ARTICLE - Red Cell Biology & its Disorders

Stem cell factor and erythropoietin-independent production of cultured reticulocytes

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Supplementary methods:

Culture conditions: All cultures were performed at 37C, in 7% CO_2 and in 5% oxygen.

Pluripotent stem cell culture: hiPSCs were maintained undifferentiated in chemically-defined conditions essentially as described by Chen et al. using E8 medium (1). Cells were grown on Vitronectin (life technologies) and passaged using EDTA every 3-4 days depending on their confluence stage.

Differentiation of iPSCs into erythroid cells using the PSC-RED protocol: Composition of the IMIT and R6 media and of all supplements are provided in **Tables S1 and S2**.

On day -1: Three-day-old iPSC colonies were dissociated with 5mM EDTA to generate small clumps that yield colonies of about 50 cells on day 0. Clumps were plated at 1–2 × 10^5 cells/well in 2mL/well of E8 medium on vitronectin-coated six-well plates and allowed to attach overnight.

On day 0: Differentiation was induced by replacing the E8 medium with IMIT medium containing **supplement 1**.

On day 2, 6x concentrated **supplement 2** was added.

On day 3, the cells were dissociated with Tryple-Select, centrifuged, and plated in IMIT medium containing **supplement 3** at 1x10^5 cells/mL in a 6-well plate (3mL/well).

On day 6, the cells were centrifuged and plated at 5.10^5/mL in IMIT medium containing supplement 3 excluding SB431542 but including 30nM of UM171 (or with UM729 at 500 nM). An additional dose of supplement 3 (provided from a 6x concentrated stock) was added on day 8.

On day 10, the cells were centrifuged and re-suspended at 0.66 × 10^5 cells/mL in IMIT medium containing the STIF supplement.

On day 17, the cells were centrifuged and plated at 2 × 10^5 cells/ mL in IMIT plus GM-CSF and the SED supplement.

Note: cells obtained on day 17 (i.e., day-17 HPCs) from kit or kitjak2 mutant lines give rise to self-renewing erythroblasts when cultured as described in the self-renewing erythroblasts section.

On day 24, the cells were centrifuged and plated at 2 × 10^5 cells/mL in IMIT containing the SER supplement. RU486, a Dex antagonist, is included in the SER supplement to block residual traces of Dex

On day 31, the cells were centrifuged and plated in R5 medium containing the SER2 supplement.

On day 38, the cells were centrifuged diluted to $0.5x10^6$/mL and maintained in pure R6 medium for up to 8 days.

Cells cultured between days 6 to 38 were diluted to $0.5x10^6$/mL in the appropriate media and supplement whenever their concentrations reach more than about $1.5x10^6$cells/mL. Cytokine concentrations between days 6 and 38 were refreshed by addition of 6x concentrate of the appropriate supplement every other day. 6x concentrates of each supplements were made by multiplying by 6 the concentrations of cytokines and small molecules indicated in Table S2. Concentrated supplements were used to keep the concentration of cytokine and small molecules high enough at all times without having to spin the cells and without overly increasing the cell culture volume.

1x and 6x concentrates of supplements 1, 2, 3, 4, STIF, SED and SER were prepared in IMIT. SER2 supplement was prepared in R6. All centrifugations were performed at 250g for 5 to 10 minutes (depending on volume). All culture vessels used were tissue culture-treated. Experiments were generally performed in 6-well plates, but flasks were used to obtain larger volumes.

HPLC analysis: Cells were washed twice with PBS and lysed in water by 3 rapid freeze-thaw cycles in dry-ice and in a 37C water bath. Debris was eliminated by centrifugation at 16 000g and the lysates stored at -80°C. HPLC were performed as described by Fabry et al. (2) Briefly, a few mL of lysate containing about 50 μg of protein in about 100 μL of 40% acetonitrile and 0.18% TFA was filtered and loaded on a VYDAC C4 column. The globins were then eluted with increasing concentration of acetonitrile during a period of about 80 minutes. The starting elution buffer was programmed to be 80% buffer A and 20% buffer B and to rise to 50% buffer B in 50 minutes. Buffer A = 36% acetonitrile and 0.18% TFA and buffer B = 56% acetonitrile and 0.18% TFA. Globin chain elution was monitored by measuring O.D. at 220 nm.

CRSPR editing: A nucleic acid solution containing 1ug of capped, polyadenylated cas9 mRNA substituted with modified Uridine (CLEAN-Cap cas9 mRNA, cat # L-7206 from Trilink Biotechnologies) were mixed with 4 ug of synthetic sgRNA (Sigma technologies) and 4μg of a 200-mer oligonucleotides HDR donor in 15 ml of H20. About 150,000 iPSCs dissociated with Accutase were resuspended in 15 ml of buffer P (100mM phosphate buffer pH 7.4; 15mM $NaHCO_3$; 2 mM glucose, 12mM $MgCl_2$) and mixed with 15 ml of the nucleic acid solution in a 1 cm 1/16 of an inch inner diameter electroporation chamber made of platinum-cured silicone tubing. The tube was then subjected to two 10 msec pulses of 115V using a NEPA21 (Nepagene) electroporator hooked up to Cell-Porator Voltage Booster (Life technology) resulting in a 600V pulse. Subclones were then isolated and screened for the presence of the desired mutations by amplifying and sequencing PCR fragments representing the regions of interest. In some experiments, the PCR fragments were pre-screened by digestion with an appropriate restriction enzyme. To obtain heterozygous clones, donor HDR oligonucleotides coding for

the wt sequences were mixed-in with the mutated donor HDR oligonucleotide. Sequences of the sgRNA and HDR donor DNA are provided in **Table S4**.

Methyl-cellulose assays: Assays were performed as suggested by the manufacturer using MethoCult™ SF H4636 (Stem Cell Technology, Vancouver, CA). For each replicate cells were tested in duplicated humidified 35mm dishes. Colonies were counted on day 14.

Cell morphology: Erythroid differentiation and enucleation were also assessed microscopically by Rapid Romanowsky staining (3) of cytospin preparations using the HEMA-3 kit from Fisher Scientific according to the manufacturer instructions. Cell sizes were estimated on a Nikon TE-2000S microscope using the NIS Element F software provided by the manufacturer.

RBC filtration: 99.5% pure populations of enucleated RBCs were generated by filtration of the cells obtained after 10 days of differentiation of self-renewing erythroblasts through PAL Acrodisc 25mm WBC filters as recommended by the manufacturer. Filtered cRetics were stored for up to one month with little signs of hemolysis in Alsever's solution (Sigma).

Cell enumeration and viability: Cells were counted with a Luna-FL dual channel Automated Cell Counter (Logos) using acridine orange to visualize the live cells, and propidium iodide to exclude the dead cells. Alternatively, cells were counted using a Cytek Aurora flow cytometer. Apoptosis was detected by staining with Annexin V-FITC and DAPI.

Enucleation: The enucleation rate was measured using the DRAQ5 DNA nuclear stain (ThermoFisher) after exclusion of dead cells with Propidium Iodide or DAPI. The cells were analyzed with a Cytek Aurora flow cytometer and the FlowJo software.

Supplementary figure legends

Figure S1: Medium requirement

A: During the PSC-RED protocol the cell number doubles on average every 48 hours, leading to > 200,000-fold amplification.

B: Graph illustrating medium consumption during the 38 days of the PSR-RED protocol to produce about 5.10^9 RBCs (the number of RBCs present in 1 mL of blood), in low density cell culture (< $2x10^6$/mL). More than 99% of the cell culture medium is consumed after day 37 during the PSC-RED protocol. Increasing the cell culture density helps decrease the volume of culture medium, but not the amount of cytokines, because cytokines are generally internalized and are not recycled.

Figure S2: Insertion of the D816V mutation in the kit gene using CRSPR/cas9

Top: Three nucleotide substitutions were introduced into the donor oligonucleotide DNA. A C to G transversion which destroys the PAM was introduced to increase the overall rate of mutation by preventing re-mutation of CRSPR modified alleles after homologous recombination. Two nucleotide substitutions were also introduced to change codon 816 from GAC (ASP) to GTG (valine). Complete sequence of the donor oligonucleotide is provided in the methods section.

Middle: Chromatograms illustrating the sequences of clone B34 and A4 which were selected for further studies.

Bottom: Protein sequence of the alleles that we generated. Clone B34 is homozygous for the D816V mutation. Allele A of clone A4 carries the D816V allele. Allele B contains a 1 base insertion that leads to a frame shift at position 816 resulting in a stop codon at position 820.

Figure S3: Romanowsky staining of IPSCs differentiated according to the PSC-RED protocol:

iPSCs O1 (controls) were differentiated according to the PSC-RED protocol. iPSCs A4 (kitD816V hemizygous) and B34 (kitD816V homozygous) were differentiated with the same protocol but SCF was omitted at all steps. About 100,000 cells were collected at days 10, 17, 24, 31, 38 and 45, cytospined on poly-lysine treated slides, stained using the Romanowsky protocol. Cells progressively differentiate into mature erythroid cells.

Figure S4: Flow cytometry analysis of control, A4 and B34 iPSCs during the PSC-RED protocol

A: A 15-color flow cytometry assay was used to monitor erythroid differentiation using the PSC-RED protocol. Cells were segmented into 8 major populations according to expression of 10 markers using flowSOM. Heatmap illustrates the expression of the 10 markers used to define the 8 populations. The scatterplots illustrate the evolution of each population. The Ery3 and Ery4 populations become dominant much earlier in the A4 and B34 clones.

B: The same experiment was repeated on later passage iPSCs with new lots of antibodies. FlowSOM was used using the same parameters to segment the data into 8 cell populations. As in A, populations of erythroid cells arise earlier in the kitD816V mutated clones.

C: Dotplots illustrating expression of 11 markers during PSC-RED erythroid differentiation of iPSC O1 (grown in the presence of SCF and Epo) and of iPSC clones A4 and B34 (both grown in the absence of SCF).

Figure S5: Methyl cellulose assays

iPSCs O1 (controls) were differentiated according to the PSC-RED protocol. iPSCs A4 (kitD816V hemizygous) and B34 (kitD816V homozygous) were differentiated with the same protocol but SCF was omitted at all steps. Respectively, 1,500, 3,000 and 10,000 cells were collected at days 10, 17, 24 and plated in duplicate into methyl-cellulose. Colonies were counted after 14 days of incubation. Histograms summarize the results of two experiments. The total number of colonies decreases from day 10 to day 24. No significant differences in the myeloid potential between the cells derived from the O1, A4 and B34 iPSC lines was detected.

Figure S6: D816V heterozygous clones

A: iPSCs O1 and O2 were transfected with cas9 mRNA, an sgRNA and two oligo donors' DNA: a mutated and an unmutated oligo. MboI was used to identify 96 clones having at least one V816 allele. For both O1 And O2 about 20% of the clones were positive for the MboI restriction sites. Of those, about half were heterozygous for the D816V mutation. Top line: wild type genomic sequence; middle: donor oligonucleotide coding for a D at position 816. Bottom line: mutated donor oligonucleotide coding for a V at position 816. Bottom chromatogram illustrates the sequence of one of the heterozygous clones selected for further studies.

B. Representative growth curves illustrating the proliferation of clones heterozygous for the D816V mutation isolated from donors O1 (clone D14) and O2 (clone CP) during the PSC-RED protocol (n=2) as compared to clone A4.

C: Bargraph illustrating enucleation rate of the same clones as evaluated by staining with Draq5 (n=2).

D: Dotplots illustrating expression of 11 markers during PSC-RED erythroid differentiation of kit D816V iPSC A4, O1 and O2 in the absence of SCF.

Figure S7: kitD816V SREs grown in DI conditions (Dex and IBMX but no cytokines).

