SUPPLEMENTAL INFORMATION

Mitochondrial Localization and Moderated Activity are Key to Murine Erythroid Enucleation

Running Title: Mitochondria Regulate Erythroid Enucleation (Limit: 50 characters)

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SUPPLEMENTAL METHODS

Mice

C57BL/6 mice between 8-12 weeks old were used for all experiments. Protocols were approved by the Institutional Animal Care and Use Committee of the Icahn School of Medicine at Mount Sinai.

Seahorse Analysis

FACS-purified erythroblasts or RBCs were seeded into Cell-Tak treated Seahorse XF- 96 cell culture plates (Agilent, Cat# 101085-004) at $1x10^6$ cells/well. Plates were then subjected to the Seahorse XFe96 Analyzer and analyzed with the Seahorse XF glycolysis stress test kit (Agilent, Cat# 103344-100) and program.

Enucleation Assay

MMP-low or -high, or total Gate 3 ($CD45^-$, $TER119^+$, $CD44^{low}$, $TMRE^+$) erythroblasts were FACS purified and plated in untreated 96-well plates at a density of $1x10^6$ cells/mL with 100µL of StemSpan (Stem Cell Technology, Cat# 09650) + 2% FBS. Wells were then

processed and TER119^{+} cells were analyzed by flow cytometry to determine viability (DAPI), mitochondrial activity (TMRE; 100 nM; Sigma, Cat# 87917), and enucleation (DRAQ5; 5 µM; ThermoFisher Scientific, Cat# 62251) at indicated times or up to 18 hrs post plating. For experiments using small molecule inhibitors, drugs were added at the beginning of plating at the indicated concentration.

For substrate exclusion, DMEM minus glucose, glutamine, and sodium pyruvate (Gibco, Cat# A14430-01) was used with 2% FBS (minimal media). Glucose (10 mM; Gibco, Cat# A24940-01), glutamine (4 mM; Gibco, Cat# 25030-081), sodium pyruvate (4 mM; Gibco, Cat# 11360-070) or dimethyl α-ketoglutarate (1 mM) and methyl pyruvate (1 mM) were added separately as indicated for 18 hrs. Viability (DAPI), mitochondrial activity (TMRE), and enucleation were analyzed by flow cytometry.

ImageStream

Bone marrow (BM) cells or FACS-sorted erythroblasts of indicated stages were processed and stained with TER119-PE or TER119-PB and DRAQ5. For analyzing mitochondria, antibody stained cells were incubated with MitoTracker Green (100 nM) for 20 min at 37°C. Live stained or fixed cells were acquired with the ImageStream X (Mark II). Data was processed and analyzed using IDEAS 6.2 software (Amnis/EMD Millipore) and FlowJo (v10). Erythroblasts were identified by gating on in-focus (RMS-based) single cells expressing TER119 and displaying DRAQ5 fluorescence. In experiments using total BM, erythroblasts at each stage were further resolved based on cell size (TER119 morphology mask) and nuclear size (DRAQ5 morphology mask). For analyzing the correlation between MMP and enucleation, BM cells stained with TER119-PB, CD44-PECy7, DAPI, and TMRE were FACS-sorted for MMP-low or -high Gate 3 erythroblasts and then fixed with freshly prepared 4% formaldehyde solution for 20 min at RT, followed by two washes in PBS. Fixed cells were stained with DRAQ5 and TER119-PB before acquisition with ImageStream X.

Enucleating erythroblasts were identified based on Δ_{nuc}Centroid metric measuring nuclear displacement (the distance, delta centroid X/Y, between DRAQ5 mask and TER119 mask), as in[1]. Centered nuclei defined by Δ_{nuc}Centroid < 0.5; and polarized nuclei defined by Δ_{nuc}Centroid between 0.5 - 1.0 and enucleating cells were defined by Δ_{nuc}Centroid > 1.

Localized or evenly distributed mitochondria were determined based on the circularity metric and Δ_{mito}Centroid, with localized mitochondria displaying low circularity and high Δ_{mito}Centroid values. The circularity metric measures the average distance from the center to the boundary of the morphology-masked mitochondria divided by the variation of the distance, and assigns a higher value based on how close the overall shape is to a circle. To identify erythroblasts at early stage of mitochondrial localization, peak intensity instead of morphology mask of mitochondria was applied. The delta mito-centroid metric measures the distance between the centers of the mitochondrial mask and the TER119 mask.

Live Cell Imaging, Immunofluorescence and Confocal Microscopy

FACS purified erythroblasts were cytospun (Cytospin 3, Thermo Shandon) onto glass slides or retronectin pre-coated Ibidi chamber slides (250rpm, 3min) and fixed with 4% formaldehyde solution for 10min at RT. After three washes with 1 X PBS for 3 min each, cells were permeabilized and blocked using 0.2% Triton X-100/5% BSA solution for 30min. The slides were washed three times with PBS for 3 min each and incubated with the indicated antibodies and concentrations in PBS + 1% BSA overnight at 4°C. Slides were then washed three times and incubated with corresponding fluorescence conjugated secondary antibody (1:500) in PBS + 5% BSA for 1 hr at RT. Slides were mounted with Fluoroshield Mounting Medium containing DAPI (Abcam, Cat# ab104139). For live imaging of mitochondria, cells were first stained with TER119-PE, MitoTracker Green (100 nM; Invitrogen, Cat# M7514), and DRAQ5 (5 µM; ThermoFisher Scientific, Cat# 62251). Total nucleated erythroblasts ($TER119^+$, $DRAQ5^+$) were FACS purified and seeded into retronectin pre-coated Ibidi chamber slides for confocal analysis. Images for fixed and live cells were acquired on an LSM 880 Airyscan (Zeiss) with a 100x/1.46 Oil DIC objective for up to four hours. For live cells, Ibidi chamber slides were kept at 37°C with 5% CO_2 in IMDM (Gibco, Cat# 12440-053) + 2% FBS. Zen software was used to acquire and process images, while Fiji was used to analyze and quantify images [2,3].

Image Analysis

Mitochondria Localization

Original gray scale images or gray scale images from split channels of RGB images captured by confocal microscopy were analyzed using Fiji / ImageJ software (v. 2.1.0/1.53.c)[2]. Circular regions of interest were manually drawn to circumscribe erythroblasts based on the boundaries of TER119 expression. Within the region of interest, mitochondria (TOM20) distribution around the cell was determined by Azimuthal Average plugin (https://imagej.nih.gov/ij/plugins/azimuthal-average.html) pre-installed in Fiji /ImageJ software, which integrates the fluorescence intensity around the ring of radius resulting in a plot of integrated intensity versus angle. The plot was exported as a continuous variable table (Y: normalized integrated intensity/ X: angle of circumference, 1-360) for further analysis. The parameters for calculating Azimuthal Average were set as the following: Outer radius (pixels) was determined by the circumference of the region of interest; Inner radius (pixels) was set to include all TOM20 fluorescent signal within the ring between the outer and inner radius; Starting Angle (0), Integration Angle (180), Number of Bins (360).

