PIEZO1 activation delays erythroid differentiation of normal and hereditary xerocytosis-derived human progenitor cells

Alexis Caulier,[1,2*] Nicolas Jankovsky,[1*] Yohann Demont,[3] Hakim Ouled-Haddou,[1] Julien Demagny,[4] Corinne Guitton,[5] Lavinia Merlusca,[2] Delphine Lebon,[1,2] Pascal Vong,[1] Aurélien Aubry,[1] Agnès Lahary,[6] Christian Rose,[7] Sandrine Gréaume,[8] Emilie Cardon,[1] Jessica Platon,[1] Halima Ouadid-Ahidouch,[9] Jacques Rochette,[1,10] Jean-Pierre Marolleau,[1,2] Véronique Picard[11] and Loïc Garçon[1,4,10]

[1]EA4666 HEMATIM, Université Picardie Jules Verne, Amiens; [2]Service des Maladies du Sang, CHU Amiens, Amiens; [3]Unité de Thérapie Cellulaire, CHU Amiens, Amiens; [4]Service d'Hématologie Biologique, CHU Amiens; [5]Service de Pédiatrie Générale, CHU Bicêtre, AP-HP, Le Kremlin-Bicêtre; [6]Laboratoire d'Hématologie, CHU Rouen, Rouen; [7]Service d'Oncologie et d'Hématologie, Hôpital Saint Vincent de Paul, Lille; [8]Etablissement Français du Sang (EFS) de Normandie, Bois-Guillaume; [9]EA4667 Laboratoire de Physiologie Cellulaire et Moléculaire, Université Picardie Jules Verne, Amiens; [10]Laboratoire de Génétique Moléculaire, CHU Amiens, Amiens and [11]Laboratoire d'Hématologie, AP-HP, Le Kremlin-Bicêtre, France

**AC and NJ contributed equally to this work.*

 doi:10.3324/haematol.2019.218503

Received: February 11, 2019.

Accepted: August 9, 2019.

Pre-published: August 14, 2019.

Correspondence: *LOÏC GARÇON* - garcon.loic@chu-amiens.fr

Supplemental methods

In vitro primary erythroid cell culture protocol

In vitro erythroid differentiation was driven in IMDM-based medium (Biochrom, Merck-Millipore) containing 5% human plasma (gift from Etablissement Français du Sang, EFS), 10 µg/mL human recombinant insulin (Sigma-Aldrich), 250 µg/mL human holo-transferrin (Sigma-Aldrich), 1% penicillin-streptomycin (PAN™-Biotech), 2U/mL unfractionated heparin, as described by Giarratana et al.[1] Maturation was promoted following sequential cytokine stimulation: recombinant erythropoietin (EPO, Epoetin beta 3U/mL), human stem cell factor (hSCF 100 ng/mL, Miltenyi), recombinant human interleukin-3 (rhIL-3, Miltenyi), and dexamethasone (DXM 10^{-6}M, Sigma-Aldrich) from day 0 to 7. DXM and rhIL-3 were removed from day 7, and EPO was maintained alone till the end of the culture. For synchronized cultures, cells were cultured in the same medium without EPO until $CD36^{+}/GPA^{-}$ cell sorting on FACS ARIA II (BD Biosciences) at day 7 as previously described (Suppl. Fig. 2B).[2] Cells were plated at 10^5/mL at day 0, 4 and 7, $2.5x10^5$/mL at day 10, $5x10^5$/mL at day 13, and $>10^6$/mL at day 17. Erythroid differentiation was followed using flow cytometry and cytology after May-Grünwald-Giemsa staining using standard procedure. A differential count of 100 erythroblasts was performed using a LEICA microscope.

Multiparametric Flow-Cytometry (MFC) analysis of antigen expression and cell sorting

For flow cytometry, 10^5 cells were stained in 200µL 1X PBS with antibodies listed in Suppl. Table 1. 7AAD staining was used to gate live cells. For PIEZO1 staining, $2x10^5$ cells were first stained in ice for 1h with 1/100 dilution of rabbit Anti-PIEZO1 antibody (15939-AP, Proteintech®) in 1X PBS, 3% BSA, 2mM EDTA. After washing, secondary staining was performed in ice for 1h using 1/200 dilution of IgG goat anti-rabbit (H+L) Alexa Fluor 647-labelled antibody (Thermo Fisher scientific) in the same buffer. Acquisition was performed on a MacsQuant 8-colours flow cytometer (Miltenyi). Data analysis was performed using FlowJo (FlowJo v10, TreeStar Inc.). For synchronized experiments, $CD36^{+}/GPA^{-}$ cells were sorted on FacsAriaII device (BD Biosciences). For cell cycle analysis, 10^6 cells were centrifuged at 300g for 5 min, and resuspended in 1mL cold 70% ethanol and 1mL 1X PBS-1%glucose for overnight fixation at 4°C. After 1 wash in 1mL 1X PBS-1%glucose, cell permeabilization was made in 0.1mL 1X PBS + 0.05% Tween-20 containing 1µg/mL DAPI for 30 min in darkness, at room temperature. Cells were then washed in 1X PBS and resuspended in 0.1mL 1X PBS + 1µg/mL DAPI.

Live imaging flow cytometry (IFC)

Intracellular calcium concentration was evaluated by live IFC using an ImageStream®^{X} Mark II (Amnis/EMD Millipore). $2x10^6$ cells were stained with 2µM Fluo4-AM probe (Thermo Fisher scientific) in 1mL serum-free α-MEM for 30min at 37°C, then washed 2 times in 1X PBS, and resuspended in 50µL calcium-containing or calcium-free 1X PBS. Calcium measurement was performed in Ca^{2+}-containing and Ca^{2+}-free 1X PBS as sheath liquid. For NFAT nuclear translocation assessment, UT7/EPO cells underwent overnight EPO and serum starvation in α-MEM. After 1X PBS washing, $2x10^6$ cells were resuspended in 1mL serum-free α-MEM, and

exposed to 10µM YODA1 or DMSO for 15min at 37°C. Then cells underwent fixation and permeabilization using eBiosciences™ FoxP3 / Transcription Factor Fixation/Permeabilization kit (Thermo Fisher scientific), according to manufacturer's instructions. Cells were then labeled for 1h on ice with NFATc1-PE antibody and 1µg/ml DAPI staining in permeabilization buffer, washed in 1X PBS and resuspended in 50µL 1X PBS before ImageStream®^{X} run. Nuclear translocation was evaluated by calculating the similarity score value of NFATc1 and DAPI fluorescent signals, using the Amnis IDEAS® software as previously published.[3]

Western blot analysis

Proteins were separated on a 15% Polyacrylamide gel in Tris-Glycine buffer (Thermo Fisher scientific) and transferred to Nitrocellulose membranes (Sigma-Aldrich). Membranes were blocked for 1h with 5% (w/v) non-fat dry milk TBS-Tween 0.1% buffer. Membranes were incubated with the primary antibody solution overnight at 4°C in 5% (w/v) non-fat dry milk TBS-Tween 0.1% for non-phospho antibody or 5% (w/v) BSA TBS-Tween 0.1% for phospho antibody, and horseradish peroxidase-conjugated secondary antibody was incubated for 1h at room temperature. After extensive wash, blots were visualized with chemoluminescent reagents (Super Signal Pico, ThermoFisher Scientific) in ChemiDoc Universal HoodII device (Bio-rad). Antibodies are listed in Suppl. Table 3.

Confocal immunofluorescence imaging

For Confocal immunofluorescence imaging, a minimum of $2x10^5$ cells were suspended in a PBS 1X (Dutcher), 3% Bovine Serine Albumine (Sigma-Aldrich), 2mM EDTA (Sigma-Aldrich) staining buffer. Cells were stained with either a PIEZO1 Rabbit antibody or a Rabbit Isotype control antibody (details of antibodies specification are described in Suppl. Table 3) 1h on ice. Cells were then washed and stained with Goat anti-Rabbit (H+L) Alexa Fluor-647 conjugated antibody before being Cytospun at 400rpm during 4mn on Cytospin3 (Shandon). Images were acquired directly on LSM780 (Zeiss).