A: Day-17 HPCs derived from the A4 and B34 iPSCs lines were differentiated according to the PSC-RED protocol omitting SCF and placed in culture with only Dex and IBMX (DI conditions) to assess their self-renewal potential. In parallel experiments, the day-17 HPCs were differentiated into mature erythroid according to the PSC-RED protocol until day 45 (omitting SCF). Flow cytometry using a 15-color assay was performed weekly between day 17 and day 52 on the cells cultured in DI conditions. the A4 and B34 day-17 HPCs downregulated CD49f, CD45, CD38 and CD34 and upregulated expression of CD36, CD71, and CD235a (Figure S7A). This transition from an HPC to an erythroblast phenotype, resembling iPSC-derived late CFU-Es and pro-erythroblasts, was almost completed by day 24. After that time point the antigen profiles of both the A4 and B34 cells remained constant until day 45.

B: Romanowsky staining supported these findings. Day-17 HPCs grown in DI condition exhibit a CFU-E/pro-erythroblast morphology at day 45.

Figure S8: Viability of the A4 and H5 SREs:

A4 and H5 SREs were cultured in DI conditions for about 135 days. The blue curve illustrates the cumulated cell number. The pink curve the viability assessed by propidium iodide staining. The viability was very high, generally 98 to 99% until senescence. A dip of the viability to about 90% was observed whenever the cells were allowed to reach concentrations above 2.10^6 cells / mL.

Figure S9: Production and FACS analysis of the jak2V617F mutant

A: Design of the donor oligonucleotide: Sequence of the wild-type (wt) and modified sequences.

B: Chromatograms illustrating the sequence of the region around jak2 position 657 in A4; H5 and H12 cells.

Ther A4 line has no modification at this position (homozygous 657V); H12 is homozygous for 657F and H5 is heterozygous for 657F and for a 2 amino acid deletion at position 655 and 656.

C: Dotplot illustrating expression of 11 markers during PSC-RED erythroid differentiation of iPSCs A4, G19 and H5 in the absence of SCF and Epo.

Figure S10: Flow cytometry analysis of the SREs

A: Dotplots illustrate the expression of 11 markers of A4 D816V erythroblasts that had been in culture for 93 days. CD90, 49f, 38 and 34 are barely expressed; CD31 and 45 are expressed at low levels; CD43, 36, 71 and 235a are expressed at high levels in almost all cells. CD117 (the SCF receptor) is expressed at a high level but only in about two-thirds of the cells.

B: left: Bargraph illustrating the expression of 11 markers in kitjak2 lines G19 and H12 (n=2). Right: HUDEP-2 cells analyzed with the same antibody panel express much higher levels of CD34, 90 and 45, more consistent expression of CD117 and lower expression of CD235a. HUDEP-2 are blocked at an earlier stage of differentiation than the kitjak2 cells.

C: Romanowsky staining of the A4, H5, H12 and H12 SREs grown in DI conditions for about 100 days. Cells have very similar morphology during their self-renewing period. See figure S7 for micrograph of the A4 cells at an earlier passage.

Figure S11: SRE differentiation

A: H5 SREs grown in DI conditions were differentiated as described in Figure 5A and expression of CD36 and CD71 was analyzed by flow cytometry at days 3, 6, 9 and 11. Expression of CD36 and CD71 decrease over time.

B: Micrographs illustrating the morphology of differentiated H5 SREs at day 3, 5, 7 and 9. As expected from the FACS analysis, the cells exhibit the morphology of increasingly mature erythroid precursors.

C: Scatterplot illustrating the average ± S.D. (n=3) of cell growth and viability during A4 SREs differentiation assessed after acridine orange and propidium iodide staining.

D: Apoptosis analysis: differentiating A4 SREs were analyzed by FACS after staining with DAPI and annexin V-FITC. Bargraph illustrates the average ± S.D. (n=3) percentage of apoptotic cells at days 3, 5, 7 and 10.

E: Dotplots illustrating the expression of CD235a, CD43, CD36 and CD71 during A4 SREs differentiation. The pattern of differentiation of the A4 SREs is very similar to that of the H5 SREs.

Figure S12: Apoptosis analysis

Differentiating H5 SREs were analyzed by FACS after staining with DAPI and annexin V-FITC. Doplots illustrate the results. Data are summarized in Figure 5E.

Figure S13: Late passage SRE differentiation

A: Graphs illustrating the enucleation rates of the A4, H5, G19, H11 and H12 SREs observed at various passage numbers. SREs retain their ability to enucleate at a high rate even when close to senescence. The blue and green dots with a black outline were fed every two days during the differentiation.

B: Romanowsky stain illustrating the morphology of mature erythroid cells and cRetic obtained after 11 days of differentiation from early or late passage A4 SREs (top row). The two bottom rows illustrate the result of slightly larger scale differentiation experiments in which we purified the cRetics on day 11.

C: FACS analysis illustrating typical morphology (FSC-H and SSC-H) and draq5 analysis at the end of the differentiation.

Figure S14: copy-number analysis by low-pass sequencing

Genomic DNA was sequenced at a depth of 1 to 2 x and analyzed using the CNVkit software. Graphs illustrate that PB MNCs O1 and O2, passage 38-40 iPSC O1, A4, H5, H12, G19 (derived from iPSC O1), and passage 28 iPSCs O2 D816V exhibit no detectable copy number variants greater than 1Mb (the limit of detection for this read depth). By contrast, transformed cells, HUDEP-2 and K562 cells, cultured for a long period of time exhibit a high level of aneuploidy.

IMIT
IMDM with 1mM Glutamine
Methyl-β-Cyclodextrin 0.1mg/mL
Trolox 50μM
Insulin 10μg/mL
Optiferrin 20 μg/mL
FeIII-EDTA 4μM
Gibco Lipids (1.5X)
Ethanolamine 0.01%
R5
RPMI 1640
L-ascorbic acid 220 μM
Insulin 10μg/mL
Optiferrin 20 μg/mL
FeIII-EDTA 4μM

Table S1: Media composition

Day 1	2	3 to 10	10 to 17	17 to 24	24-31	31 to 38
S1	**S2**	**S3**	**STIF**	**SED**	**SER**	**SER 2**
Activin A 5ng/mL	Activin A 5ng/mL					
Inhibitor VIII 2µM	Inhibitor VIII 2µM					
Wnt3A/5A 5ng/mL each	Wnt3A/5A 5ng/mL each					
BMP4 10ng/mL	BMP4 20ng/mL	BMP4 20ng/mL				
VEGF 10ng/mL	VEGF 30ng/mL	VEGF 30ng/mL				
	b-Estradiol 0.4ng/mL	b-Estradiol 0.4ng/mL				
		SB431542 3µM day 3 only				
		Heparin 5µg/mL	Heparin 5µg/mL			
			GM-CSF 20 ng/mL day 17 only			
bFGF 10ng/mL	bFGF 10ng/mL	bFGF 20ng/mL	bFGF 5ng/mL			
	SCF 20ng/mL	SCF 30ng/mL	SCF 15ng/mL	SCF 100ng/mL	SCF 50ng/mL	SCF 10ng/mL
		TPO 10ng/mL	TPO 10ng/mL			
		IGF2 10ng/mL	IGF2 10ng/mL			
		UM171* 30nM day 6 only	UM171* 30nM			
		IBMX 50µM	IBMX 30µM			
				EPO 4U/mL	EPO 4U/mL	EPO 4U/mL
				Dex 1mM		
		*: UM171 can be replaced with 500nM UM729			RU-486 1µM	RU-486 1µM

Table S2: Supplements composition.

Antibody	color	company	cat#
CD11b	PE-CY5	Invitrogen	15-0118-41
CD11c	PE-CY5	Biolegend	301609
CD14	PE-CY5	Invitrogen	15-0149-42
CD15	PE-CY5	Biolegend	323013
CD16	PE-CY5	Biolegend	302009
CD19	PE-CY5	ebioscience	15-0199-42
CD56	PE-CY5	Invitrogen	15-0567-42
CD123	BV605	Biolegend	306026
CD36	AF488	Biolegend	336232
CD71	SB780	Invitrogen	78-0719-42
CD235A	BV421	BD	562938
CD31	BV805	BD	742013
CD34	BUV563	BC	748740
CD38	PE-CY7	Invitrogen	25-0389-42
CD43	BUV395	BD	743616
CD45	APC-Fire750	Biolegend	304062
CD45RA	BV510	BD	563031
CD49f	BV711	BD	740793
CD90	PE/Daz-zle594	Biolegend	328134
CD117	efluro450	Biolegend	313235
CD133	BV480	BD	747837

Table S3: Antibody panel

mutation	type	direction	sequence
kit D816V	sgRNA	antisense	5'AGAATCATTCTTGATGTCTC
jak2V617F	sgRNA	sense	5'AATTATGGAGTATGTGTCTG
kitD816V mutated	HDR donor	sense	5'ACCTAATAGTGTATTCACAGAGACTTGGCAGCCAGAAA-TATCCTCCTTACTCATGGTCG-GATCACAAAGATTTGTGATTTTGGTCTAGCGAGAGTGATCAAGAATGATTCTAATTATGTGGTTAAAGGAAACGTGAG-TACCCATTCTCTGCTTGACAGTCCTG-CAAAGGATTTTTAGTTTCAACTTTCGATAAAAATTGT
kitD816VWT	HDR Donor	sense	5'ACCTAATAGTGTATTCACAGAGACTTGGCAGCCAGAAA-TATCCTCCTTACTCATGGTCG-GATCACAAAGATTTGTGATTTTGGTCTAGCGAGAGACATCAAGAATGATTCTAATTATGTGGTTAAAGGAAACGTGAG-TACCCATTCTCTGCTTGACAGTCCTG-CAAAGGATTTTTAGTTTCAACTTTCGATAAAAATTGT
jak2V617F	HDR donor	antisense	5'CTGAATTTTCTATATAAACAAAAACAGATGCTCTGA-GAAAGGCATTAGAAAGCCTGTAG-TTTTACTTACTCTCGTCGCCGCAAAAGCAGACTCCATAATTTAAAACCAAATGCTTGTGAGAAAGCTTGCTCATCATACTT-GCTGCTTCAAAGAAA-GACTAAGGAAAAAAAAAAGTACAAAGAATTGTTGTTTGACTGTTG

Table S4: Sequence of the sgRNA and HDR donor oligonucleotides used to generate the kitD816V and jak2V617F lines.