Data processing, analysis, and interpretation:

Data tables generated from the previous step were preprocessed for correction to exclude software generated artefactual values of zero (NaNs) by replacing these values with the mean of its neighbor values. Data were normalized to the highest fluorescence value for each cell to a scale of 0-1 in order to dismiss day-to-day variance in intensity.

The normalized integrated intensities were then used to calculate Localization Index or Shannon Entropy. The leading assumption behind the analysis is that localized mitochondria would exhibit only a few peaks of intensity across their azimuth compared to those evenly distributed around the nucleus. Each of the two methods measures the distribution of mitochondria from a different perspective. Localization Index describes the width, height, the number of the mitochondrial intensity peaks and the distance between peaks. This method is sensitive to parameter (baseline) setting. Shannon Entropy measures the extent of randomness of peaks in a non-bias way without taking account for detailed property of the peaks [2,3].

Localization Index

The normalized data tables were imported to Prism and Area Under the Curve function was applied. The Area Under the Curve within "peaks of intensity" (AUC_Peak) over the total Area Under the Curve (AUC_total) of normalized integrated intensities was calculated, and then normalized to the total number of peaks (N_Peak). The same baseline was set for all cells per experimental group, determined by the lowest value just above background signal for the majority of cells in each experiment group. The peaks of intensity were defined as: height minimal 30% unless indicated otherwise **(Figs. S1C, S1D, S3F)** of the distance from the minimum to the maximum intensity above baseline; width > 5 degree. In order to account for the separation of peaks, the number of peaks per cell was further normalized to the fraction of Peak Separation over 180 degrees, which is the shortest distance (width of angle) between the centers of each pair of peaks around the circle. Less peak separation results in reduced value of normalized peak number ($N\_Peak_{Norm.}$).

$(N\_Peak_{Norm.}) = N\_Peak- (1 - Peak\ Separation_{1-2}/180) - (1 - Peak\ Separation_{2-3}/180) \ldots\ldots - (1- Peak\ Separation_{n-1-n}/180) - (1- Peak\ Separation_{n-1}/180)$.

The resulting Localization Index (LI) was thus formulated as:

$LI = (AUC\_Peak/AUC\_total) / N\_Peak_{Norm.}$ (**Supplemental** Figure S1C)

Shannon Entropy

The computation of Shannon Entropy was performed using MATLAB (MathWorks, v. R2019a). The null hypothesis is that the distribution of mitochondrial at different maturation stages or upon treatments will not show changes in mean entropy. Practically, the entropy of the signal can describe the amount of "surprise" (information) within the signal with higher levels of entropy corresponding to higher uncertainty (randomness) within signal (https://pure.mpg.de/rest/items/item_2383164_3/component/file_2383163/content) . Shannon entropy was calculated with the 'wentropy' function of the wavelet toolbox of MATLAB (MathWorks, R2019a) https://www.mathworks.com/help/wavelet/ref/wentropy.html. Since the integrated intensity signals of localized mitochondria will distribute less randomly around a cell's azimuth, they should show a consistent decrease in entropy compared to evenly distributed mitochondria.

The value of Shannon entropy decreases with decreasing random distribution of fluorescence peak intensity. The values of Localization Index (LI) ranges between 0 and 1. Higher LI value represent fewer number of fluorescence signal peaks, each occupying greater proportion of total fluorescence signaling.

Fluorescence Intensity Quantification

For determination of H3K9me3 abundance based on fluorescence, nuclei were first delineated based on DAPI staining. The intensity (raw integrated density metric) of H3K9me3 fluorescence within the nucleus was then calculated in Fiji on a per cell basis.

Nucleus Size

Thresholding for DAPI fluorescence was performed to delimit the nucleus. Area and perimeter of thresholded nuclei was determined using the analyze particles function in Fiji. For experiments determining the impact of motor proteins on mitochondrial localization, nuclear area was plotted against Localization Index and the coefficient of determination based on linear regression was determined.

Mass Spectrometry

An average of 1.2 x 10^6 FACS purified MMP-low and -high orthochromatic erythroblasts were used per sample with a total of 3 biological replicates. In brief, cells pellets were lysed, and protein was subjected to reduction, alkylation, and trypsin digestion (1:20 enzyme to substrate ratio). Samples were then analyzed on the mass spectrometer (Orbitrap Fusion Lumos ETD).

JC-1 Staining

BM cells were stained with markers identifying erythroblasts stages as described previously. After primary antibody staining, BM cells were incubated with JC-1 (2.5 µM) for 10 min at 37°C. Mitochondrial activity was quantified based on the ratio of erythroblasts with JC-1 aggregates (405 nm/610 nm) to monomers (488 nm/530 nm) within each gate. To help establish threshold between aggregates and monomers, a second set of samples were treated with BAM15 (20 µM) prior and during flow acquisition.

Human CD34 Purification and Culture

Peripheral blood was obtained from the New York Blood Center and $CD34^+$ cells were isolated by positive selection using the human CD34 Microbead kit from Miltenyi Biotec (Catalog No.130-046-702) as per manufacturer's instructions. Cells were cultured in Iscove's Modified Dulbecco's Medium (Gibco, Catalog No. 12440-053) supplemented with 3% human AB serum (Sigma, Catalog No. H4522-20mL), 2% FBS, 10 µg/mL insulin

(Sigma, Catalog No. I9278-5mL), 3 U/mL heparin (Sigma, Catalog No. H3393-10KU), and 200 µg/mL human holo-transferrin (Sigma, Catalog No. T4132-100 mg). The cell culture procedure comprised of 3 phases. In phase I (Days 0-6), $CD34^+$ cells were cultured at a concentration of 10^5 cells/mL in the presence of 10 ng/mL rh SCF (R&D systems, Catalog No. 255-SC), 1 ng/mL rh IL-3 (R&D systems, Catalog No. 203-IL), and 3 U/mL recombinant erythropoietin (Epogen Epoetin Alfa). In phase II (Days 7-11), IL-3 was taken out from the medium. In phase III (Days 12-21), SCF was taken out and transferrin was re-introduced at a concentration of 1 mg/mL. The concentration of cells was adjusted to 10^6 cells/mL on Day 11 and maintained accordingly during subsequent days. The cells were cultured in the presence of 5% CO_2 at 37°C. Slides for TOM20 staining were prepared using cells at indicated days of culture. For staining, 100,000 cells were taken in 25 µL medium and stained with the indicated antibodies for 30 min on ice in the dark. Cells were then washed once with 2% FBS/PBS solution and resuspended in the same buffer and stained with DRAQ5 and DAPI. The flow gating was performed on GPA^+ cells [4].