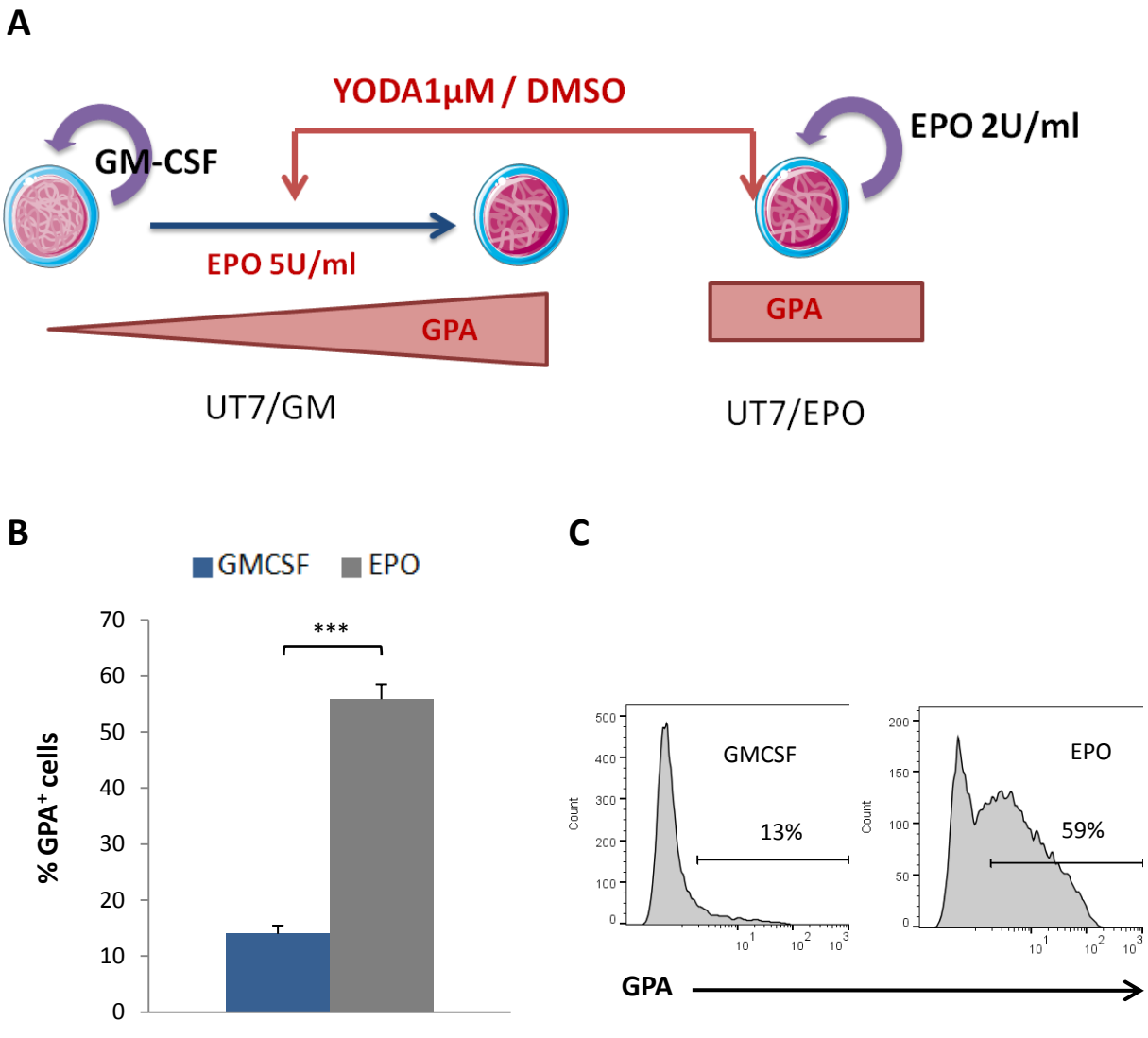
Quantitative reverse transcriptase–polymerase chain reaction (RT-qPCR)

RNA isolation was performed using mini or microRNA Qiagen kit. Gene expression was assessed by RT-qPCR, using SYBR® Green or TaqMan™ technology on QuantStudio7 device (Applied Biosystem). Relative expression levels were calculated by normalization to *HPRT* or *GAPDH* expression. Primers were designed using online PrimerBlast tool, and sequences are detailed in Suppl. Table 4.

Reagents

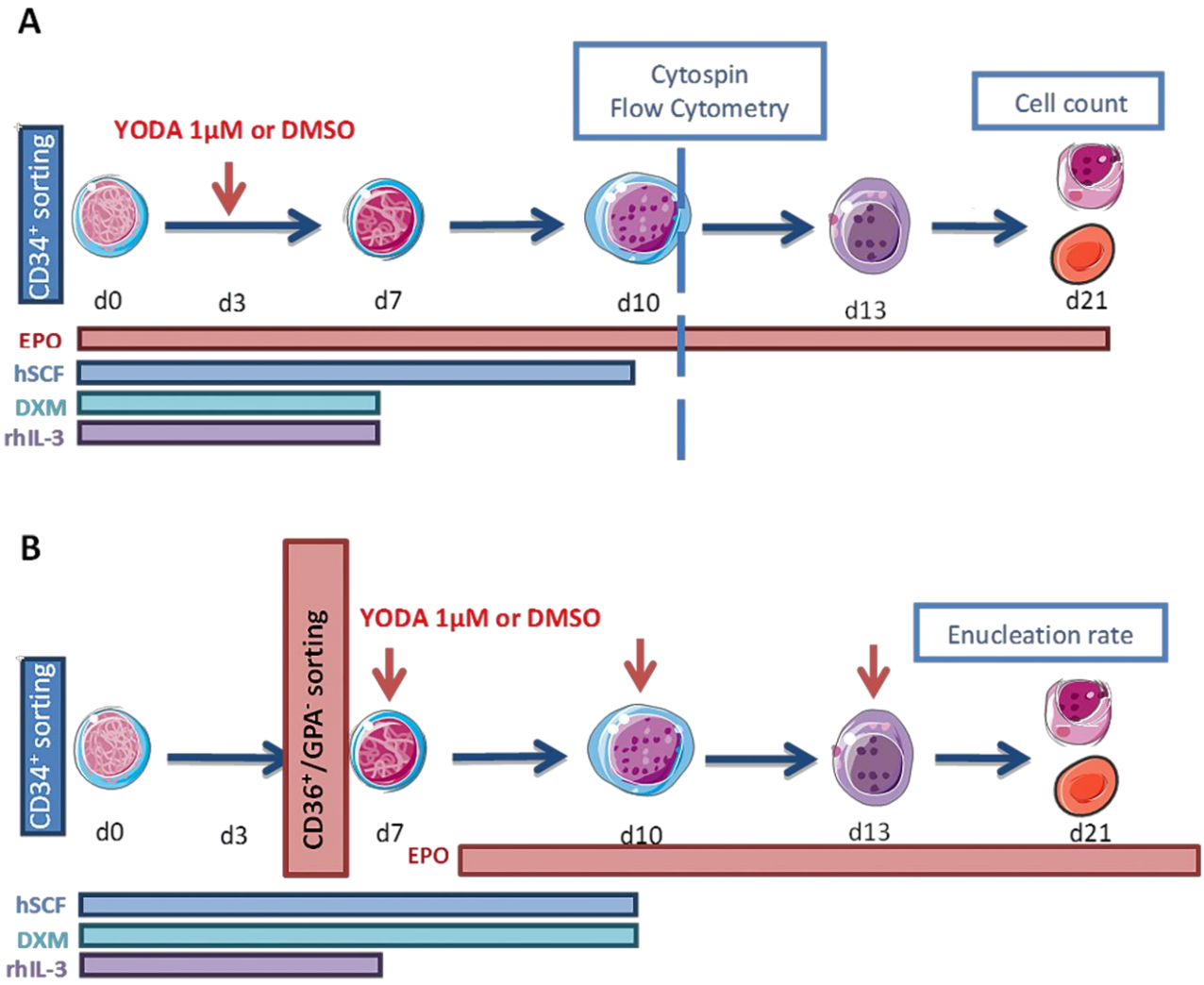
YODA1, Bapta-AM, Thapsigargin, EGTA, Ionomycin and UO126 were purchased from Sigma-Aldrich. Senicapoc was purchased from MedChemExpress.