Reagent	Provider	Catalog Number
IMDM with 1mM Glutamine	Biochrom	FG0465
RPMI 1640 with 1mM Glutamine	Gibco	61870
StemSpan SFEM	StemCell Technologies	09650
Methyl-β-Cyclodextrin	Sigma	C4555
Trolox	Sigma	238813
Insulin	Sigma	I9218
Chemically defined Lipids 200X	Gibco	11905
Ethanolamine	Sigma	E0135
L-ascorbic acid	Sigma	A8960
Optiferrin	FisherScience	NC9954311
FeIII-EDTA	Sigma	E6760
BMP4	R&D Systems/Biotechne	314-BP
VEGF165	Peprotech	100-20
Wnt3A	R&D Systems/Biotechne	5036-WN
Wnt5A	R&D Systems/Biotechne	645-WN
Activin A	Peprotech	120-14
GSK3β Inhibitor VIII	Calbiochem/EMD Millipore	361549
bFGF	Peprotech	100-18B
SCF	Peprotech	300-07
β-Estradiol	Sigma	E2758
TPO	Peprotech	300-18
IGF1	Alfa Aesar	BT-106
IGF2	Alfa Aesar	BT-107
SB431542	Tocris/Biotechne	1614
UM171	Stemcell Technologies	72912
IBMX	Sigma	I5879
PDGF AB	Peprotech	100-00AB
ANGPTL5	R&D Systems/Biotechne	6675-AN
CCL28	Peprotech	300-57
Heparin	Sigma	H3149
EPO	Amgen	NDC 55513-126-10
Dexamethasone	Sigma	D4902
RU486	Sigma	M8046
FLT3L	Peprotech	300-19
GM-CSF	Peprotech	300-23

Table S5: Reagents

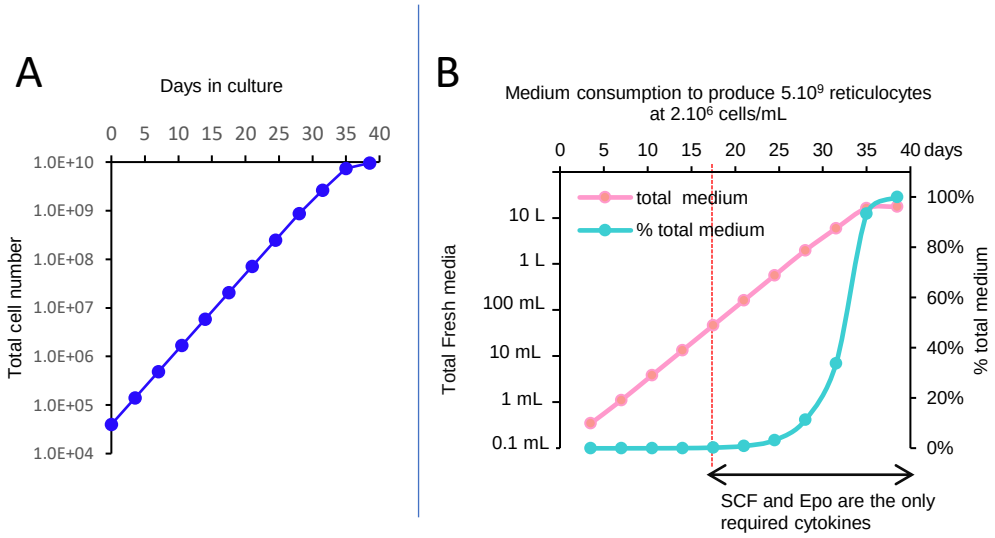

A
Days in culture
0 5 10 15 20 25 30 35 40
1.0E+10
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
Total cell number
B
Medium consumption to produce 5.10⁹ reticulocytes
at 2.10⁶ cells/mL
0 5 10 15 20 25 30 35 40 days
total medium
% total medium
10 L
1 L
100 mL
10 mL
1 mL
0.1 mL
Total Fresh media
100%
80%
60%
40%
20%
0%
% total medium
SCF and Epo are the only
required cytokines

Figure S1

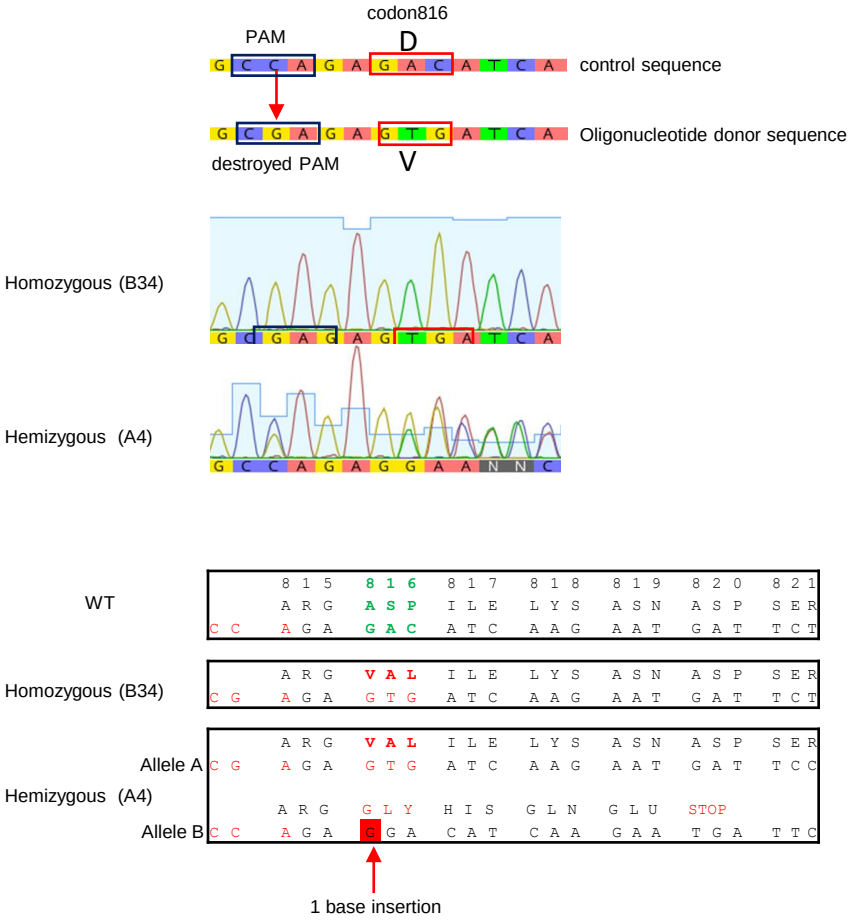
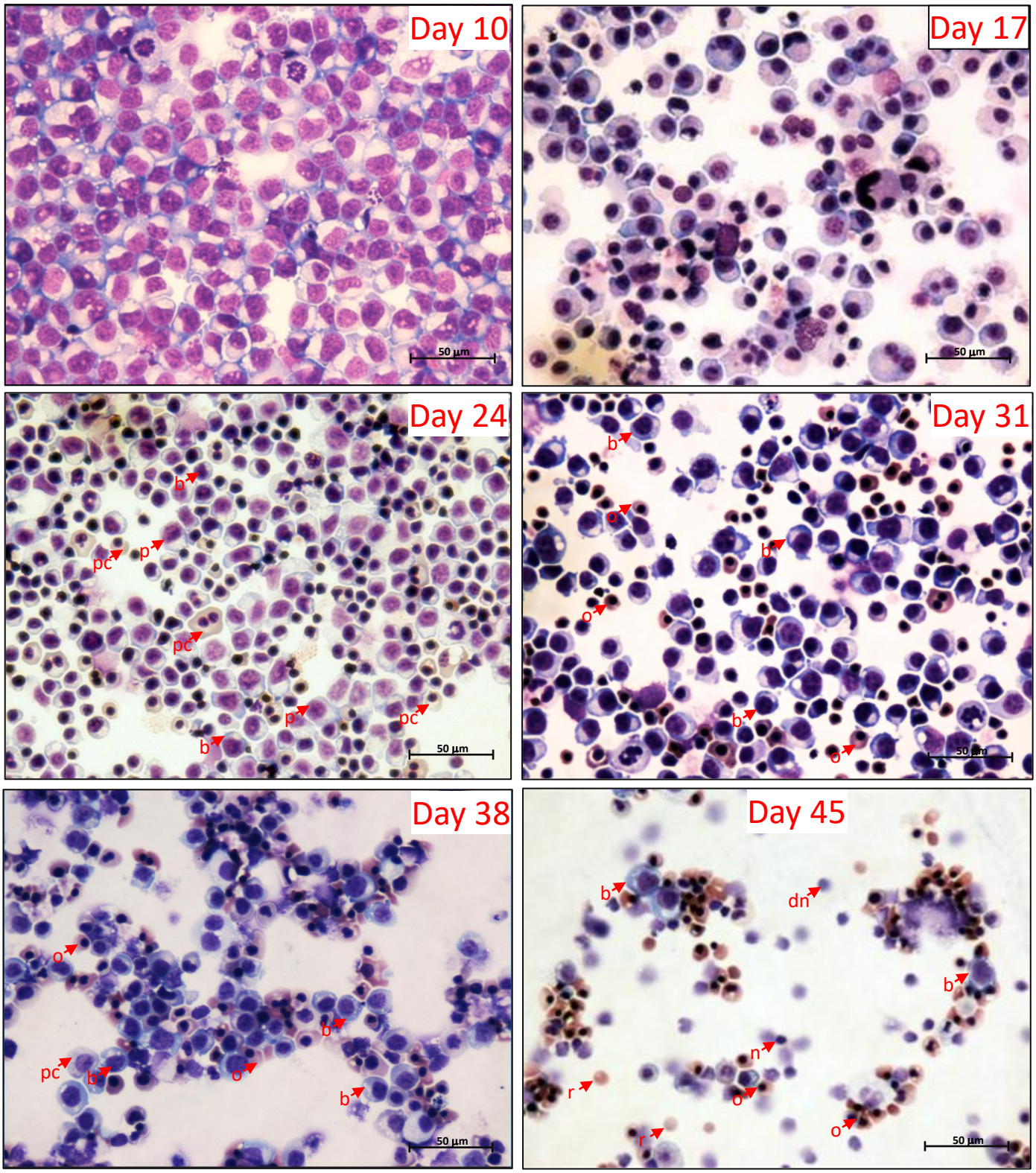

codon816
PAM
D
control sequence
Oligonucleotide donor sequence
destroyed PAM
V
Homozygous (B34)
Hemizygous (A4)
WT
Homozygous (B34)
Allele A
Hemizygous (A4)
Allele B
1 base insertion

Figure S2

Wildtype (donor O1)

