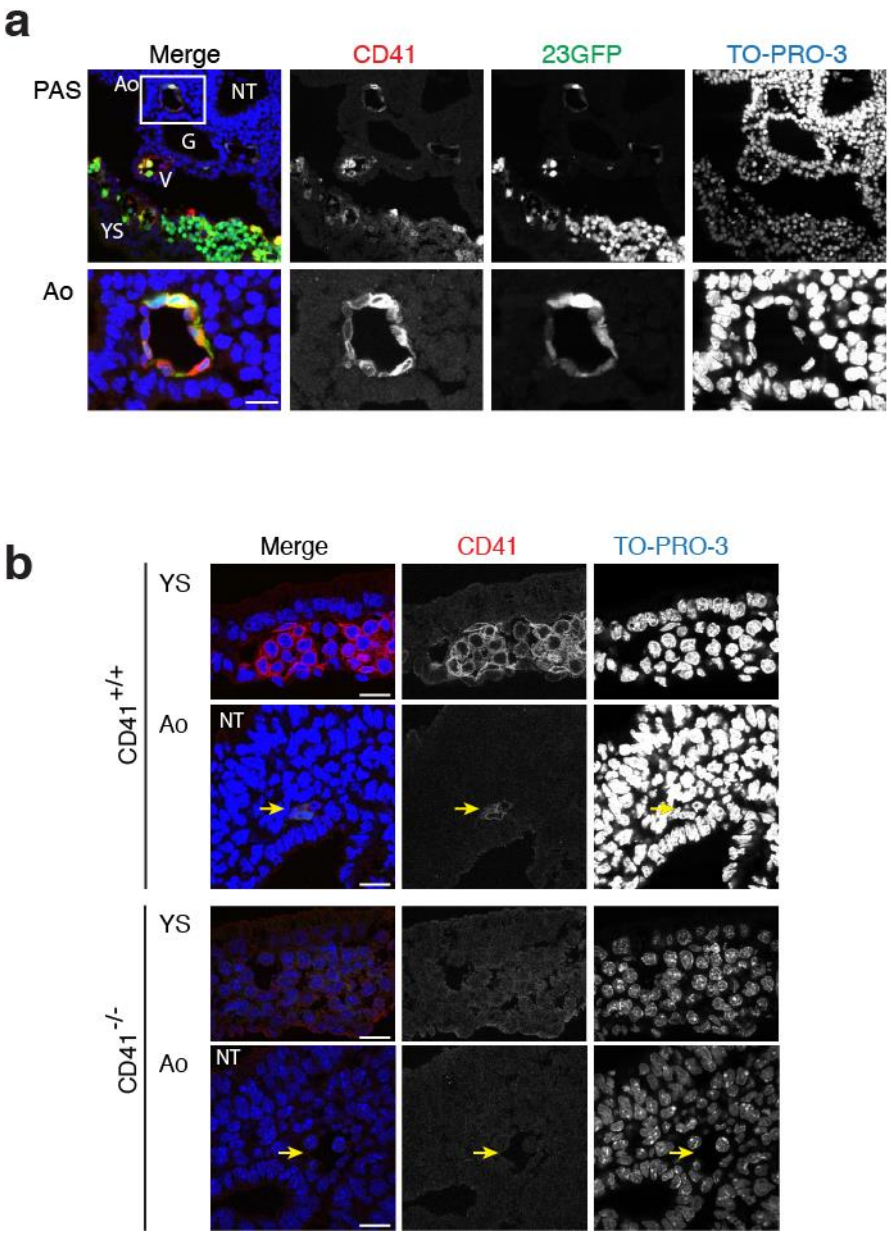
Supplementary Figure S3. Haematopoietic and endothelial progeny can be generated from single E7.5 Flk1^{+}VE-Cadh$^{-/lo}$CD41^{-} 23GFP^{+} mesodermal cells. (a) Representative sort plots and re-analysis for E7.5 mesoderm. Numbers indicate the average percentage ± SD of

events within the sort gates from 7 independent experiments. Representative post-sort reanalysis percentages are shown. (b) Frequency of cells with haematopoietic, endothelial and combined haematopoietic and endothelial potential in cultured 23GFP$^+$ and 23GFP$^-$ Flk1$^+$VE-Cadh$^{-/lo}$CD41$^-$ mesoderm populations after 4 days co-culture on OP9 stroma. Shown are the frequencies of, and number of wells positive for haematopoietic and/or endothelial cells over the total number of wells analyzed. Data are compiled from 2 independent experiments. (c) Representative images of endothelial networks stained by CD31 expression (blue). Scale bar = 50 μm. Arrowhead indicate representative semi-adherent haematopoietic cells. (d) Summary of timelapse imaging of E7.5 cells on OP9 stroma. Cells were recorded for 7 days, and tracked using Timms Tracking Tool for 4 days. The frequency of cells giving rise to haemogenic 23GFP$^+$ colonies is shown. Two of the 23GFP$^-$ sorted cells that gave rise to haematopoietic colonies were GFP$^+$ at the start of the movie.