Supplemental figure 1



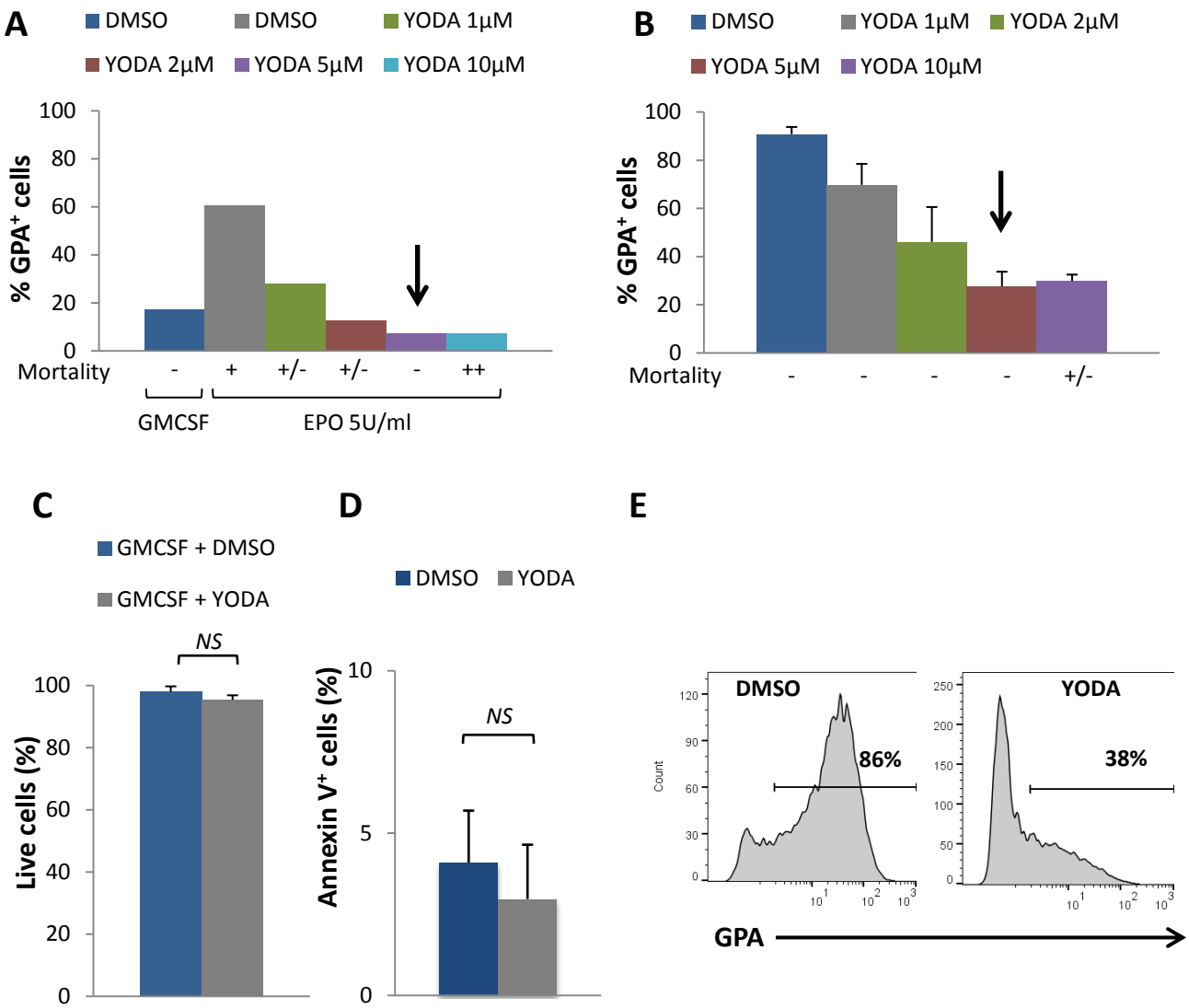
Caulier A. et al.

Supplemental figure 2



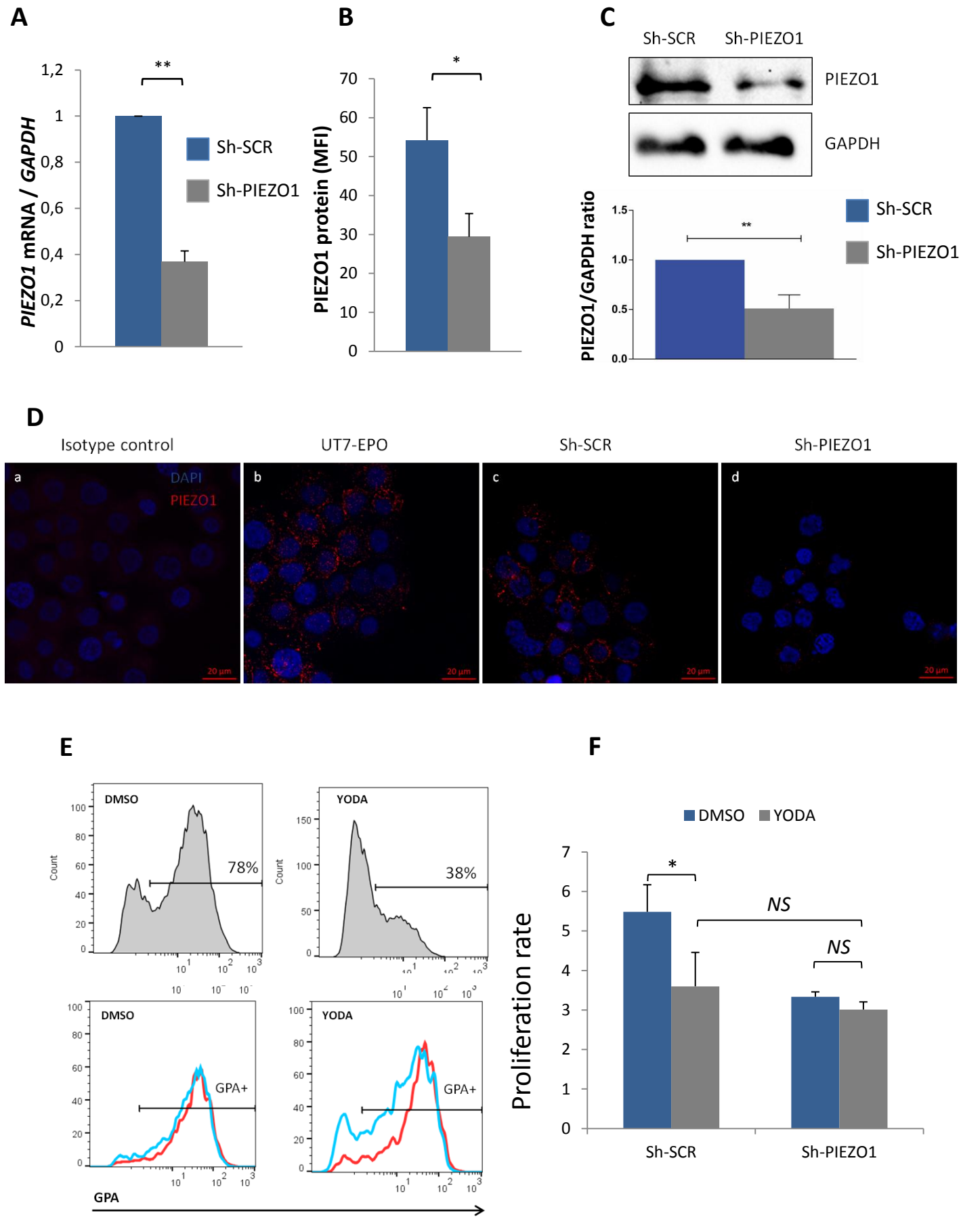
Caulier A. et al.