Fetal Liver Cells

To stain isolated erythroblasts, E14.5 fetal livers were dissociated into phosphate-buffered saline by gentle trituration using a cutoff P200 tip, followed by overnight fixation in 4% paraformaldehyde in PBS at RT. Cells were permeabilized in 0.3% Triton-X-100 in PBS for 10min at RT, blocked in blocking buffer (3% BSA/1% goat serum in PBS) overnight at 4°C. Erythroblasts were incubated with primary antibody diluted in blocking buffer overnight 4°C. Cells were stained with secondary antibodies diluted in blocking buffer for 2 hrs at RT. Stained erythroblasts were deposited onto coverslips using a Thermo-Fisher Cytospin 4 at 900 to 1000 rpm for 3 min, mounted on slides using Prolong Gold mounting medium. Images were acquired on a Zeiss LSM 780 laser scanning confocal microscope with a 100x/1.4 numerical aperture objective, zoom 2. Collected images were processed using Adobe Photoshop and Illustrator.

ATP Assay

FACS-sorted orthochromatic erythroblasts were treated with BAM15 for 4 hrs . Cells were then collected and subjected to analysis with the ATP Bioluminescence Assay kit HS II (Roche) per manufacture's instruction. Bioluminescence was read using the EnSpire Plate Reader (PerkinElmer).

Ki67 Staining

FACS purified orthochromatic erythroblasts (MMP-low and -high) were pelleted (2000 rpm, 6 min) and fixed with 4% paraformaldehyde in the dark for 30 min at 4°C. Fixed erythroblasts were washed two times in 2% FBS/PBS and permeabilized with 0.15% Triton X-100 for 15 min. Cells were then incubated with anti-Ki67 antibody (1:100 dilution)

for 1hr at RT. Finally, cells were washed twice and resuspended in 2% FBS/PBS containing DAPI (1 µg/mL) and analyzed by flow cytometry.

Immunoblotting

FACS purified erythroblasts (MMP-low and -high) were pelleted and lysed in 1X RIPA buffer (Boston Bioproducts, Cat# BP-115) for 5-10 min at 4°C and then centrifuged at 14,000 g for 15 min at 4°C. Protein samples were resolved on 15% or 4-20% SDS- PAGE gels using equal cell numbers in each well. These were then transferred to nitrocellulose membrane and probed by specific antibodies as recommended by manufacturer. The membrane was then washed with 1X TBS-T (0.1%) and developed using ECL substrate (Millipore, Cat# WBKLS0500) and X- ray films (Thermo Scientific, Cat# 34090).

Flow Cytometry and Sorting

To identify and purify erythroblast populations, bone marrow (BM) cells were obtained from flushing tibia and femurs with IMDM + 2% FBS. Cells were filtered, washed with 2% FBS/PBS, and stained with TER119-FITC, CD44-PB, and CD45-APC (1:100 dilution) in 2% FBS/PBS for 10 min at RT. After staining with primary antibodies cells were washed, incubated with 100 nM TMRE for 15 min at 37°C, washed, and finally resuspended in IMDM + 2% FBS containing DAPI (viability 1 µg/mL) and DRAQ5 (5 µM). For flow cytometry analysis and analysis of the enucleation assays, stained cells were acquired on the BD LSRII. For erythroblast purification, stained cells were FACS-sorted with the BD Influx. For experiments analyzing MMP-low and -high fractions, orthochromatic erythroblasts displaying TMRE fluorescence intensities up to the 20^{th} percentile (MMP-low) and above the 80^{th} percentile (MMP-high) were analyzed or FACS-sorted. For analysis with the probes JC-1 (2.5 µM) and MitoTracker Green (100 nM), cells were incubated with probe after primary antibody staining for 20min at 37°C.

Statistics

Statistical analyses were carried out using GraphPad Prism (GraphPad). Student's t-test was carried out for differences between 2 groups unless specified. For comparisons between more than 2 groups, one-way ANOVA with Tukey's multiple comparisons test was carried out. All experiments were carried out 3 times independently with 2 or 3 technical replicates unless specified. Error bars indicate S.E.M. or S.D. and have been indicated in the figure legends. The sample size (number of mice used) is mentioned in the figure legends.

MATLAB Code:

```
% Read the data
G_data = G3_orig;
```

```
len = size(G_data,2);
G_Cop= G_data;

% Data preprocessing: Remove 0s and NaNs from the data
for i = 1:len
    for j = 2:size(G_data,1)-1
        if G_data(j,i) == 0 || isnan(G_data(j,i))
           G_Cop(j,i) = (G_data(j-1,i)+G_data(j+1,i))/2;
        end
    end
     if G_data(1,i)==0 || isnan (G_data(1,i))
        G_Cop(1,i) = G_Cop(2,i);
    end
    if G_data(360,i)==0 || isnan (G_data(360,i))
        G_Cop(360,i) = G_Cop(359,i);
    end
end
T_G3 = G_Cop;


% Shannon entropy
for i=1:size(T_G3,2)
    G1_e(i) = wentropy((T_G3(:,i)/max(T_G3(:,i))),'shannon');
end

% Entropy Threshold (Calculated but not reported)
for i=1:size(T_G3,2)
    G1_eth(i) = wentropy((T_G3(:,i)/max(T_G3(:,i))),'threshold',0.3);
end
```

Supplemental Table 1

(*Primary antibodies used in flow cytometry, immunofluorescence, and immunoblotting*)

Antibody	***Source***	***Species***	***Clone***	***Dilution***	***Catalog Number***
FLOW CYTOMETRY					
Mouse TER119 (FITC)	BD Biosciences	Rat	TER-119	1:100	557915
Mouse TER119 (PE)	BD Biosciences	Rat	TER-119	1:100	553673
Mouse CD44 (V450)	BD Biosciences	Rat	IM7	1:100	560451
Mouse CD45 (APC)	BD Biosciences	Rat	30-F11	1:100	559864
Ki-67 (PE)	Invitrogen	Rat	SolA15	1:100	12-5698-80
IMMUNOFLUORESCENCE					
TOM20	Santa Cruz	Rabbit	FL-145	1:100	sc-11415
Tri-Methyl-Histone H3 (Lys9)	Cell Signaling	Rabbit	D4W1U	1:100	13969S
Tubulin-Microtubule Marker	Abcam	Rat	YOL1/34	1:100	ab6161
VDAC1/Porin	Abcam	Mouse	20B12AF2	1:100	ab14734
GOLM1	Novus Biologicals	Rabbit	------	1:100	NBP1-77079
IMMUNOBLOTTING					
Acetyl-Histone H3 (Lys27)	Cell Signaling	Rabbit	D5E4	1:1000-1:2000	8173T
Acetyl-Histone H3 (Lys9)	Cell Signaling	Rabbit	C5B11	1:1000-1:2000	9649T
Histone H3	Cell Signaling	Rabbit	D1H2	1:1000-1:2000	4499T

CD49d (α4 integrin), CD235a (Glycophorin A), and CD233 (Band 3) Primary Antibodies were kind gifts from Dr.Xiuli An's laboratory (New York Blood Center).