Supplementary Figure S4. (a) Principal component projections of 77 E8.5 (5-9sp; n=1), 301 E9.5 (23-28sp; n=2), 356 E10.5 (31-36sp; n=3 except CD45$^+$ haematopoietic cells n=1) and 69 E11.5 (n=1) single cells. Data from the other developmental stages are greyed out for clarity. (b) Pair-wise Pearson correlations were calculated between all genes for all cells at all time points. Positive correlations (red) occur when genes are co-expressed (or not expressed) in individual cells whereas negative correlations (blue) result from the expression of one gene and the absence/reduced expression of the other gene in the same cell.

Swiers et al. Figure S5



Supplementary Figure S5. (a) CD41 immunostaining (red) and +23GFP (green) expression on 10 µm cryosections through the posterior region of an E8.5 (12 sp) +23GFP transgenic embryo. Individual channels and merged image are shown. Boxed region is shown at higher magnification in lower panels. V = vitelline, Ao = dorsal aorta, YS = yolk sac , G = gut, NT = neural tube. Nuclear stain in blue, scale bar = 20 µm. (b) CD41 immunostaining (red) and nuclear stain (blue) on 10 µm sections of the YS and posterior region of the dorsal aorta of E8 (4sp) $CD41^{+/+}$ and $CD41^{-/-}$ embryos[57]. No CD41 expression can be detected in $CD41^{-/-}$ embryos (arrow); stage-matched wildtype embryos clearly show CD41

expression in the YS and dorsal aorta (arrow). NT = neural tube, nuclear stain in blue, scale bar = 20 μm.

Supplementary Tables

Supplementary Table S1. Antibodies and Conjugates used in this study

Antibody	Fluorochrome	Clone	Supplier	Catalogue number	Dilution
CD31	purified	MEC13.3	BD Pharmingen	553370	1:1000
Goat anti rabbit IgG (H+L)	Alexa Fluor 555		Life Technologies	A21428	1:200
Goat anti rat IgG (H+L)	Alexa Fluor 555		Life Technologies	A21434	1:200
Goat anti Rat IgG AP			Southern biotech	3050-04	1:250
Runx1			gift Jessel lab		1:1600
ToPro-3			Life Technologies	T3605	1:5000
CD41	purified	MWReg30	BD Pharmingen	553847	1:40
VE-Cadherin	purified	11D4.1	BD Pharmingen	555289	1:40
CD19	APC	1B3	BD Pharmingen	550992	1:100
CD41	PE	MWReg30	BD Pharmingen	558040	1:100
CD45	APCCy7	30-F11	BD Pharmingen	557659	1:100
	APCeFluor780		ebioscience	47-0451	1:100
	APC		BD Pharmingen	559864	1:100
	PE		BD Pharmingen	553081	1:100
Flk1	APC	Avas 12alpha1	BD Pharmingen	560070	1:100
Gr1	biotinylated	RB6-8C5	BD Pharmingen	553125	1:400
Hoechst 33258			Life Technologies	H3569	1:10,000
Mac1	PE	M1/70	BD Pharmingen	557397	1:100
Streptavidin	PECy7	SA1012	Life Technologies	SA1012	1:100
Ter119	biotinylated	TER119	BD Pharmingen	553672	1:100
VE-Cadherin	Alexa Fluor 647	ebioBV13	ebioscience	51-1441	1:100

Supplementary Table S2. Cytokines used in this study

Cytokine	Working Concentration	Supplier	Catalogue Number
Il7	50 U/ml	Peprotech	217-17
Flt3l	10 ng/ml	Peprotech	250-31L
SCF	10 ng/ml	Peprotech	250-03
VEGF	50 ng/ml	Peprotech	450-32

Supplementary Table S3. Taqman assays used in the study

Gene	Gene Name	Assay number	25 cell	Single cell
Atp5a1	**Atp5a1**	Mm00431960_m1	x	x
Atp5a1	**Atp5a1**	Mm01257366_m1		x
ActB	**Bactin**	Mm00607939_s1	x	
Cbfb	**Cbfb**	Mm00491551_m1		x
VE-Cadherin	**Cdh5**	Mm00486938_m1		x
Erg	**Erg**	Mm01214246_m1		x
Etsrp71	**Etv2**	Mm00468389_m1		x
Fli1	**Fli1**	Mm00484409_m1		x
Gata2	**Gata2**	Mm00492300_m1	x	x
Gata3	**Gata3**	Mm00484683_m1	x	
Gfi1	**Gfi1**	Mm00515855_m1	x	x
Gfi1b	**Gfi1b**	Mm00492318_m1		x
hHex	**Hex**	Mm00433954_m1		
Hrpt1	**Hprt1**	Mm00446968_m1	x	
		Mm01545399_m1		x
CD41	**Itga2**	Mm00439768_m1		x
CD61	**Itgb3**	Mm00443980_m1		x
Flk1	**Kdr**	Mm01222421_m1		x
Lmo2	**Lmo2**	Mm01281680_m1		x
Lyl1	**Lyl1**	Mm00493219_m1		x
Meis1	**Meis1**	Mm00487664_m1	x	x
C-Myb	**Myb**	Mm00501741_m1	x	x

Supplementary References

56. Mirshekar-Syahkal, B. *et al.* Dlk1 is a negative regulator of emerging hematopoietic stem and progenitor cells. *Haematologica* **98**, 163-171 (2013).
57. Emambokus, N.R. & Frampton, J. The glycoprotein IIb molecule is expressed on early murine hematopoietic progenitors and regulates their numbers in sites of hematopoiesis. *Immunity* **19**, 33-45 (2003).