Supplemental figure 3

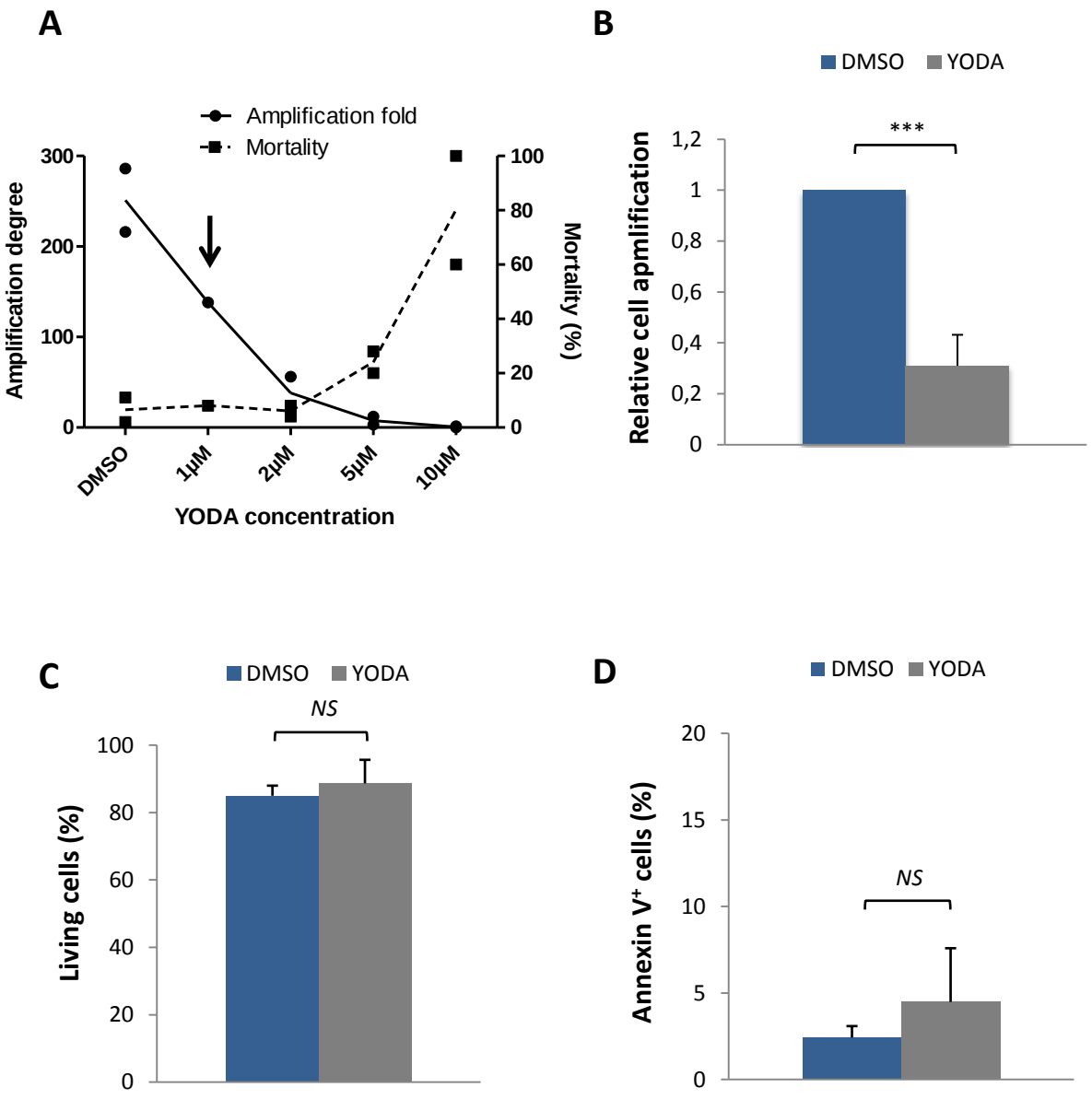


Caulier A. et al.

Supplemental figure 4

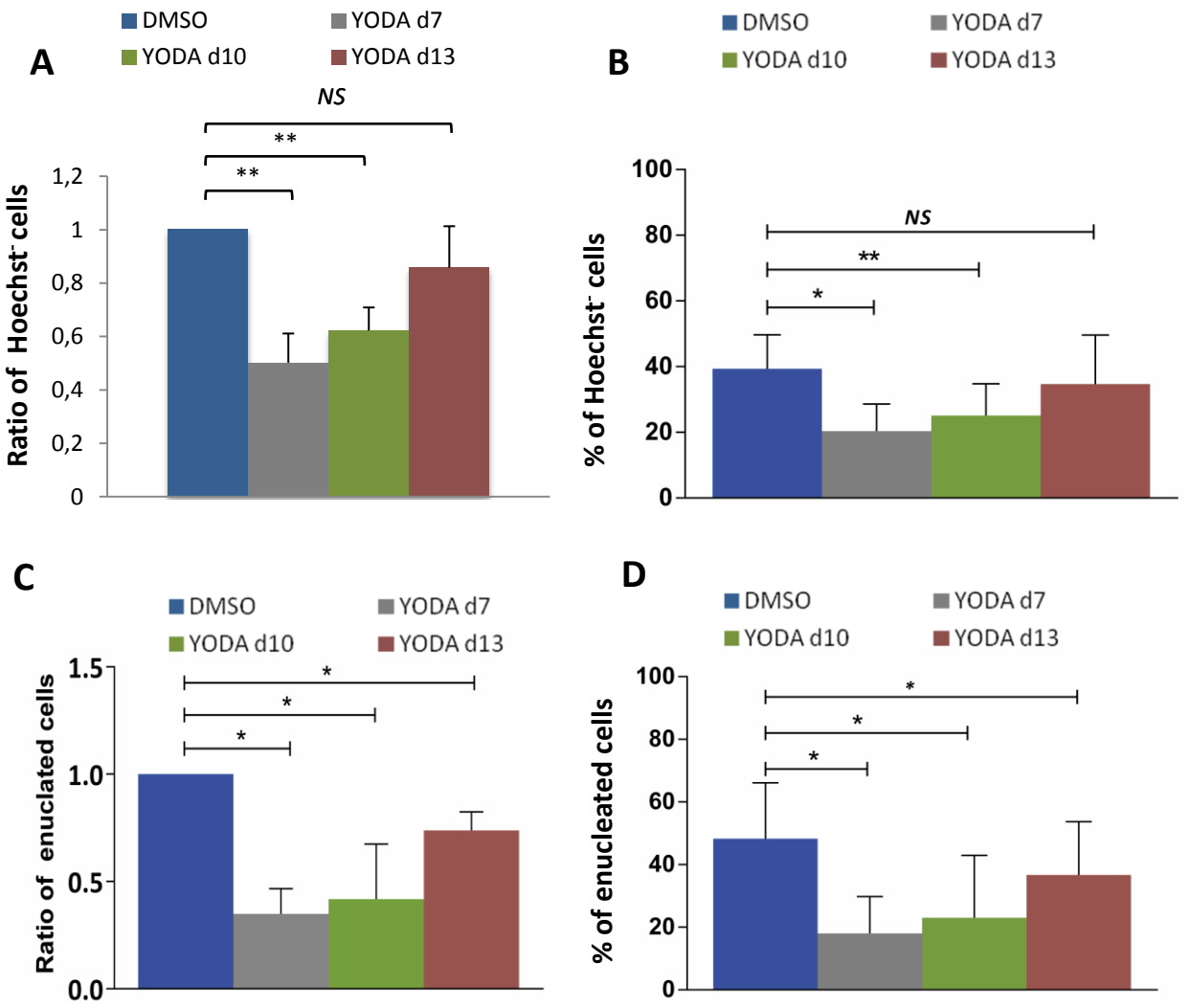


Supplemental figure 5



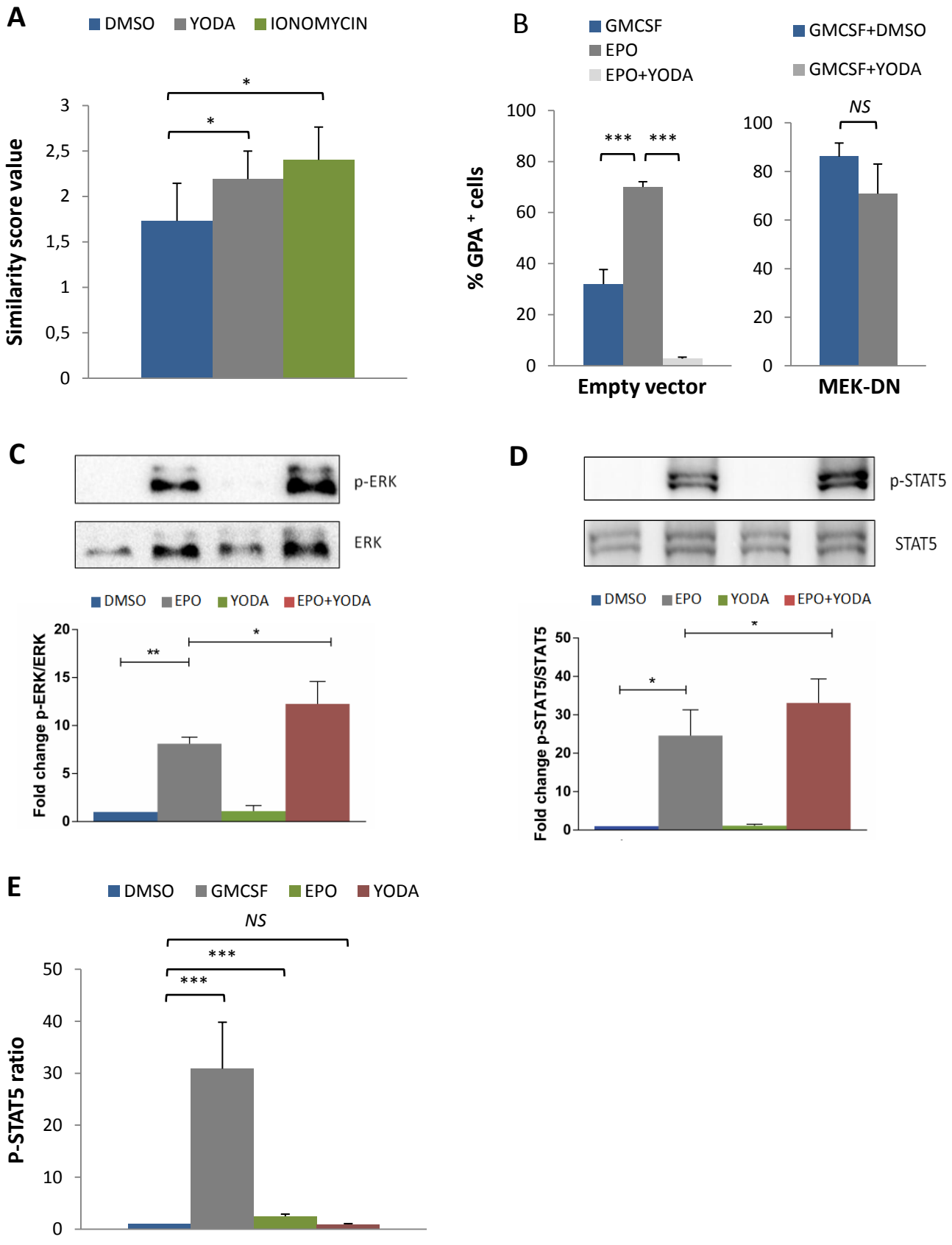
Caulier A. et al.