Supplemental Table 2

(*Secondary antibodies*)

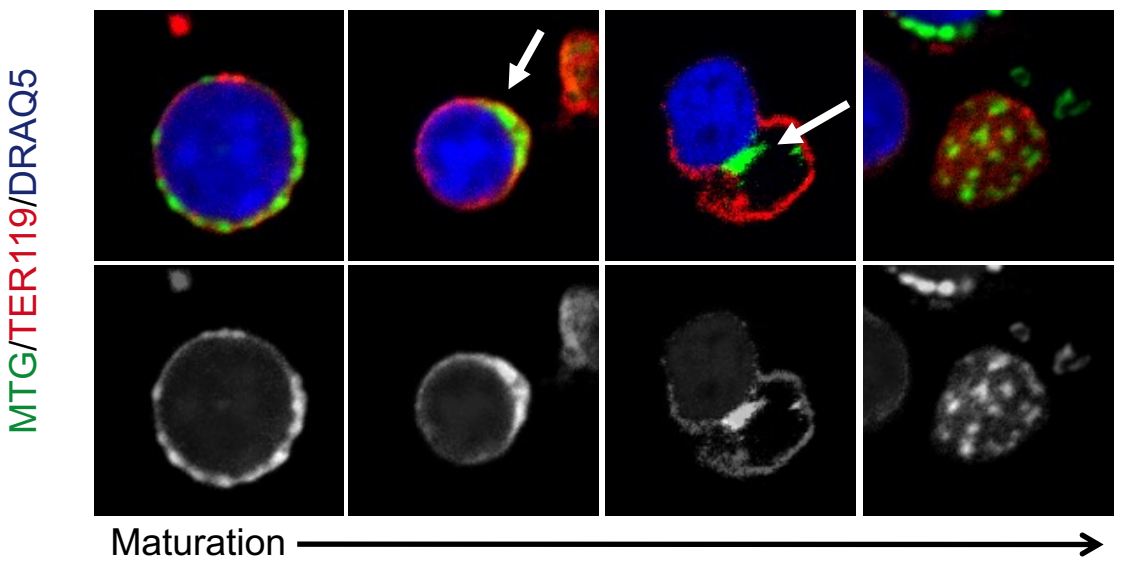
Antibody	***Source***	***Species***	***Fluorophore***	***Dilution***	***Catalog Number***
IMMUNOFLUORESCENCE					
Anti-Rabbit	Invitrogen	Goat	Alexa Fluor 594	1:400	A11012
Anti-Rat	Invitrogen	Goat	Alexa Fluor 594	1:400	A11007
Anti-Mouse	Invitrogen	Goat	Alexa Fluor 647	1:400	A21235
IMMUNOBLOTTING					
Anti-Rabbit	Jackson ImmunoResearch	Goat		1:2000-1:4000	111-035-003

Supplemental Table 3
(*Inhibitors/Activators*)

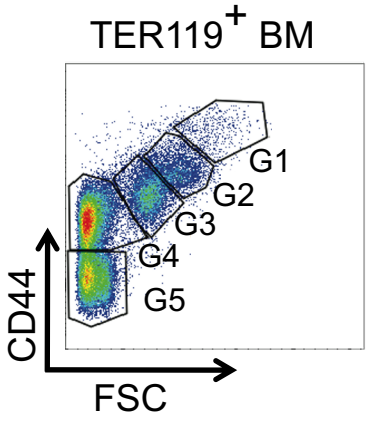
INHIBITORS			
Compound	***Inhibits***	***Source***	***Catalog Number***
Monastrol (MON)	Kinesin Eg5	Millipore Sigma	475879-1MG
Etomoxir sodium salt hydrate (Etox)	Fatty Acid Transport	Sigma	E1905
Ciliobrevin D (CBD)	Dynein	Calbiochem	250401
BAM15	Uncoupler (OXPHOS)	Millipore Sigma	SML1760-5MG
Oligomycin	ATP synthase	Sigma	75351
Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP)	Uncoupler (OXPHOS)	Sigma	C2920
Carbonyl cyanide 3-chlorophenylhydrazone (CCCP)	Uncoupler (OXPHOS)	Millipore Sigma	C2759-250MG
Rotenone (Rot)	Respiration (Complex I)	Millipore Sigma	557368
Antimycin A (AA)	Respiration (Complex III)	Millipore Sigma	A8674-25MG
SSO	Fatty Acid Translocase (CD36/FAT)	Cayman Chemicals	11211
CHC	Monocarboxylic Acid Transport (MCT)	Millipore Sigma	C2020-10G
UK-5099	Mitochondrial Pyruvate Carrier (MPC)	Millipore Sigma	PZ0160-5MG
NHI-2	Lactate dehydrogenase-A (LDH-A)	Sigma	SML1463
2-Deoxy-D-glucose (2-DG)	Glycolysis (Competitive inhibitor of Hexokinase 2)	Sigma	D8375
DON (6-Diazo-5-oxo-L-norleucine)	Glutamine analog	Sigma	D2141
AR-C155858	Monocarboxylate transporter 1/2 (MCT1/2)	TOCRIS	4960
ACTIVATORS			
Compound	***Activates***	***Source***	***Catalog Number***
Dimethyl 2-oxoglutarate (MOG)	TCA Cycle	Millipore Sigma	349631-5G
Methyl Pyruvate (MP)	TCA Cycle	Millipore Sigma	371173-25G