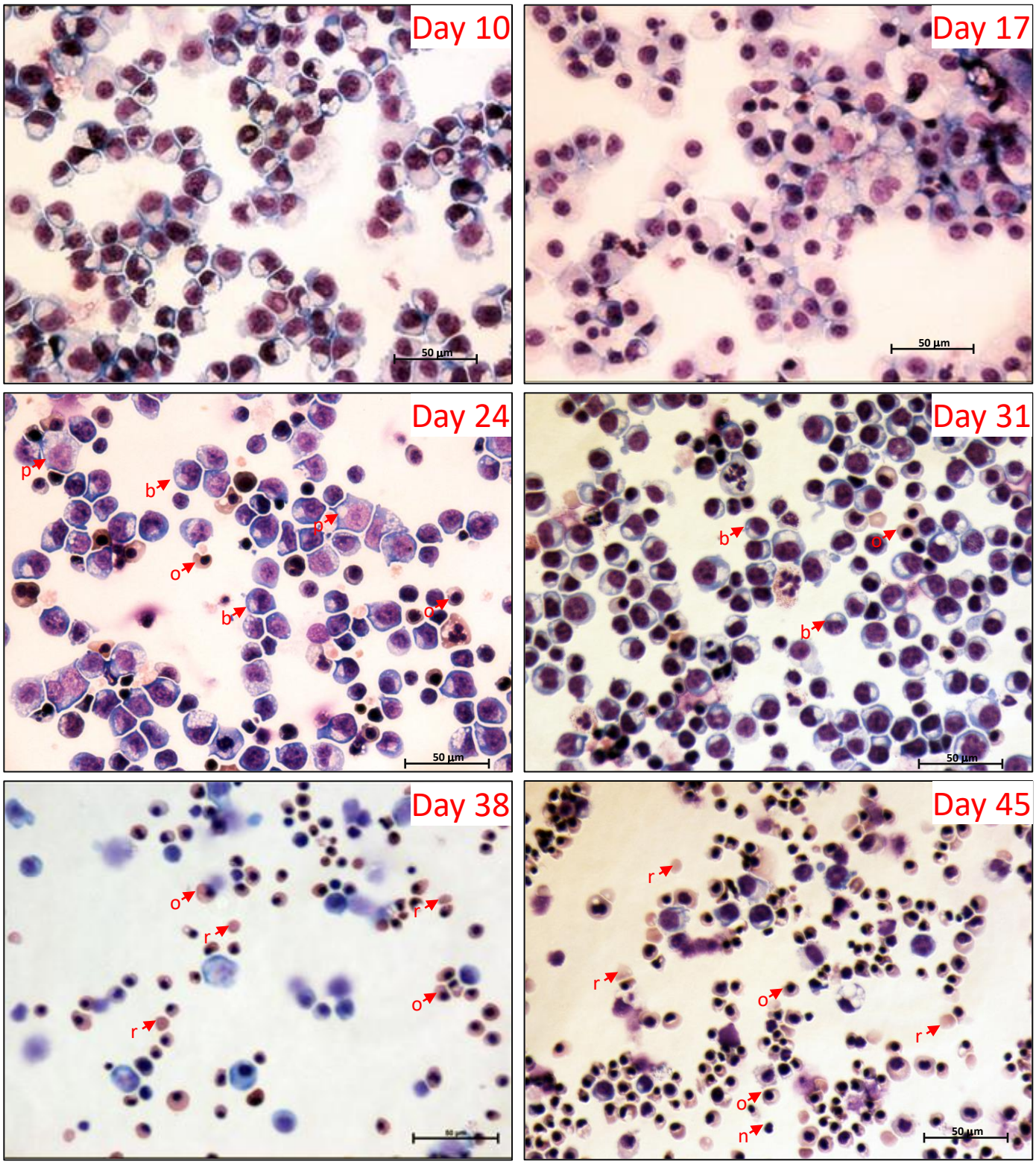


r = reticulocyte o = orthochromatic E pc= polychromatic E

b= basophilic E b= pro E n= nucleus dn= dead nucleus

Figure S3A

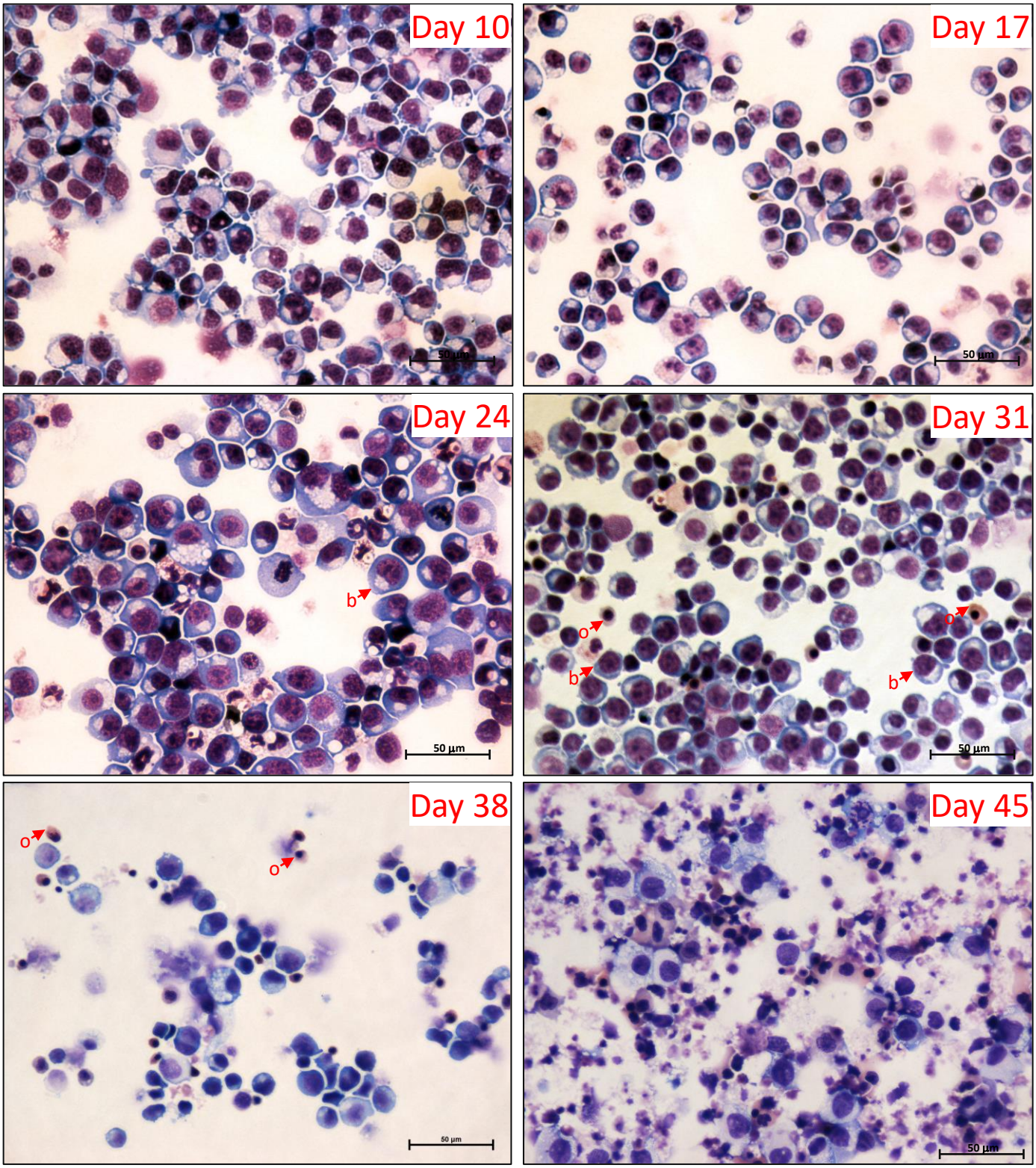
A4 (D816V) hemizygous



r = reticulocyte o = orthochromatic E pc= polychromatic E
b= basophilic E b= pro E n= nucleus dn= dead nucleus

Figure S3B

B4 (D816V) homozygous



r = reticulocyte o = orthochromatic E pc= polychromatic E
b= basophilic E b= pro E n= nucleus dn= dead nucleus

Figure S3C

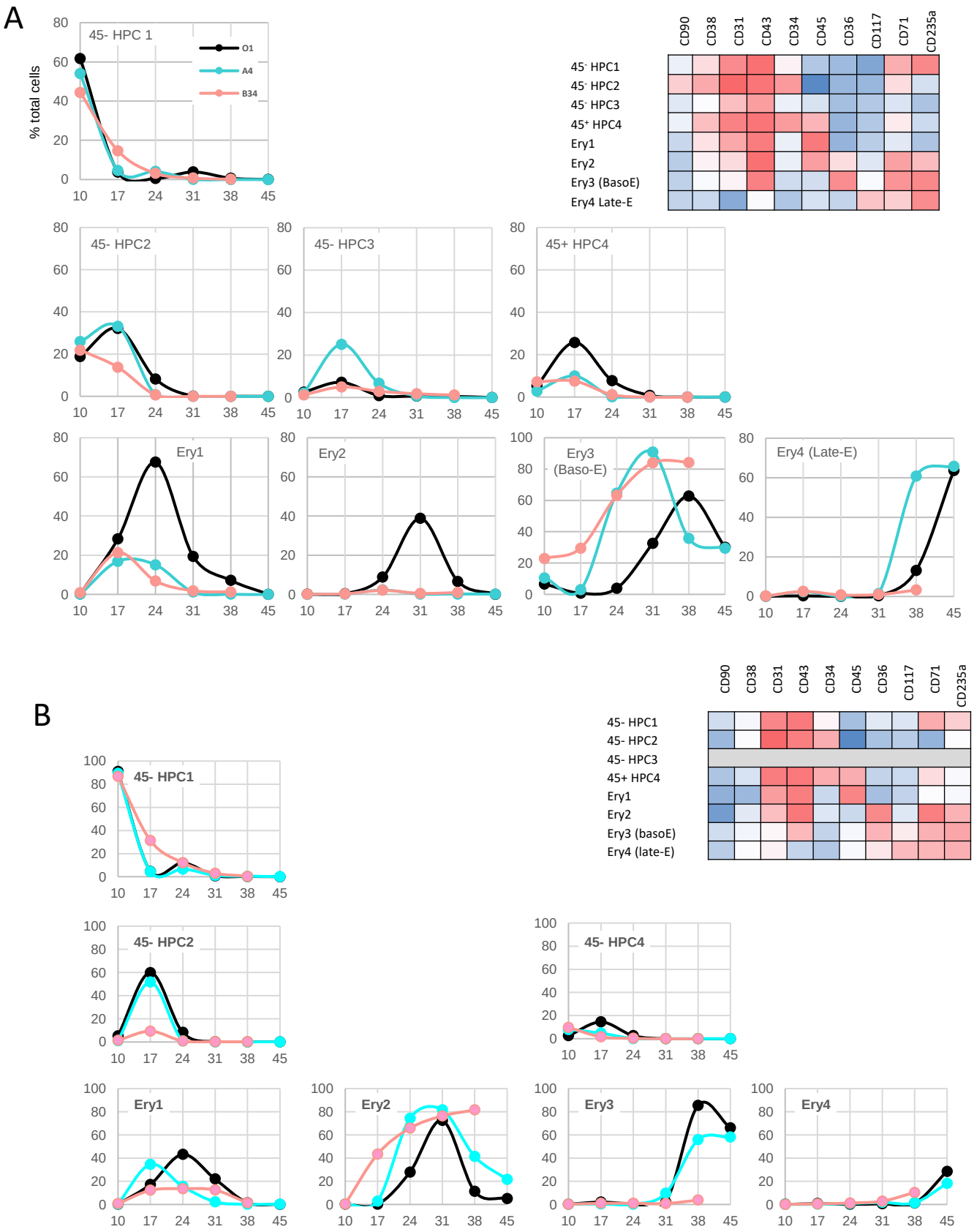

A
% total cells
45- HPC 1
O1
A4
B34
CD90
CD38
CD31
CD43
CD34
CD45
CD36
CD117
CD71
CD235a
45- HPC1
45- HPC2
45- HPC3
45+ HPC4
Ery1
Ery2
Ery3 (BasoE)
Ery4 Late-E
45- HPC2
45- HPC3
45+ HPC4
Ery1
Ery2
Ery3
(Baso-E)
Ery4 (Late-E)
B
45- HPC1
45- HPC2
45- HPC3
45+ HPC4
Ery1
Ery2
Ery3 (basoE)
Ery4 (late-E)
45- HPC4
Ery3
Ery4