Supplemental figure 6



Caulier A. et al.

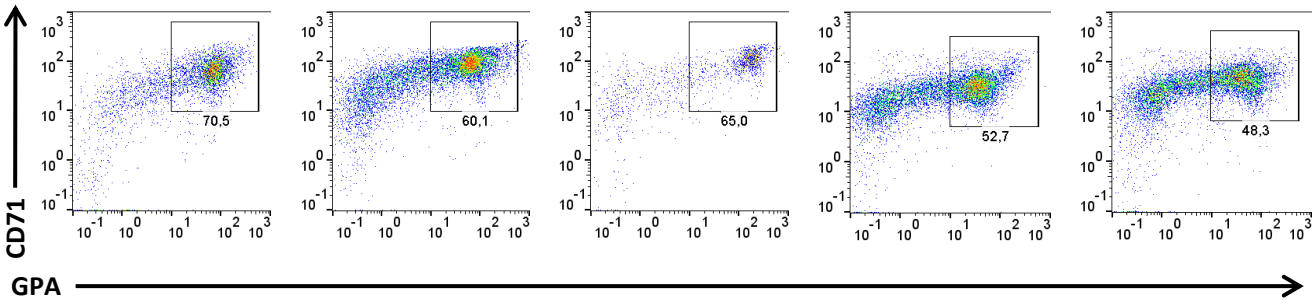
Supplemental figure 7



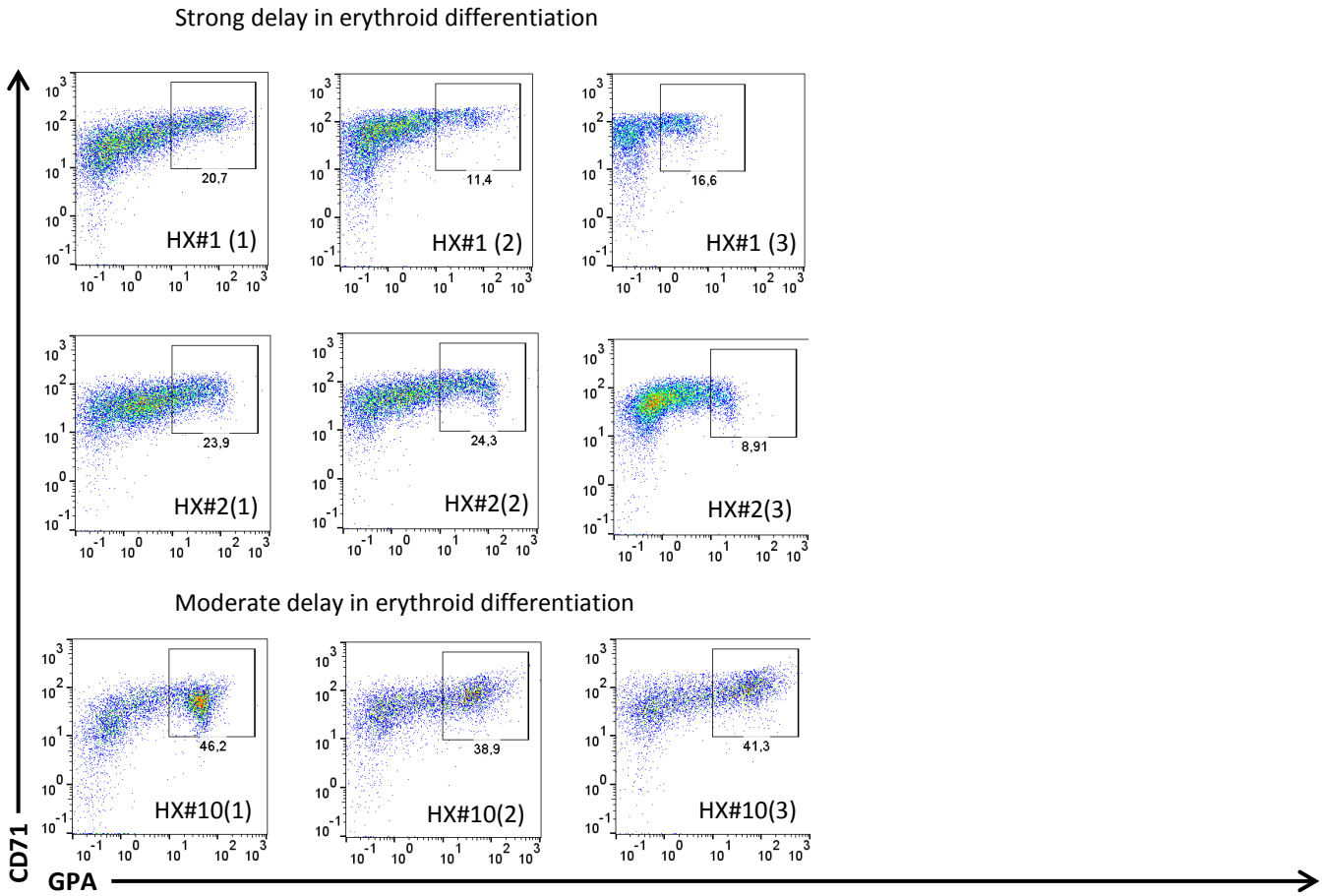
Caulier A. et al.

Supplemental figure 8

A- Erythroid differentiation from sorted control CD34⁺ cells, CD71/GPA expression at d10



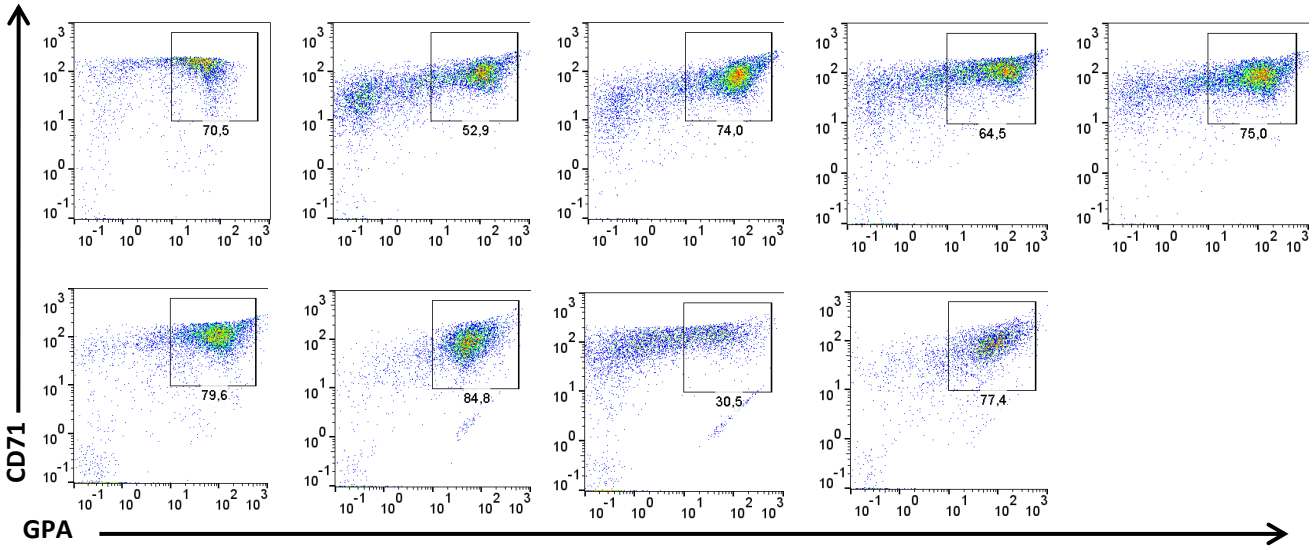
B- Erythroid differentiation from sorted HX CD34⁺ cells, CD71/GPA expression at d10