Figure S1

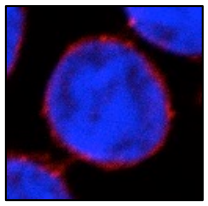
A



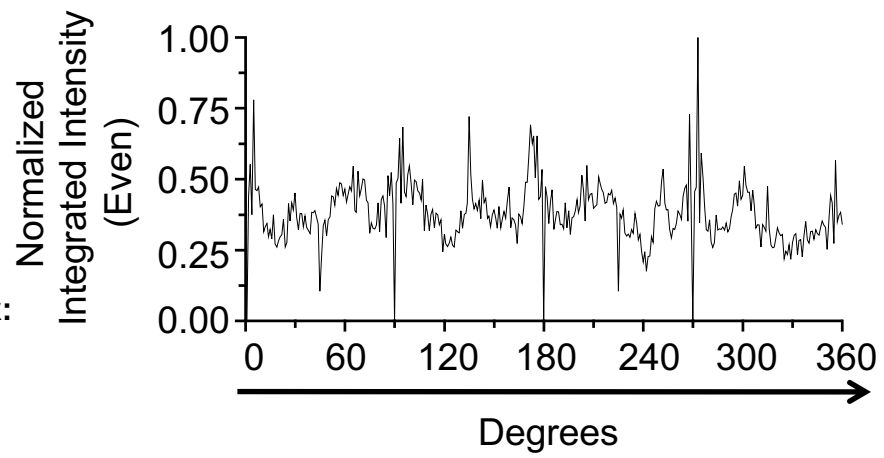
B

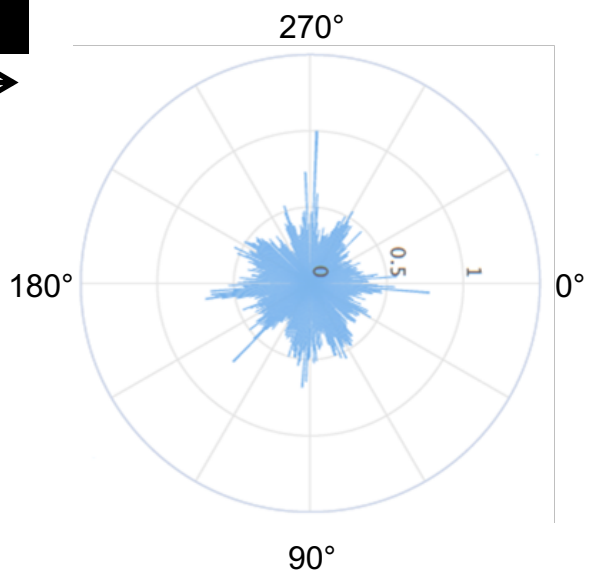


C



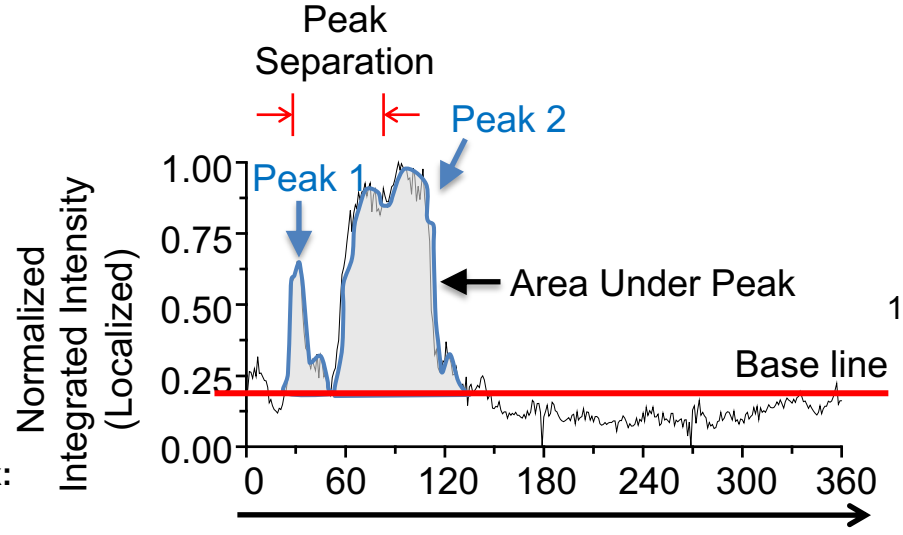
Localization Index: 0.03

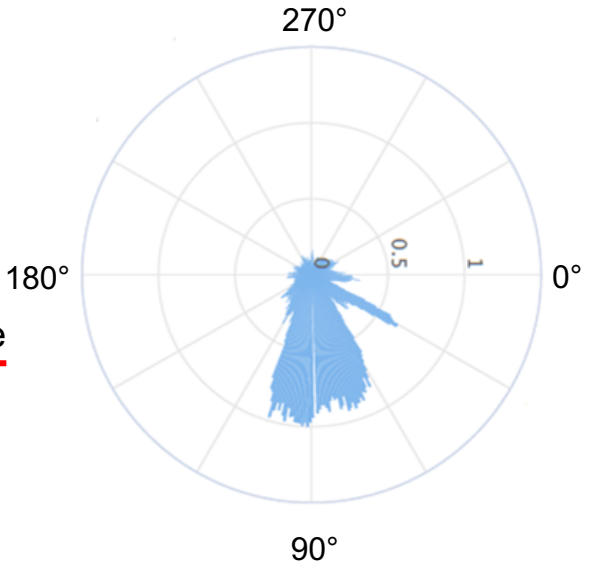




Peak Separation

Localization Index: 0.87





Localization index = (AUC_Peak / AUC_total) / $N\_Peak_{Norm.}$

$N\_Peak_{Norm.}$ = N_Peak - (1 - Peak Separation$_{1\text{-}2}$/180) - …. - (1- Peak Separation$_{n\text{-}1\ -\ n}$/180) - (1- Peak Separation$_{n\ -\ 1}$/180)