Figure S4

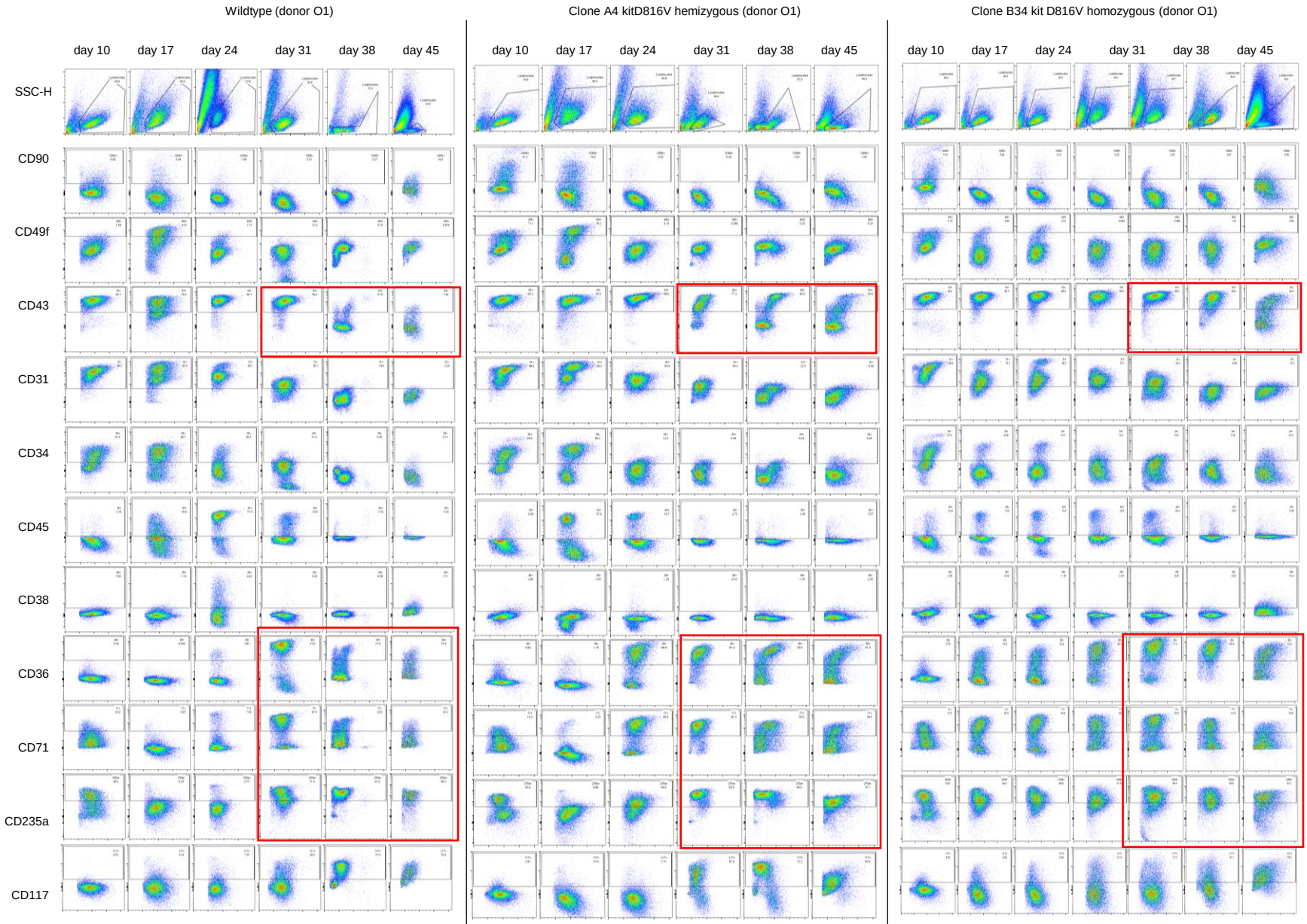

Wildtype (donor O1)
Clone A4 kitD816V hemizygous (donor O1)
Clone B34 kit D816V homozygous (donor O1)
day 10
day 17
day 24
day 31
day 38
day 45
SSC-H
CD90
CD49f
CD43
CD31
CD34
CD45
CD38
CD36
CD71
CD235a
CD117
FSC-H

Figure S4C

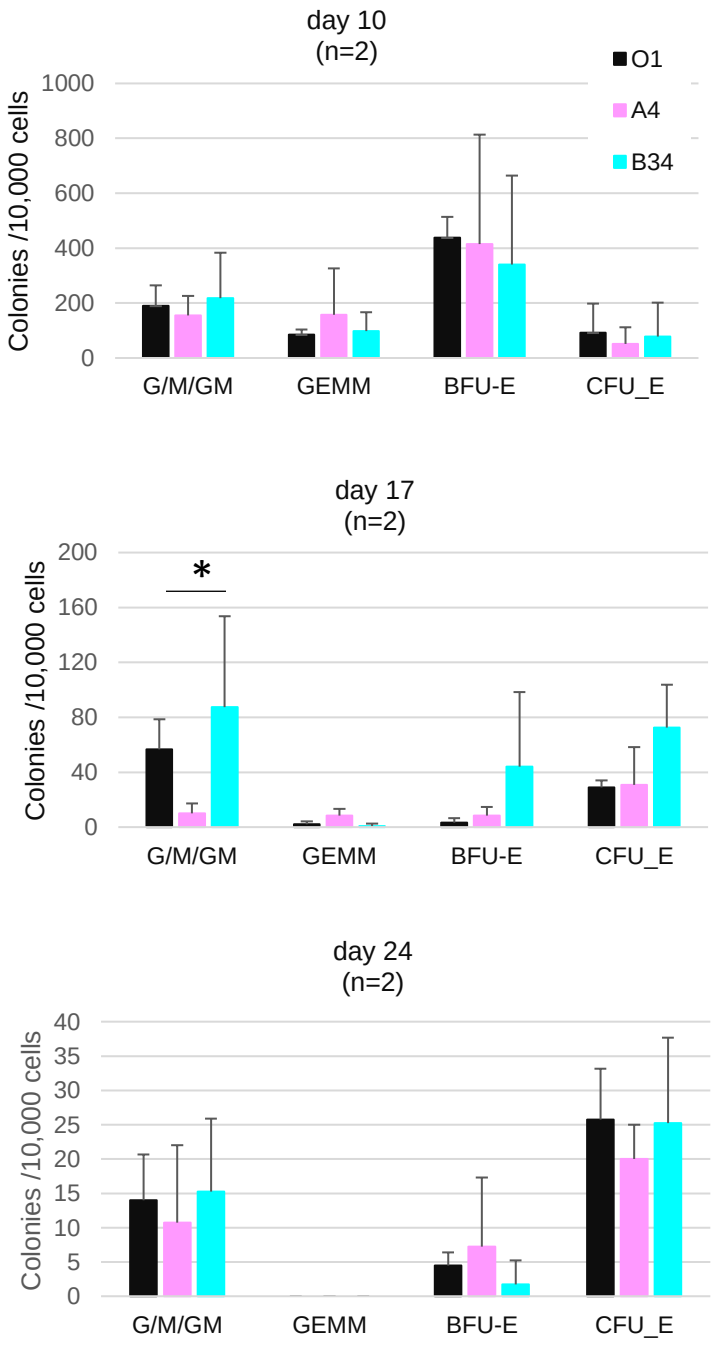
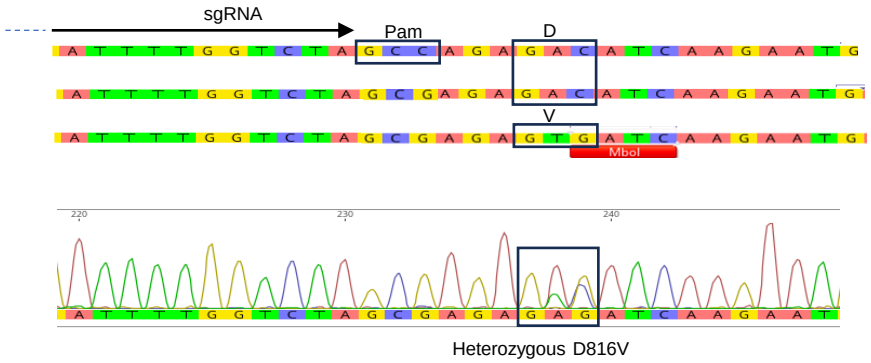
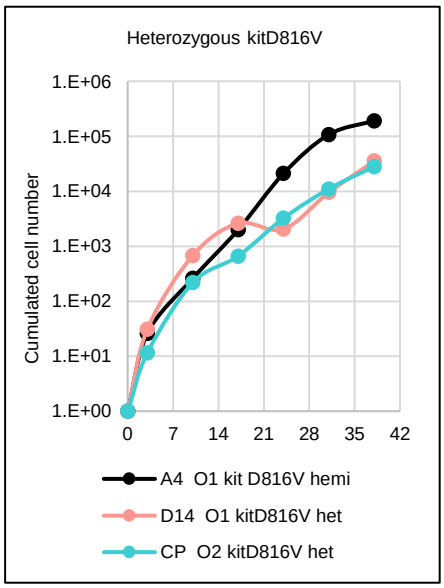

day 10
(n=2)
O1
A4
B34
Colonies /10,000 cells
1000
800
600
400
200
0
G/M/GM
GEMM
BFU-E
CFU_E
day 17
(n=2)
*
Colonies /10,000 cells
200
160
120
80
40
0
G/M/GM
GEMM
BFU-E
CFU_E
day 24
(n=2)
Colonies /10,000 cells
40
35
30
25
20
15
10
5
0
G/M/GM
GEMM
BFU-E
CFU_E

Figure S5

A



B



C

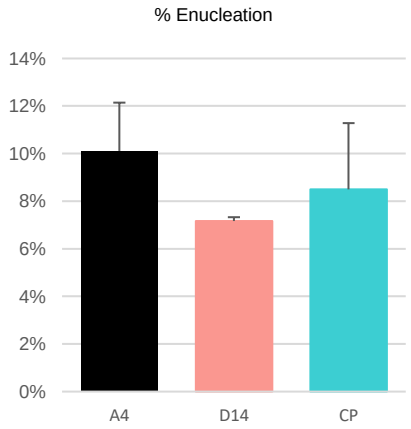


Figure S6

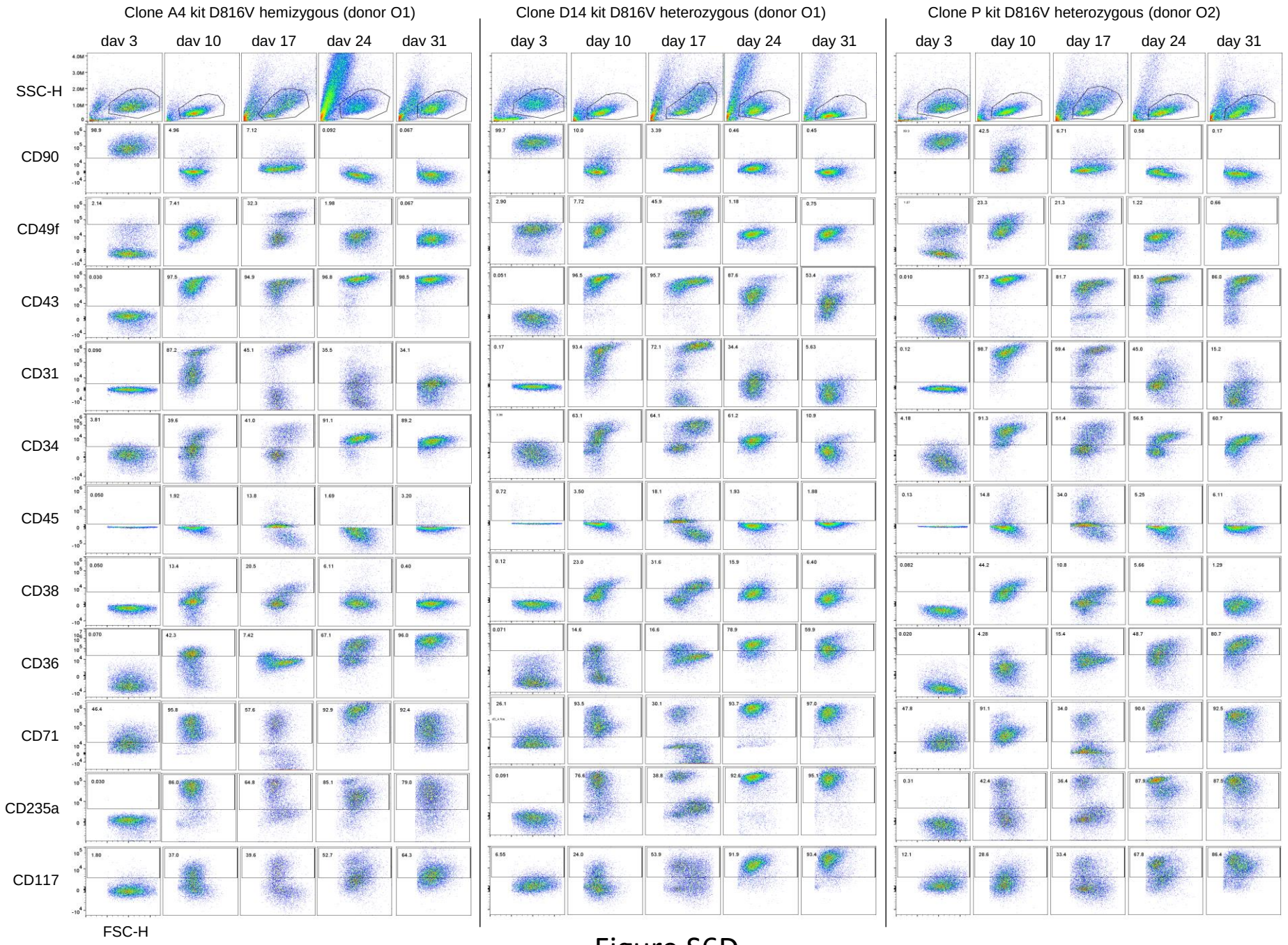
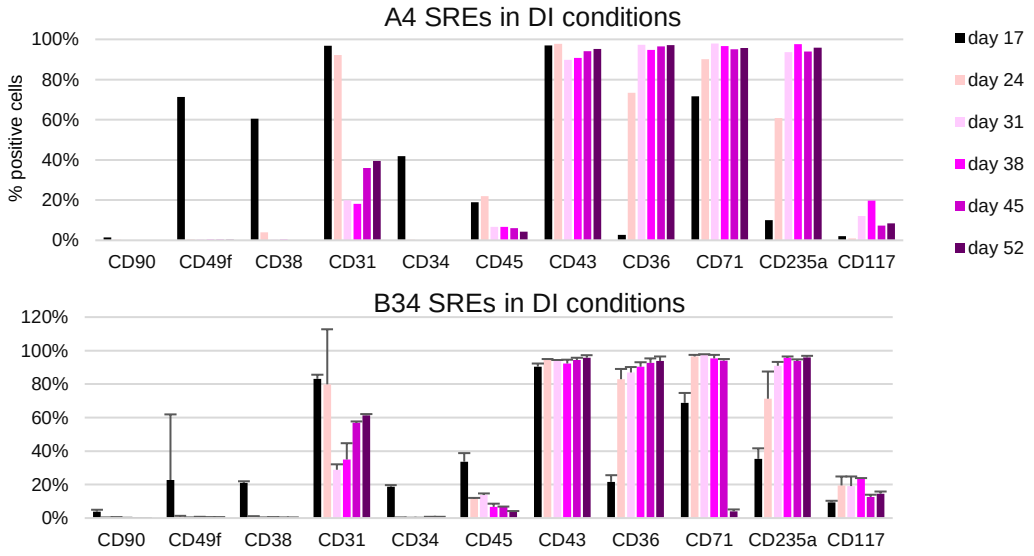