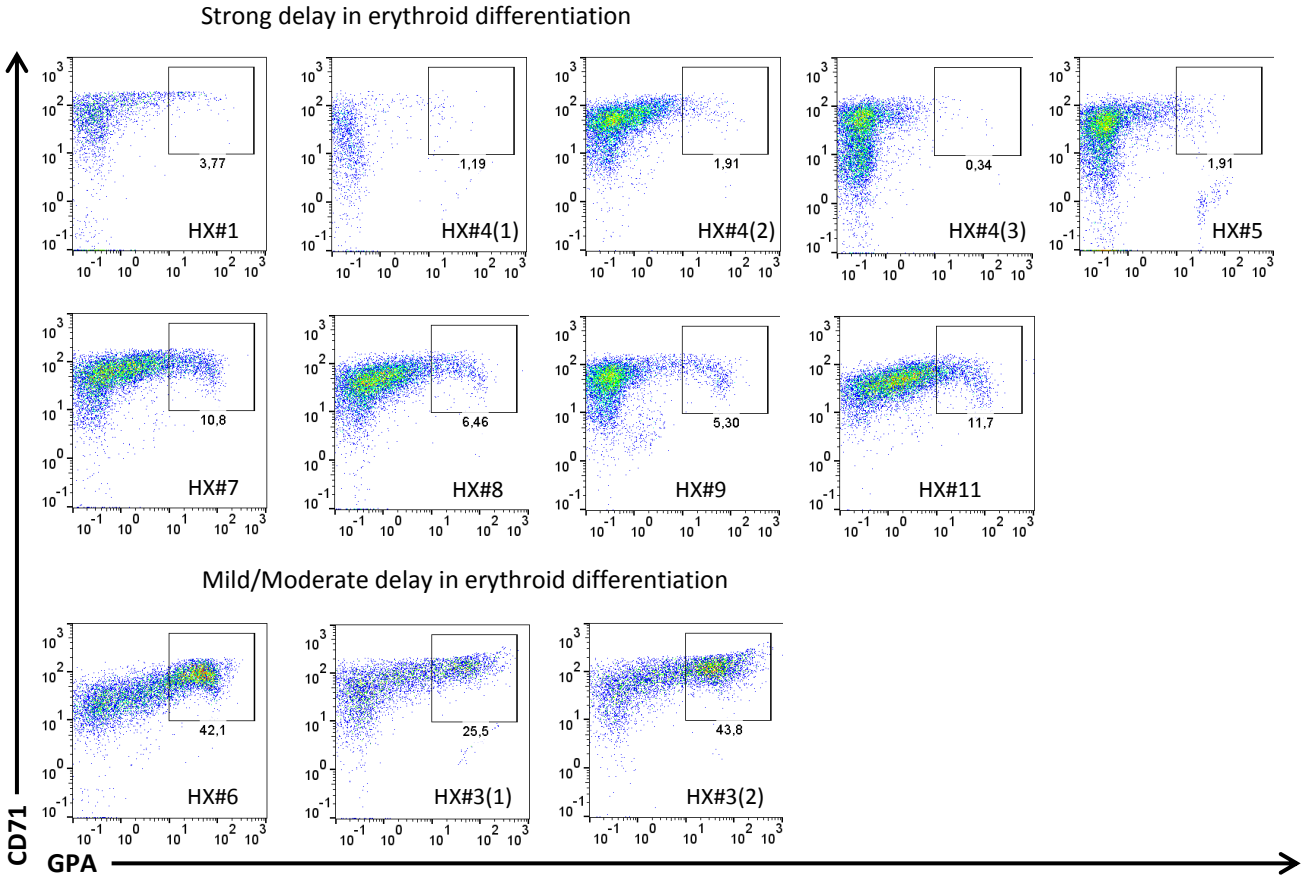
Caulier A. et al.

Supplemental figure 9

A- Erythroid differentiation from MNC, controls; CD71/GPA expression at d10

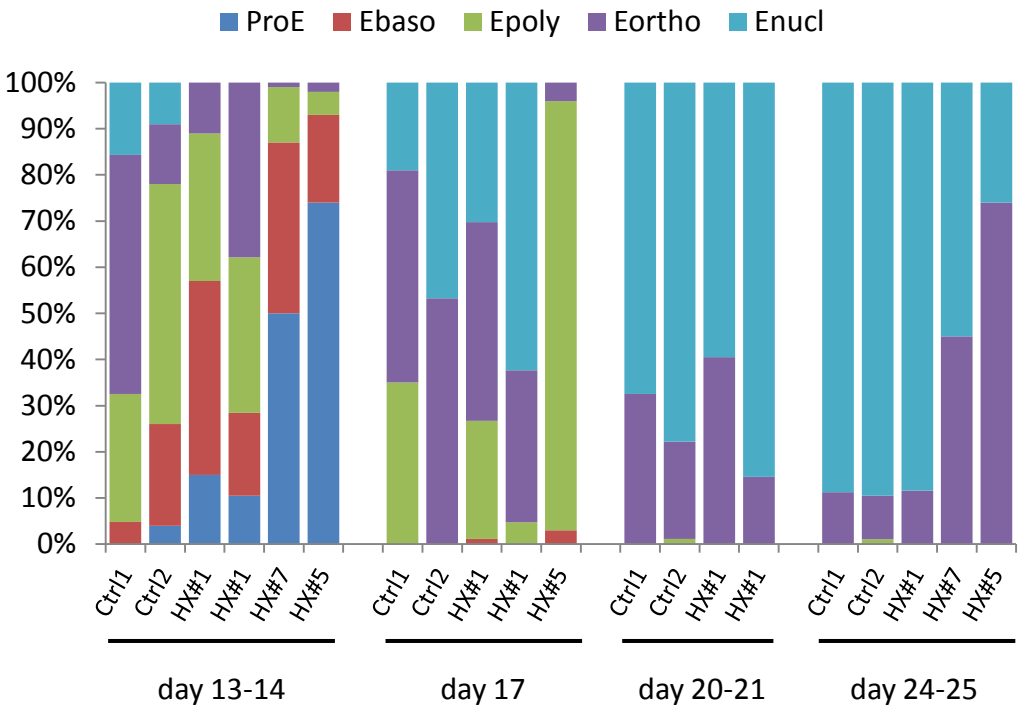


B- Erythroid differentiation from HX MNC cells, CD71/GPA expression at d10



Caulier A. et al.

Supplemental figure 10



Caulier A. et al.

Legends to supplemental figures

Supplemental figure 1. Description of UT7 cell lines.
(A) UT7/GM cell line was cultured in GMCSF-containing medium and acquired GPA expression when exposed to EPO. UT7/EPO cell line was cultured in EPO-containing medium and highly expressed GPA at steady state. 5µM YODA1 during 72 hours was used to activate PIEZO1.
(B) In UT7/GM cells, 5U/mL EPO induced GPA expression in comparison to 5ng/mL GMCSF (56±3% *vs.* 14±1.5%, p<.001).
(C) Representative MFC histograms of GPA expression in UT7/GM cells, in medium containing either GMCSF (left) or EPO (right).

Supplemental figure 2. Erythroid differentiating culture of human primary $CD34^+$ derived cells.
(A) Non-synchronized *in vitro* erythroid primary cell differentiation. After magnetic sorting, $CD34^+$ cells were cultured for 21 days in medium containing sequential cytokines. 1µM YODA1 was added from d3 to d21.
(B) Synchronized *in vitro* differentiation of primary cells.[2] After magnetic sorting, $CD34^+$ cells were cultured without EPO for 7 days, $CD36^+/GPA^-$ cells were sorted and plated in EPO-containing medium until day 21. 1µM YODA1 was added either at d7, d10, or d13 to evaluate the delay in erythroid differentiation assessed by the enucleation rate at d21.