D

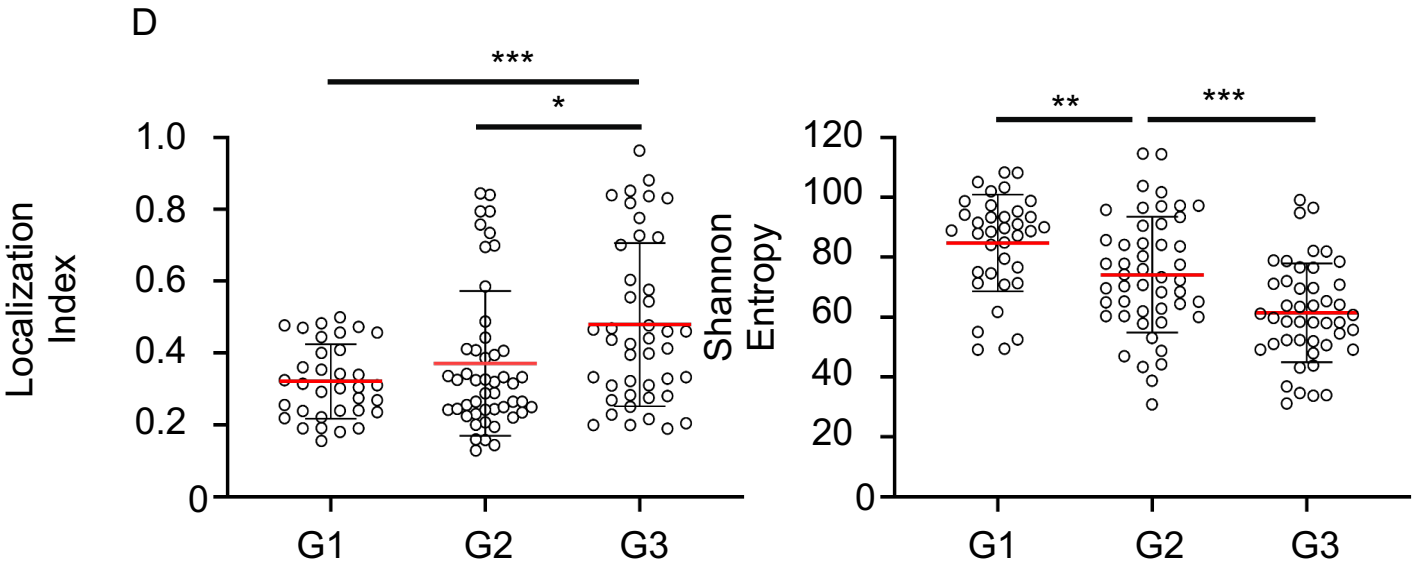
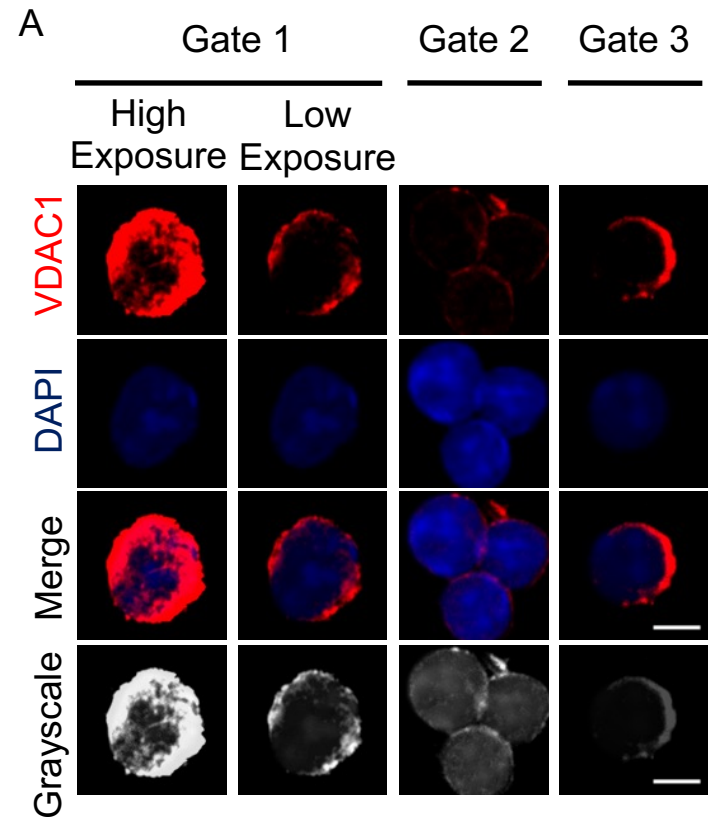
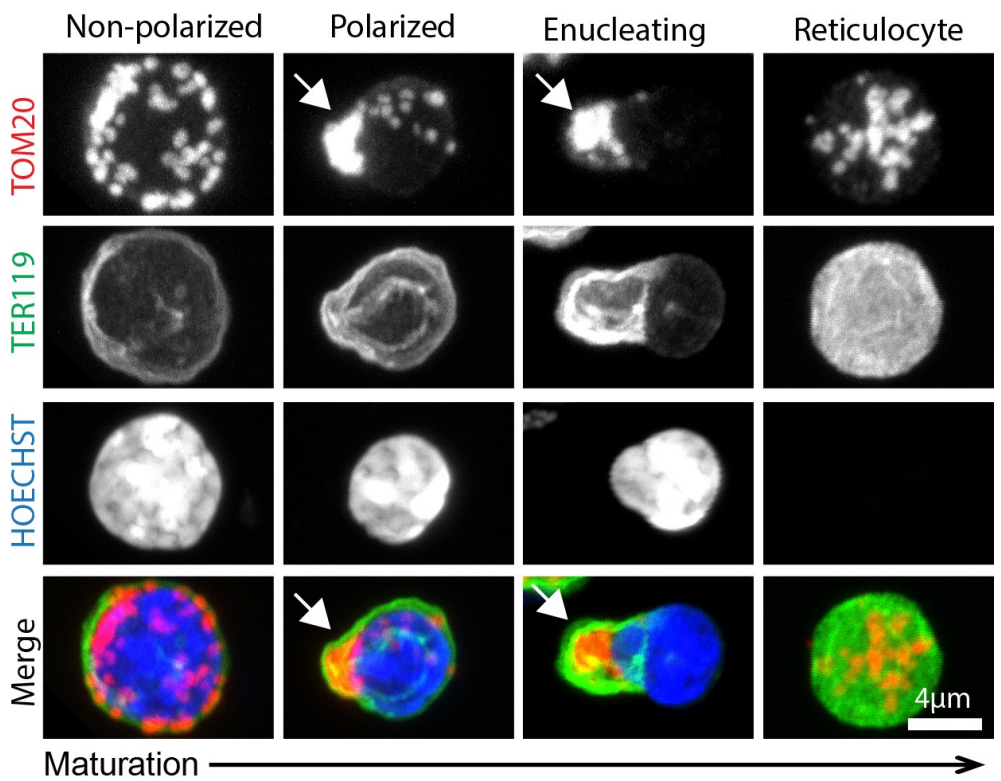
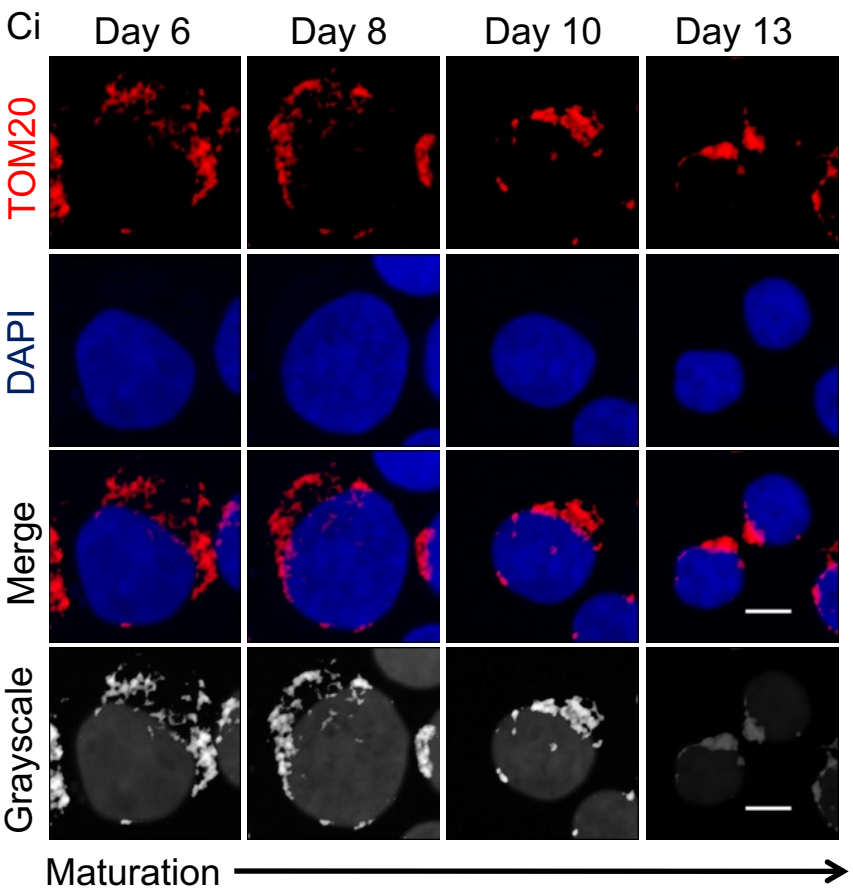