Clone A4 kit D816V hemizygous (donor O1)
Clone D14 kit D816V heterozygous (donor O1)
Clone P kit D816V heterozygous (donor O2)
day 3
day 10
day 17
day 24
day 31
SSC-H
CD90
CD49f
CD43
CD31
CD34
CD45
CD38
CD36
CD71
CD235a
CD117
FSC-H

Figure S6D

A



B

days	protocol	1	2	3-10	10-17	17-24	24-31	31-38	38 .....
No SCF no EPO	PSC-RED	S1	S2*	S3*	TIF	DI	R	R2	
No Epo No SCF	SRE	S1	S2*	S3*	TIF	DI			

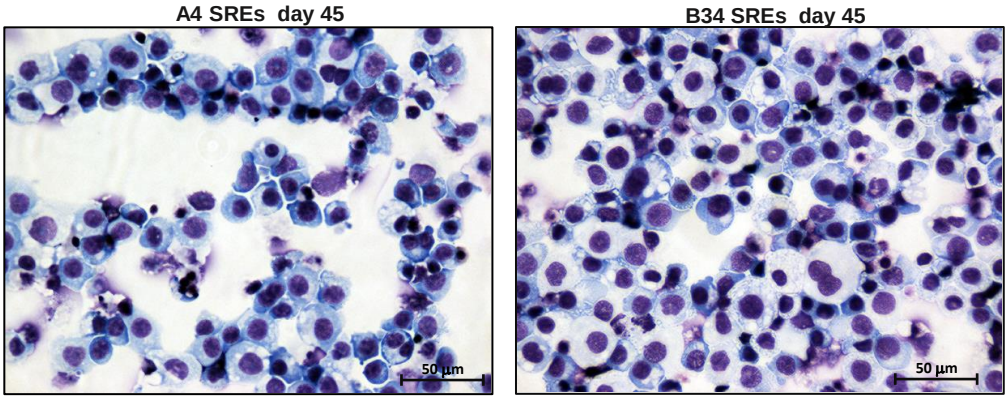
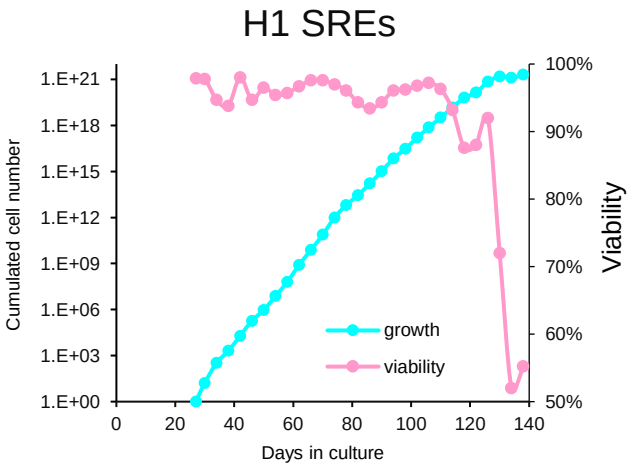


Figure S7


H1 SREs
Cumulated cell number
1.E+21
1.E+18
1.E+15
1.E+12
1.E+09
1.E+06
1.E+03
1.E+00
0
20
40
60
80
100
120
140
Days in culture
100%
90%
80%
70%
60%
50%
Viability
growth
viability

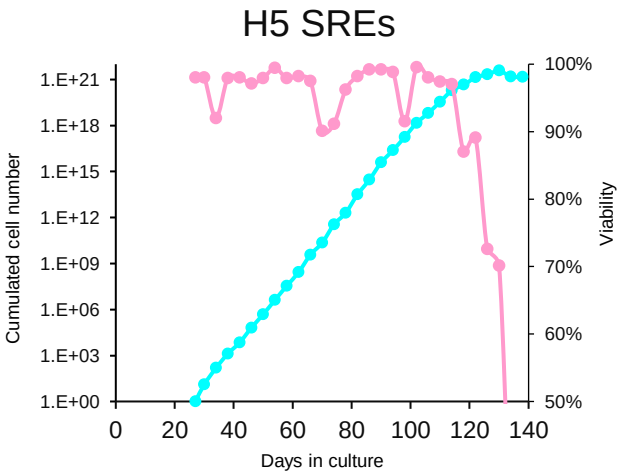
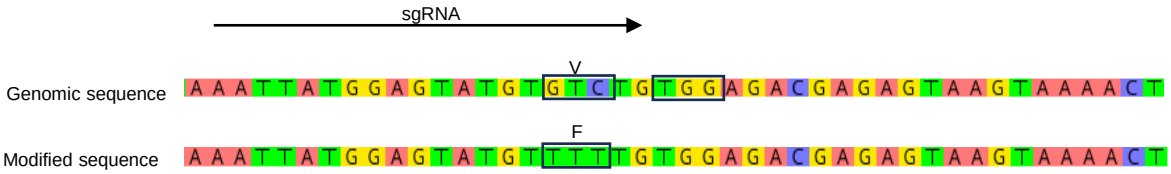
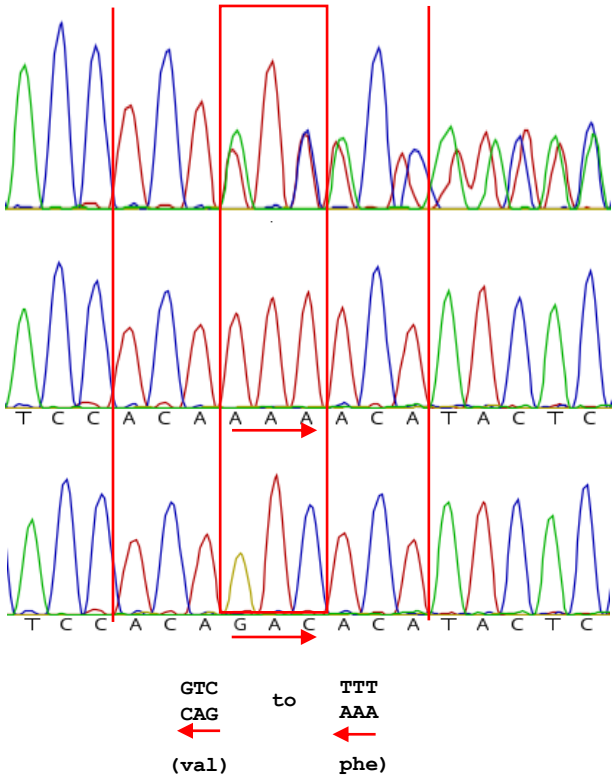

H5 SREs
Cumulated cell number
1.E+21
1.E+18
1.E+15
1.E+12
1.E+09
1.E+06
1.E+03
1.E+00
0
20
40
60
80
100
120
140
Days in culture
100%
90%
80%
70%
60%
50%
Viability

Figure S8

A



B



H5 (hemizygous jak2 657F)
jak2 del (656-657)

H12 (homozygous jak2 657F)

A4 (homozygous jak2 657V)

Figure S9

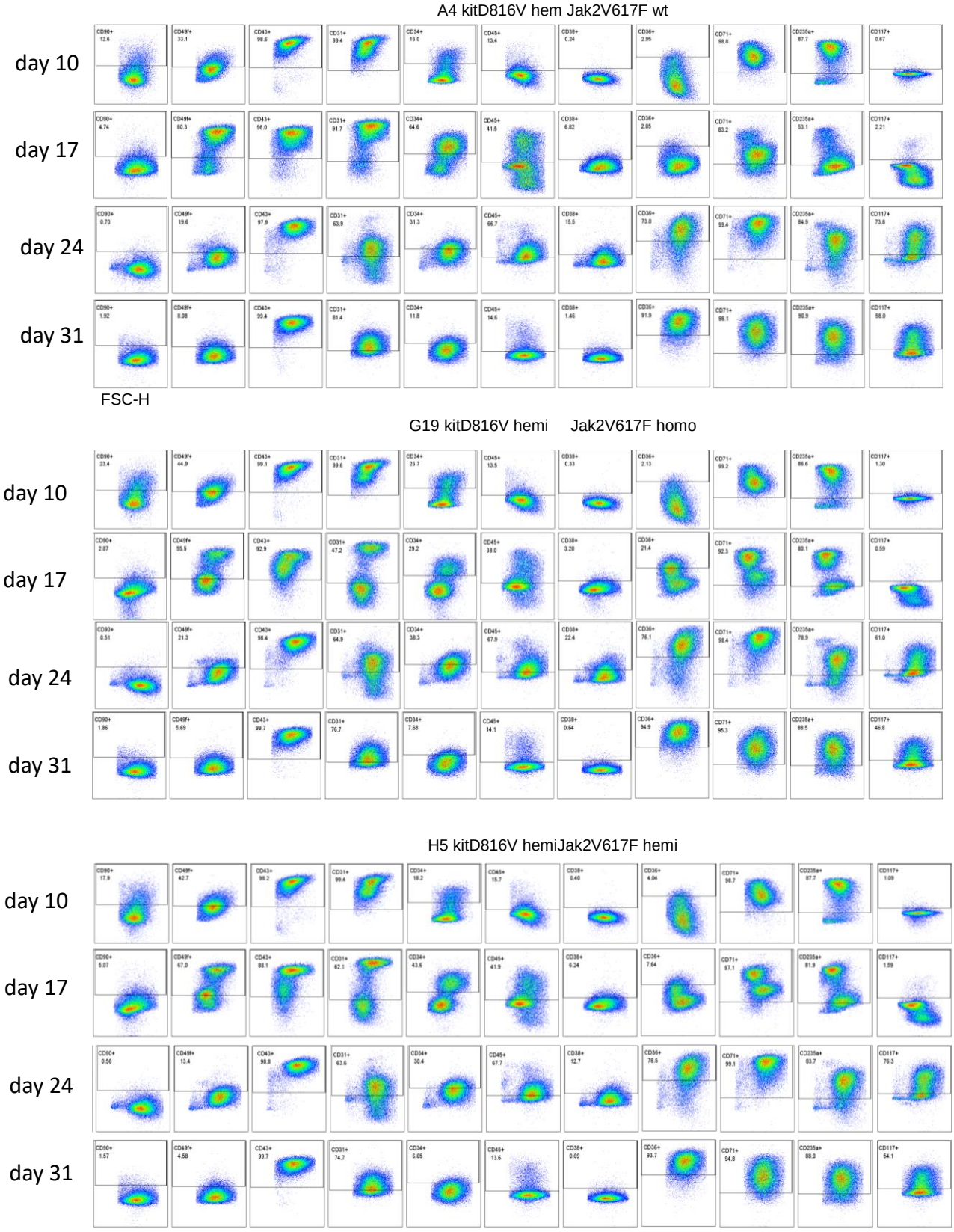

A4 kitD816V hem Jak2V617F wt
day 10
day 17
day 24
day 31
FSC-H
G19 kitD816V hemi Jak2V617F homo
day 10
day 17
day 24
day 31
H5 kitD816V hemiJak2V617F hemi
day 10
day 17
day 24
day 31