Supplemental figure 3. Effect of YODA1 on viability and phenotype in UT7 cell line.
Experiments A and B were performed in duplicate; C, D and E in triplicate; *** p<.001; **p<.01; *p<.05
(A) Increased concentrations of YODA1 from 1 to 10µM were tested in UT7/GM cells. YODA1-mediated GPA repression was dose dependent but reached its maximum at 5µM. The 10µM dose was associated with a high mortality rate assessed by trypan blue staining. We chose 5µM for further experiments in UT7 cells (black arrow).
(B) Same experiment was performed in UT7/EPO cells. The YODA1-mediated GPA repression reached a maximum at 5µM with no significant mortality. We selected this dose (black arrow).
(C) In UT7/GM cells, 5µM YODA1 did not increase mortality compared to DMSO under GMCSF condition, as assessed by trypan blue staining.
(D) In UT7/GM cells, 5µM YODA1 did not induce significant apoptosis after 24h exposure (3±1.7% with YODA1 *vs*. 4±1.6% with DMSO, p=*NS*), as assessed by Annexin V staining.
(E) Representative MFC histogram plots showing the decrease in GPA expression due to YODA1 exposure in UT7/EPO cells.

Supplemental figure 4. Effect of lentiviral Sh-PIEZO1.
Experiment D was performed in duplicate. All other experiments were performed in triplicate; *** p<.001 ; **p<.01 ; *p<.05
(A) Mean *PIEZO1* mRNA expression assessed by RT-qPCR, relative to *GAPDH*, in UT7/EPO cells expressing Sh-scramble (SCR) or Sh-PIEZO1 (63% reduction, p<.001).
(B) PIEZO1 expression was quantified at protein level on cell membrane in Sh-SCR and Sh-PIEZO1-transduced UT7/EPO cells using MFC, as described in Methods section. We observed a 45% reduction in the mean fluorescence intensity level (n=3, p<.05) after PIEZO1 knockdown.

(C) *PIEZO1* expression in UT7/EPO transduced with Sh-SCR or Sh-PIEZO1 assessed by Western blot. We confirmed the MFC data, showing a 49% reduction after Sh-PIEZO1-transduction (n=3, p<.01).
(D) Representative confocal immunofluorescence images of UT7-EPO stained with polyclonal rabbit Isotype control antibody (red; a) or polyclonal rabbit Piezo1 (red; b, c and d) and DAPI (blue). UT7/EPO were either untransfected (a and b), transfected by Sh-SCR (c) or Sh-PIEZO1 (d). The data confirmed the efficiency of the PIEZO1 knockdown.
(E) GPA expression was assessed by MFC in UT7/EPO transfected with Sh-SCR (upper) or Sh-PIEZO1 (lower) after a 72h stimulation with DMSO (left) or 5µM YODA1 (right). YODA1 induced a decrease in GPA expression in Sh-SCR (a). In Sh-PIEZO1-expressing cells (b), this phenotype was totally abolished in GFP^{high} cells (red line) (Mean % of GPA^+ cells: 95±1 *vs.* 93±2, p=*NS*) whereas the decrease was partial in cells expressing GFP at an intermediate level (blue line) (92±1 *vs.* 82±2, p<.001), showing that the phenotype depended on Sh-PIEZO1 expression level.
(F) As expected, YODA1 decreased the proliferation rate in Sh-SCR expressing UT7/EPO after a 72h exposure. In Sh-PIEZO1 expressing cells, no variation in proliferation rate was observed under YODA1 treatment but Sh-PIEZO1 alone decreased proliferation compared to Sh-SCR.

Supplemental figure 5. Effect of YODA1 stimulation on viability in primary erythroid cells.
Experiment A was performed in duplicate, n=3 in all other experiments; ***p<.001
(A) YODA1 exposure reduced cell proliferation of $CD34^+$-derived erythroid progenitors in a dose-dependent manner. A markedly increased cell mortality was observed from 5µM. 1µM was the selected dose for further experiments in primary cells.
(B) 1µM YODA1 induced a 3.2 fold reduction in cell expansion of $CD34^+$-derived erythroid progenitors (p<.001). Cells were cultured for 21 days and YODA1 was added from day 3 to 21. To overcome the heterogeneity of $CD34^+$ cells amplification between donors, fold change was calculated relative to DMSO from the same $CD34^+$ cell source for each experiment.
(C) In $CD34^+$-derived erythroid cells, 1µM YODA1 did not increase mortality at day 17 (live cells = 88±7%) comparative to DMSO (85±3%, p=*NS*), as assessed by trypan blue staining.
(D) In $CD34^+$-derived erythroid cells, no difference in apoptosis rate was observed between cells exposed to 1µM YODA1 (4.5±3%) or DMSO (2.4±1%, p=*NS*), as assessed by Annexin V staining in MFC. Experiments were carried out at day 10, after a 24h drug exposure.

Supplemental figure 6. Time-dependent effect of Piezo1 chemical activation on erythroid differentiation of human primary $CD34^+$ derived cells.
n=3 in all experiments; **p<.01 ; *p<.05
(A) Synchronized primary cells were exposed to YODA1 from day 7, day 10 or day 13, as shown in Suppl. Fig. 2B. The relative YODA1/DMSO enucleation rate was assessed at day 21 by MFC after Hoechst staining. Compared to DMSO, PIEZO1 activation using 1µM YODA1 from day 7 and 10 significantly reduced the enucleation rate (0.50±0.11% and 0.62±0.09% respectively, p<.01) whereas YODA1 added from day 13 did not (0.86±0.16%, p=*NS*).
(B) Absolute percentage of enucleation assessed by MFC after Hoechst staining. A reduced enucleation rate was observed after YODA1 stimulation from day 7 (20±8%, p<.05) or day 10 (25±10%, p<.01) but not from day 13 (35±15%, p=*NS*) compared to DMSO (39±10%).
(C) Cytological analysis after MGG staining at day 21 confirmed the relative decrease in enucleation rate when PIEZO1 was activated from day 7 (reticulocyte ratio YODA1/DMSO=

0.35±0.12, p<.05) and day 10 (0.42±0.26, p<.05). Slighter decrease was observed after YODA1 stimulation from 13 (0.74±0.1, p<.05).
(D) Absolute percentage of enuclated cells evaluated by cytological analysis, showing a decrease after 1µM YODA1 stimulation from day 7 (18±12%, p<.05), day 10 (23±20%, p<.05) and a slighter decrease from day 13 (37±17%, p<.05) compared to DMSO (48±18%).

Supplemental figure 7. YODA1 effects on signaling pathways in UT7 cell line and human primary cells.
n=3 for B,C,D,E and n=4 for A. ***p<.001 ; **p<.01 ; *p<.05
(A) 10µM YODA1 increased NFAT nuclear translocation, as shown by the significant increase in similarity score value (SS= 2.2±0.3 *vs* 1.7±0.4, p<.05). 1µM Ionomycin was used as positive control.
(B) In UT7/GM cells transduced with empty MigR, 5U/mL EPO induced GPA expression (70±2%) compared to DMSO (32±6%, p<.001). This effect was blocked with 5µM YODA1 (3±1%, p<.001). UT7/GM cells transduced with the dominant-negative MEK retrovirus (MEK-DN) exhibited higher GPA expression under GMCSF (86±6%, p<.001) and blocked YODA1 effect on GPA expression (71±12%, p=*NS*).
(C) Western blot showing ERK1/2 activation in $CD34^+$-derived erythroid cells at day 10 of culture. 5U/mL EPO induced ERK1/2 phosphorylation (fold increase in the P-ERK/ERK ratio: 8.1±1.2, p<.05) that was enhanced after co-stimulation with 2µM YODA1 (12.3±2.1, p<.05). 2µM YODA1 alone didn't phosphorylate ERK1/2 pathway (1.1±1, p=*NS*). These results confirmed the PhosphoFlow experiments.
(D) Western blot showing STAT5 phosphorylation in $CD34^+$-derived erythroid cells at day 10 of culture. 5U/mL EPO induced a strong STAT5 phosphorylation (fold increase in the P-STAT5/STAT5 ratio: 24.6±6.7, p<.05) that was stronger in the presence of 2µM YODA1 (33.1±6.3, p<.05). No STAT5 phosphorylation was observed with 2µM YODA1 stimulation in the absence of EPO (1.1±0.4, p=*NS*).
(E) In UT7/GM cells, 5ng/mL GMCSF induced STAT5 phosphorylation (fold increase in the P-STAT5/STAT5 ratio: 30.9±8.9, p<.001), much stronger than 10U/mL EPO (2.5±0.4, p<.001), whereas 5µM YODA1 alone did not (x0.93±0.12, p=*NS*). Experiments were carried out after serum and cytokine starvation for 6h. Stimulation was performed for 15min before fixation.