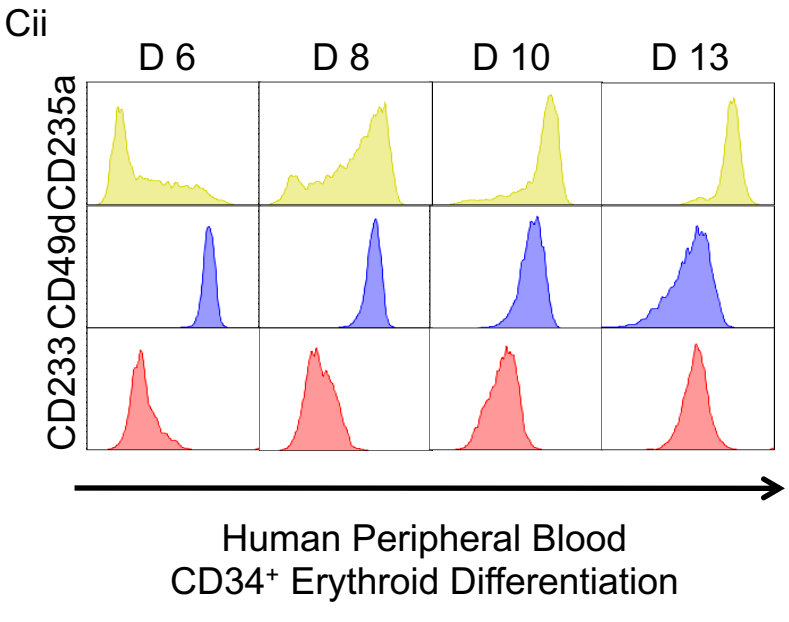
Figure S1: Mitochondrial localization index in maturing murine erythroblasts
A. Same as, and enlarged Fig. 1A. Snapshots of live confocal imaging of bone marrow erythroblasts to identify mitochondrial localization during increasing stages of erythroblast maturation. Cells were stained with DRAQ5 (nucleus, blue), MitoTracker Green (MTG; mitochondria green), and TER119 (erythroid marker, red). Arrows show mitochondrial localization. Retic; reticulocyte. **B.** Flow cytometry analysis of maturation of post-progenitor erythroblasts using forward scatter (FSC), TER119 and CD44 maturation**. C.** Left: Representative confocal images of even (top) or localized (bottom) (right) mitochondria labeled by TOM20. Middle, Right: Histogram (Middle) or radar chart (right) of Azimuthal average (integrated intensity / angle of circumference). The value of peak numbers (N_Peak) were normalized to Peak separation, the distance (width of angle) between adjacent peaks. Localization Index of mitochondria (TOM20) was determined as in Methods. Note erythroblasts with mitochondria localized in one area of the cell displayed fewer peaks including most of the fluorescence and resulting in greater Localization Index values (bottom) as compared to erythroblasts with evenly distributed mitochondria (top). **D.** The value of Localization Index (left) and Shannon Entropy (right) of freshly sorted Gate 1, 2, 3 erythroblasts [same data of G1, 2, 3 (without treatment) used as in **Figs. S4F, S4G**]. Similar results as in **Fig. 1C** with data generated from distinct set of mice. Results expressed as mean $\pm$ SD, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, student's t-test.

Figure S2


A
Gate 1
Gate 2
Gate 3
High Exposure
Low Exposure
VDAC1
DAPI
Merge
Grayscale


B
Non-polarized
Polarized
Enucleating
Reticulocyte
TOM20
TER119
HOECHST
Merge
4µm
Maturation


Ci
Day 6
Day 8
Day 10
Day 13
TOM20
DAPI
Merge
Grayscale
Maturation


Cii
D 6
D 8
D 10
D 13
CD235a
CD49d
CD233
Human Peripheral Blood
CD34⁺ Erythroid Differentiation

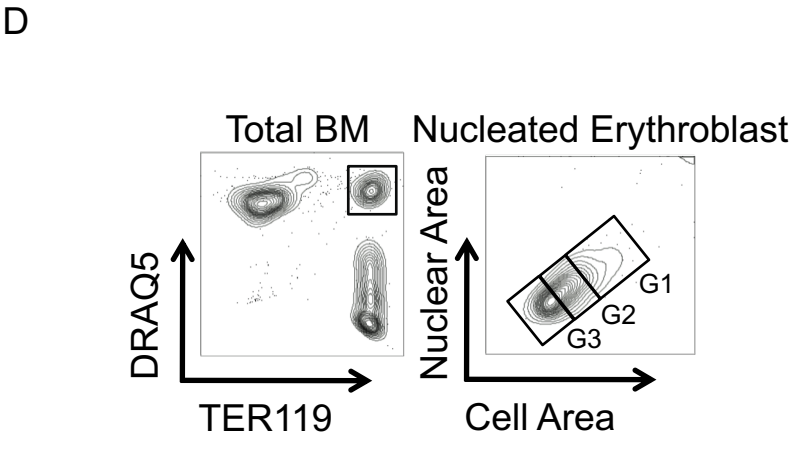
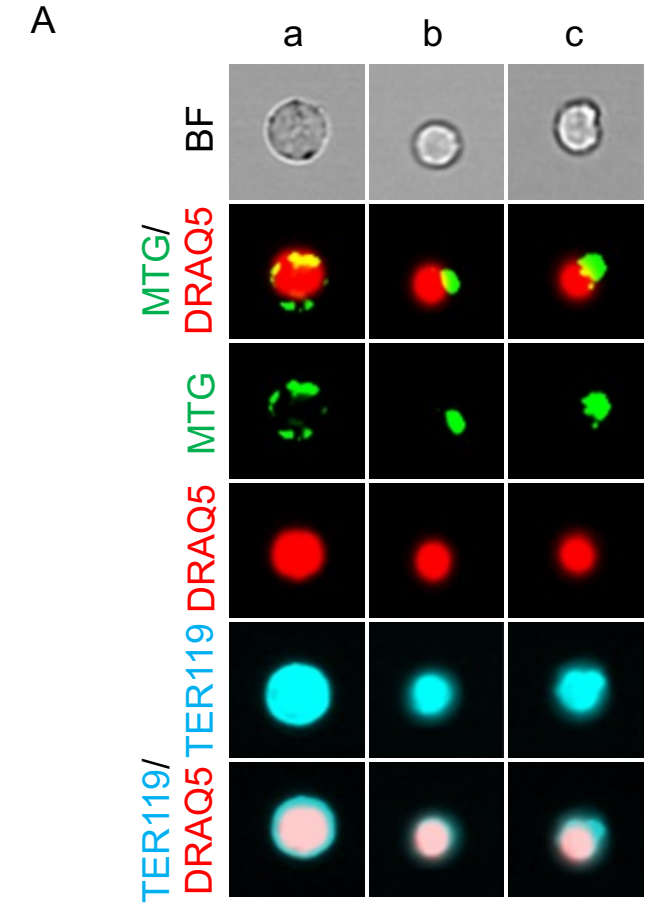