Figure S9C

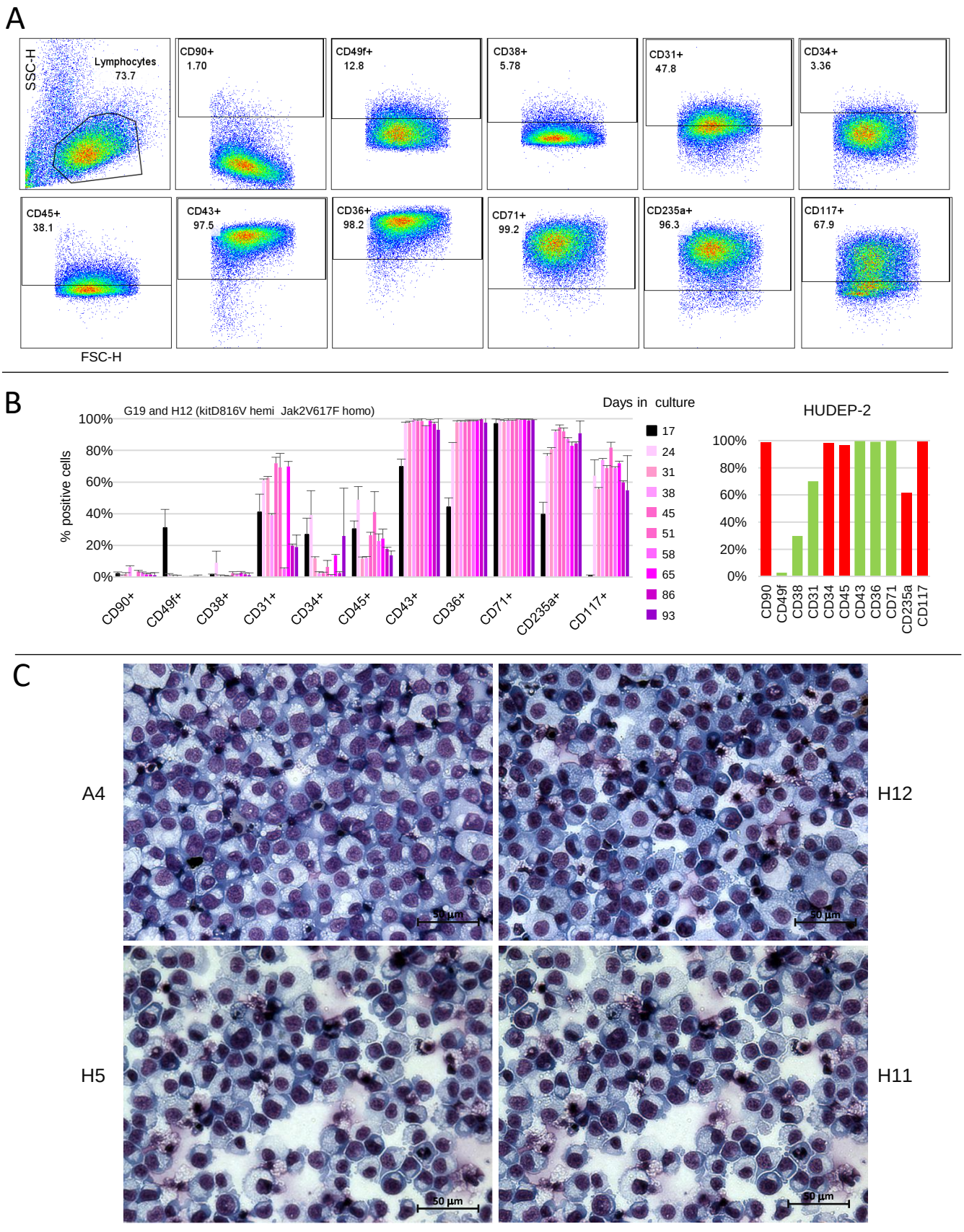

A
SSC-H
Lymphocytes
73.7
CD90+
1.70
CD49f+
12.8
CD38+
5.78
CD31+
47.8
CD34+
3.36
CD45+
38.1
CD43+
97.5
CD36+
98.2
CD71+
99.2
CD235a+
96.3
CD117+
67.9
FSC-H
B
G19 and H12 (kitD816V hemi Jak2V617F homo)
% positive cells
100%
80%
60%
40%
20%
0%
CD90+
CD49f+
CD38+
CD31+
CD34+
CD45+
CD43+
CD36+
CD71+
CD235a+
CD117+
Days in culture
17
24
31
38
45
51
58
65
86
93
HUDEP-2
CD90
CD49f
CD38
CD31
CD34
CD45
CD43
CD36
CD71
CD235a
CD117
C
A4
H12
H5
H11
50 µm

Figure S10

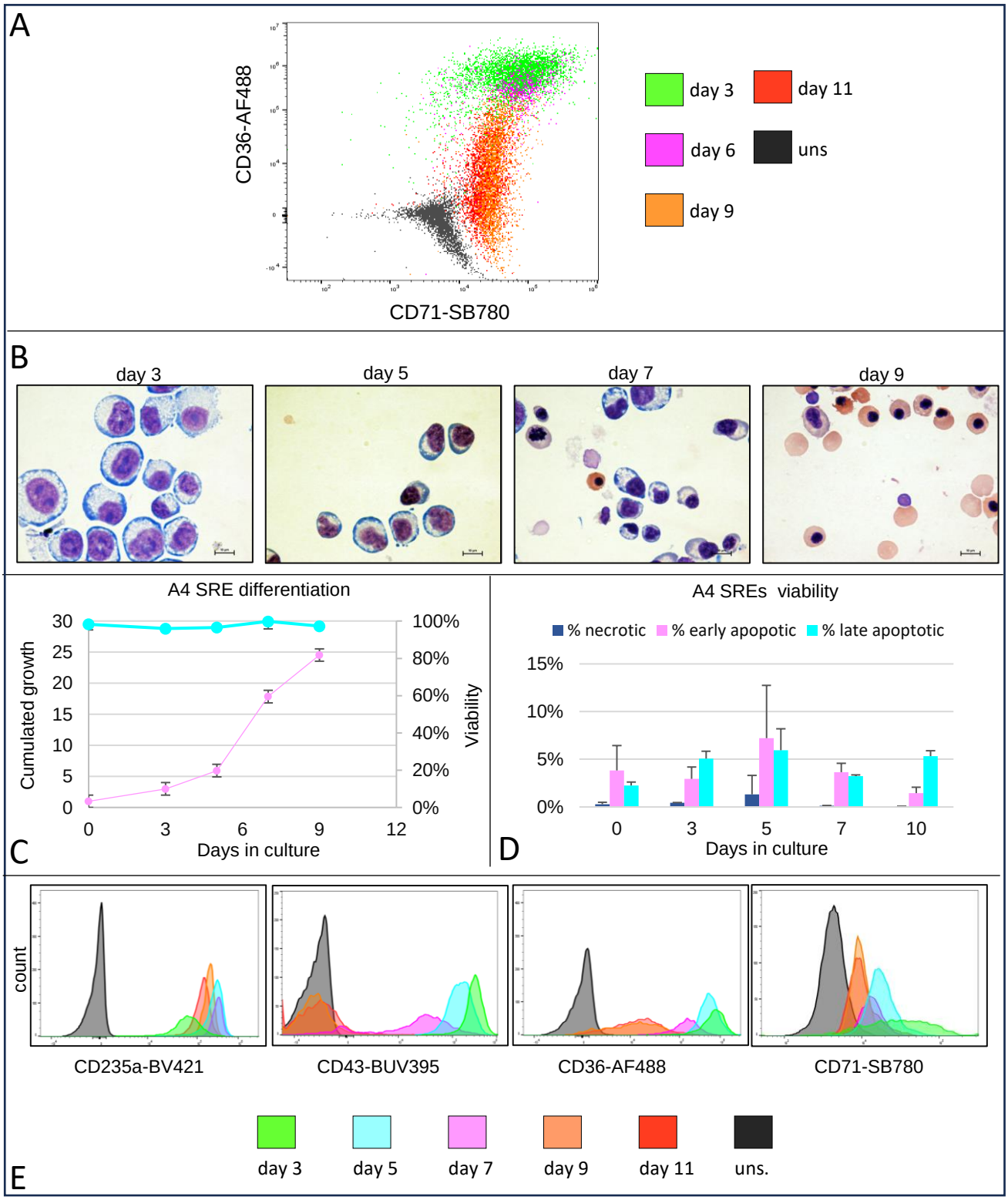

A
CD36-AF488
CD71-SB780
day 3
day 6
day 9
day 11
uns
B
day 3
day 5
day 7
day 9
A4 SRE differentiation
Cumulated growth
Viability
Days in culture
C
A4 SREs viability
% necrotic
% early apopotic
% late apoptotic
Days in culture
D
count
CD235a-BV421
CD43-BUV395
CD36-AF488
CD71-SB780
day 3
day 5
day 7
day 9
day 11
uns.
E