Supplemental figure 8. *In vitro* erythroid differentiation from normal and HX-sorted $CD34^+$ cells: dot plots showing CD71/GPA expression at day 10.
(A) $CD34^+$ cells from mobilized peripheral blood mononuclear cells (n=5) were cultured as described in Methods and in Suppl. Fig. 2A, CD71/GPA staining was performed at day 10.
(B) Same MFC dot plots at day 10 obtained from erythroid differentiation of sorted $CD34^+$ cells from HX patients. $CD34^+$ cells were collected from phlebotomy bags.

Supplemental figure 9. *In vitro* erythroid differentiation from normal and HX-derived mononuclear cells: dot plots showing CD71/GPA expression at day 10.
(A) MNC differentiation from healthy donors (n=9). Except one (diff. 8), all differentiations showed a homogeneous CD71/GPA expression pattern with a majority of $CD71^+/GPA^{high}$ mature cells.
(B) MFC dot plots at day 10 from erythroid differentiation of MNC from HX patients.

Supplemental figure 10. Kinetics of erythroid differentiation beyond day 10 in HX and control patients.

Cytological count of Pro-Erythroblast (ProE), Erythroblast Basophilic (Ebaso), Erythroblast Polychromatic (Epoly), Erythroblast Orthochromatic (Eortho) and enucleated cells (Enucl) of MNC erythroid differentiation at day 20-21/ day 24-25. Erythroid differentiation wasn't blocked since HX-derived progenitors could still enucleate, but in heterogeneous proportions according to the underlying mutations. No time points after d25 were performed so whether HX#7 or #5 reached total enucleation rate later is not known.

Supplemental tables

Sh-PIEZO1 clone	Clone ID	Targeted sequence	Position
#1	TRCN0000121969	CTCACCAAGAAGTACAATCAT	Exon #43
#2	TRCN0000141308	CCCTCTGCATTGATTATCCCT	Exon #22-#23
#3	TRCN0000142281	GCACTCCATTATGTTCGAGGA	Exon #51
#4	TRCN0000142459	GAAGACCACATCAGGTGGAA	Exon #39

Supplemental table 1. Location and targeted sequences and of the 4 Sh-RNA anti PIEZO1. Sequences were inserted in pLKO.1-CMV-tGFP vector.

Target molecule	Host species	Clone	Dilution	Isotype	Conjugated	Source
CD117	Mouse	A3C6E2	1:100	IgG1κ	PE-Vio770	Miltenyi Biotec
CD71	Mouse	AC102	1:300	IgG2aκ	PE	Miltenyi Biotec
GPA (CD235a)	Recombinant Human	REA175	1:100	IgG1	APC	Miltenyi Biotec
CD36	Mouse	AC106	1:100	IgG2aκ	FITC	Miltenyi Biotec
CD34	Mouse	8G12	1:40	IgG1κ	APC	BD Biosciences
CD45	Mouse	J33	1:40	IgG1	PC7	Beckman Coulter
CD123	Mouse	SSDCLY107D2	1:40	IgG1	Pacific Blue	Beckman Coulter
P-ERK1/2 (Thr202/Tyr204)	Mouse	20A	1:5	IgG1κ	Alexa Fluor 488 Alexa Fluor 647	BD Biosciences
P-STAT5 (Tyr694)	Mouse	47/stat5 (pY694)	1:5	IgG1κ	Alexa Fluor 647	BD Biosciences
PIEZO1	Rabbit	Polyclonal	1:100	IgG	Unconjugated	Proteintech
Isotype Control	Rabbit	Polyclonal	1:500	IgG	Unconjugated	Abcam
NFATc1	Mouse	7A6	1:5	IgG1	PE	Santa Cruz Biotechnology
Rabbit IgG	Goat	Polyclonal	1:200	N/A	Alexa Fluor 647	ThermoFisher Scientific

Supplemental table 2. Characteristics and references of antibodies used for flow cytometry and confocal imaging.

Target molecule	Host spieces	Clone	Dilution	Isotype	Conjugated	Source
ERK1/2	Rabbit	137F5	1:1000	IgG	Unconjugated	Cell Signaling
P-ERK1/2 (Thr202/Tyr204)	Rabbit	D13.14.4E	1:1000	IgG	Unconjugated	Cell Signaling
STAT5	Mouse	89/STAT5	1:1000	IgG	Unconjugated	BD Biosciences
P-STAT5 (Tyr694)	Rabbit	Polyclonal	1:1000	IgG	Unconjugated	Cell Signaling
Rabbit IgG	Goat	Polyclonal	1:2000	IgG	HRP	Sigma-Aldrich
Mouse IgG	Goat	Polyclonal	1:2000	IgG	HRP	Sigma-Aldrich

Supplemental table 3. Characteristics and references of antibodies used for Western blot.

Gene	Orientation	Sequence
GAPDH	Forward	5’- TCGGAGTCAACGGATTTGGTCG -3’
	Reverse	5’- AGGGCATCCTGGGCTACACTGA -3’
HPRT	Forward	5’- TGTAATCCAGCAGGTCAGCA -3’
	Reverse	5’- ACCCTTTCCAAATCCTCAGC -3’
Piezo1	Forward	5’- CATCTTGGTGGTCTCCTCTGTCT -3’
	Reverse	5’- GATGAGAGGGATGTGGATGCCAG -3’
GPA	Forward	5’- GGGGTGATGGCTGTTAT -3’
	Reverse	5’- CCTCACCTGACACAGACGTG -3’
GATA1	Forward	5’- AGCAGCTTCCTCCACTGCTG -3’
	Reverse	5’- CCTCACCTGACACAGACGTG -3’
GATA2	Forward	5’- TACAGCAGCGGACTCTTCC -3’
	Reverse	5’- CTCGTTCCTGTTCAGAAGGC -3’
Hbα	Forward	5’- GGTCAACTTCAAGCTCCTAAGCC -3’
	Reverse	5’- AAGAAGCATGGCCACCGAGG -3’
Hbβ	Forward	5’- CAACGTGCTGGTCTGTGTG -3’
	Reverse	5’- CACTAAGCTCGCTTTCTTGCTG -3’
Hbγ	Forward	5’- TCCTTGGGAGATGCCACAAA -3’
	Reverse	5’- GTGACCGTTTTGGCAATCCAT -3’
BMI-1	Forward	5'- CCACCTGATGTGTGTGCTTTG -3'
	Reverse	5'- ACAAGACCAGACCACTACTGAA -3'
STAT5A	Forward	5’- CTGGACTTTTCTGAAGGGGCT -3’
	Reverse	5’- AGAATAGCCGGGGGAATGTG -3’
EPO-R	Forward	5'- ATCCTGACGCTCTCCCTCAT-3'
	Reverse	5'- AGAGCGAGTTTGAAGGCCTC -3'
ALAS2	Forward	5'-AGGATGTGTCCGTCTGGTGTA-3'
	Reverse	5'- TCTCAGGCACCAGTAAGTTTCA -3'
SLC4A1	Forward	5’- GGGCTCAGATCACCGTAGAC -3’
	Reverse	5’- GTAGTCTGTGGCTGTTGCCT -3’
AHSP	Forward	5’- CTGACACTTGACTCCTTGCC -3’
	Reverse	5’- AGAGCCATCTTCAGGTCTAACAG -3’

Supplemental table 4. List of primers used for RT-qPCR.

Supplemental references

1. Giarratana M-C, Rouard H, Dumont A, et al. Proof of principle for transfusion of in vitro-generated red blood cells. Blood 2011;118(19):5071–5079.

2. Gautier E-F, Ducamp S, Leduc M, et al. Comprehensive Proteomic Analysis of Human Erythropoiesis. Cell Rep 2016;16(5):1470–1484.

3. Maguire O, Tornatore KM, O'Loughlin KL, Venuto RC, Minderman H. Nuclear translocation of nuclear factor of activated T cells (NFAT) as a quantitative pharmacodynamic parameter for tacrolimus. Cytometry A 2013;83(12):1096–1104.