Figure S11

Apoptosis analysis

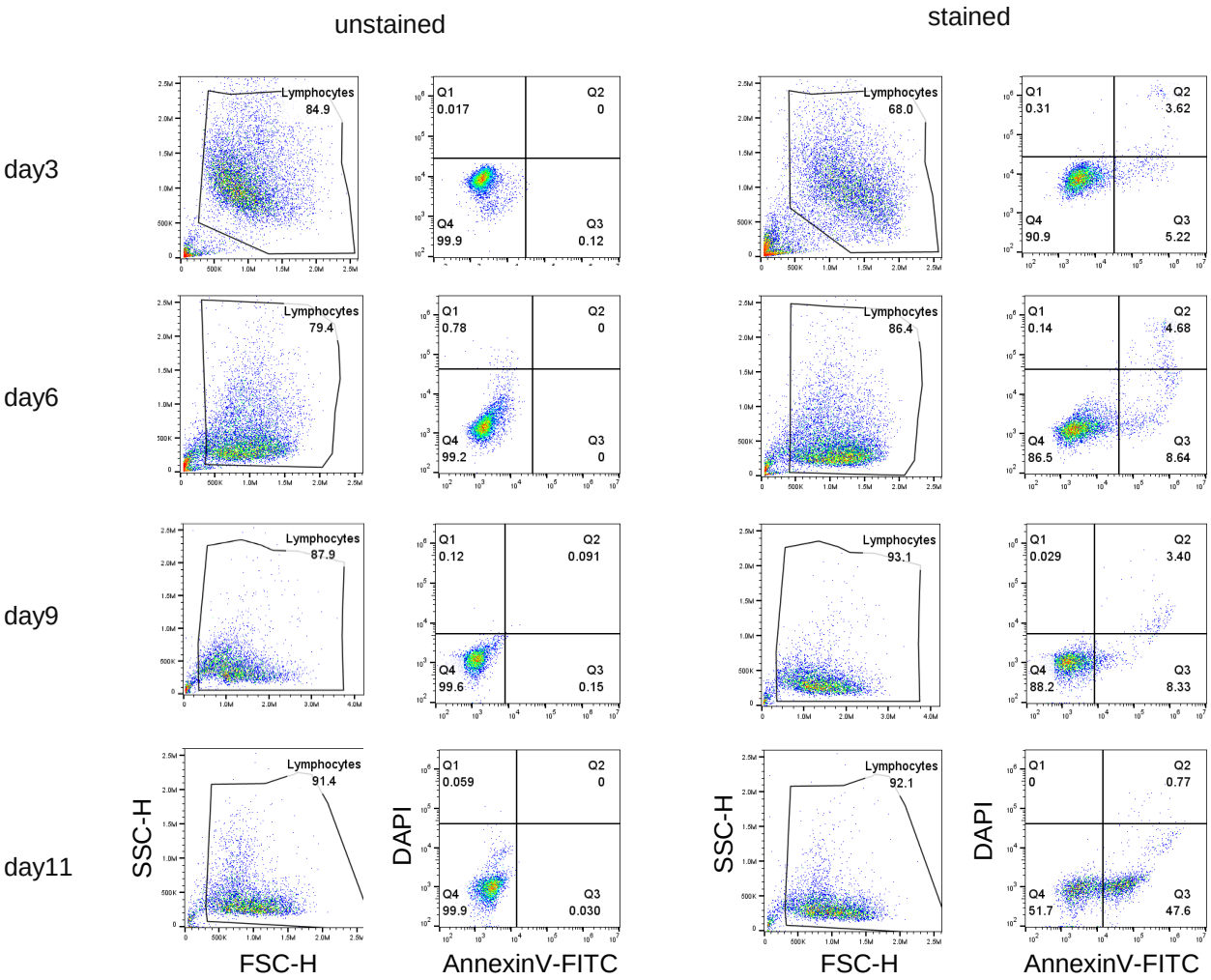
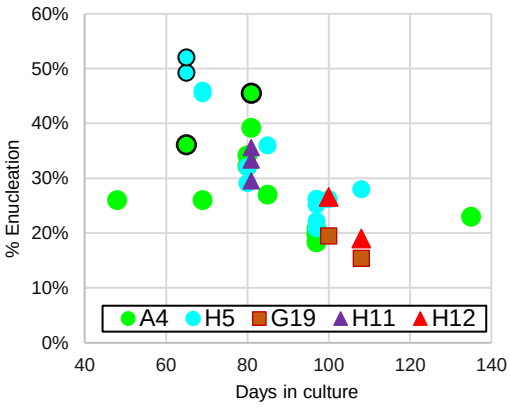


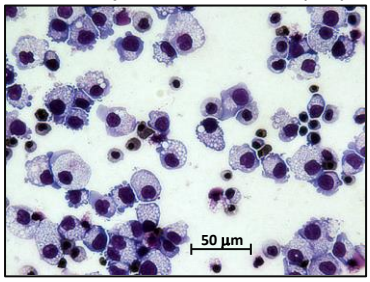
Figure S12

A

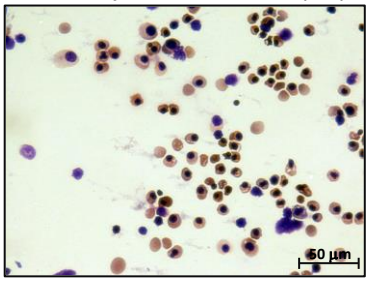


B

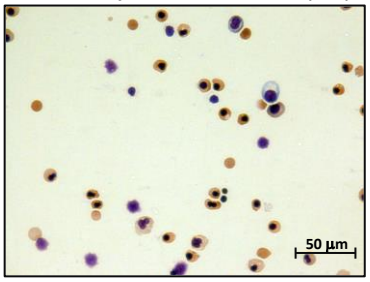
Early passage A4 SREs (day48)
after 7 days of differentiation (40x)



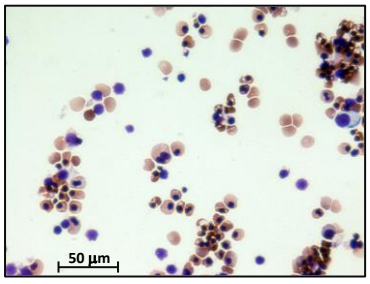
Early passage A4 SREs (day48)
after 10 days of differentiation (40x)



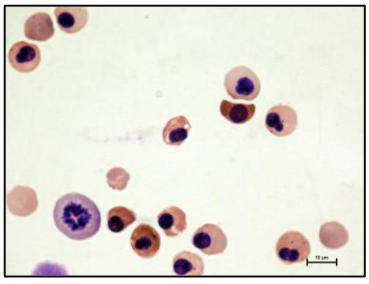
Late passage A4 SREs (day 135)
after 10 days of differentiation (40x)



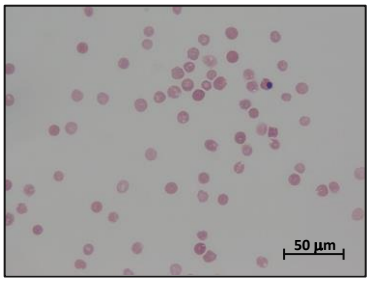
A4 SREs (day 80)
after 10 days of differentiation (40x)



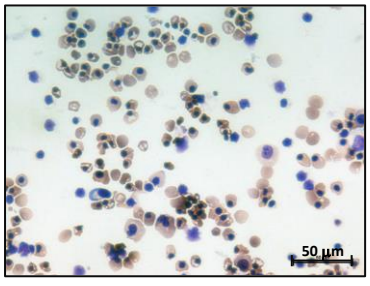
A4 SREs (day 80)
after 10 days of differentiation (100x)



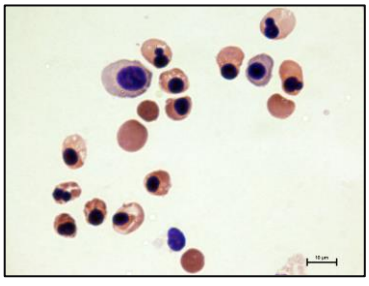
A4 SREs (day 80)
Purified cRetic (40x)



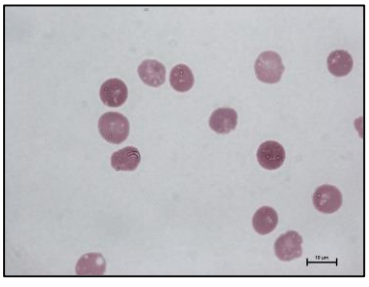
H5 SREs (day 80)
after 10 days of differentiation (40x)



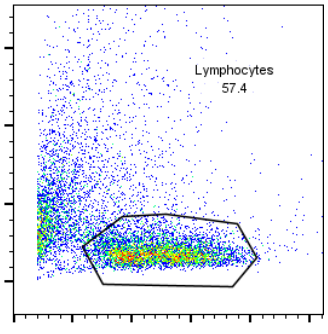
H5 SREs (day 80)
after 10 days of differentiation (100x)



H5 SREs (day 80)
Purified cRetic (100x)



C



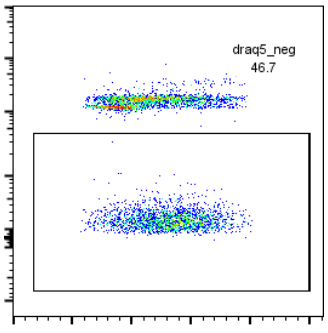


Figure S13

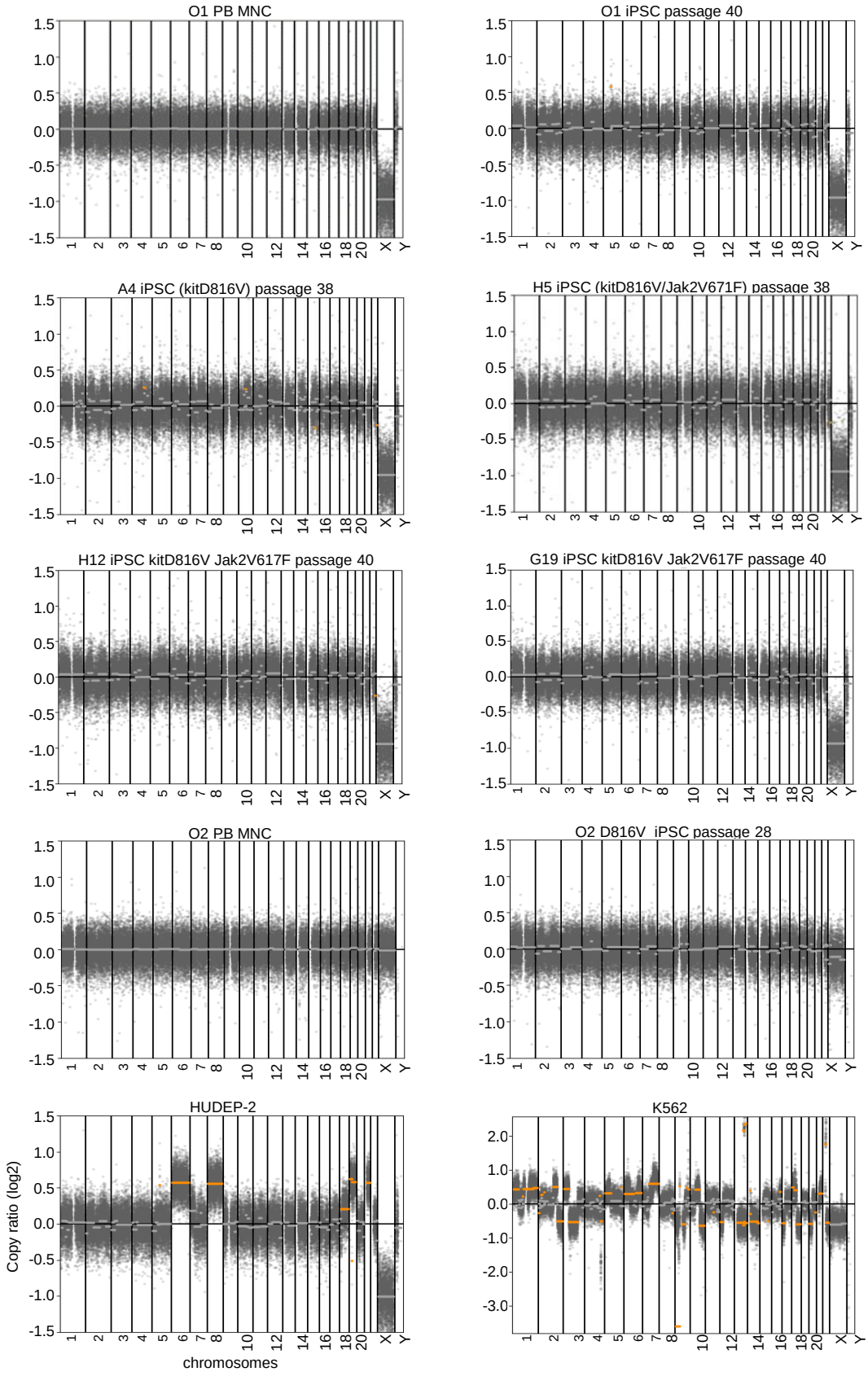

O1 PB MNC
O1 iPSC passage 40
A4 iPSC (kitD816V) passage 38
H5 iPSC (kitD816V/Jak2V671F) passage 38
H12 iPSC kitD816V Jak2V617F passage 40
G19 iPSC kitD816V Jak2V617F passage 40
O2 PB MNC
O2 D816V iPSC passage 28
HUDEP-2
K562
Copy ratio (log2)
chromosomes

Figure S14