D
Total BM
Nucleated Erythroblast
DRAQ5
TER119
Nuclear Area
Cell Area
G1
G2
G3

Figure S2: Mitochondria localize progressively behind the nucleus during terminal erythroblast maturation

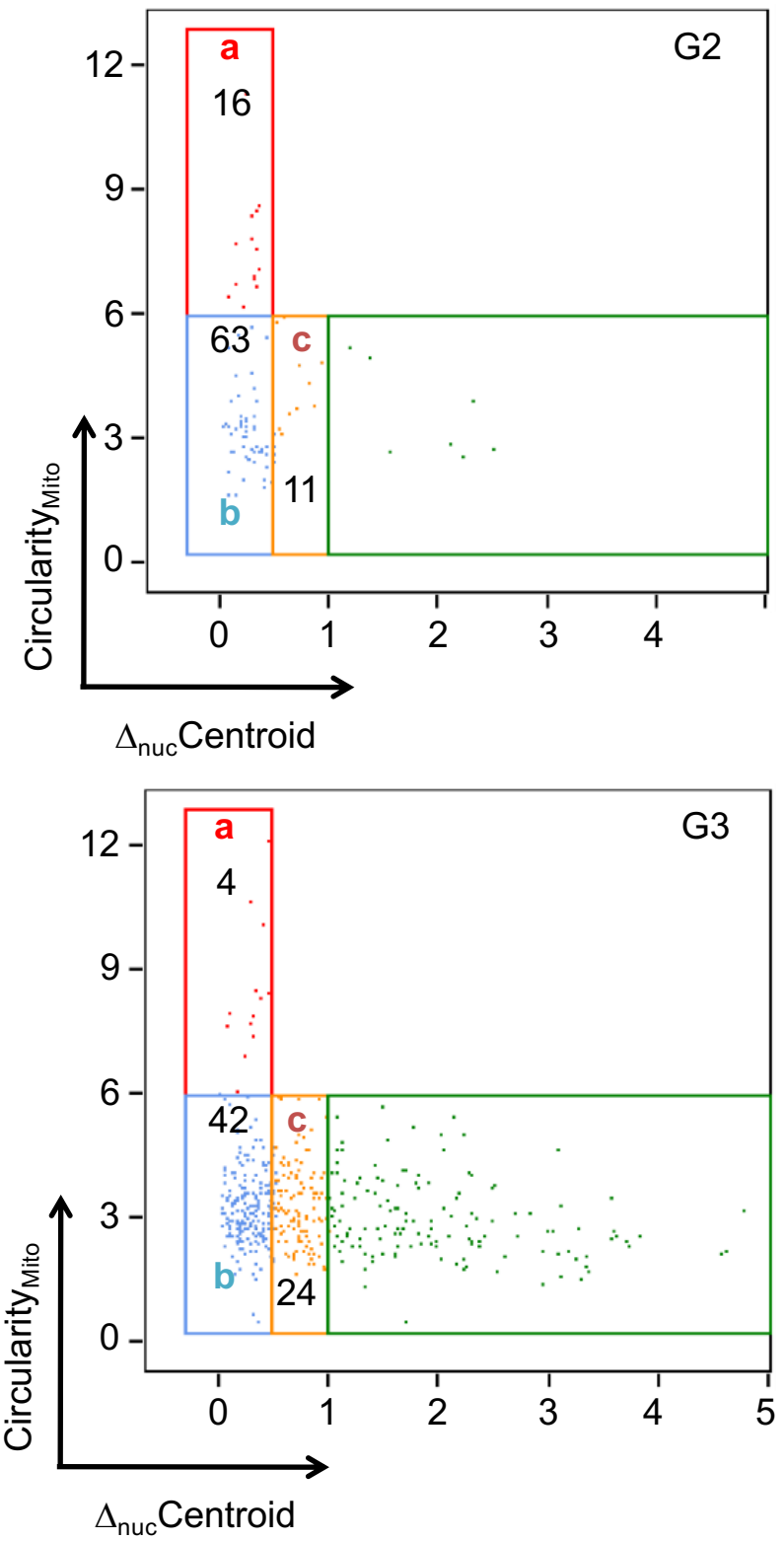
A. Specific stages of TER119$^+$ murine erythroblasts were resolved as in (**Supplemental Fig. 1B**) and fixed for confocal imaging of mitochondria (VDAC1, red), nucleus (DAPI, blue). Gate 1 is shown in both low and high exposures. **B.** Representative images of confocal analysis of mitochondrial localization in murine primary fetal liver erythroblasts stained with TOM20 (mitochondria, red), DAPI (nucleus, blue), and TER119 (erythroid marker, green) during maturation. Arrows show mitochondrial localization; retic: reticulocytes. **C. (i).** Representative images of confocal analysis of mitochondrial localization in human peripheral blood CD34$^+$ cell-derived erythroblasts *in vitro* stained with TOM20 (mitochondria, red) and DAPI (nucleus, blue) (one representative of three independent experiments) **(ii).**The human CD34$^+$ cell-derived erythroblasts cultured *in vitro* were stained with indicated antibodies to monitor erythroid differentiation. Briefly, cells in different phases of erythroid differentiation; Phase 1: Day 6 (comprising mainly proerythroblasts), Phase 2: Days 8 and 10 (comprising mainly proerythroblasts, early and late basophilic erythroblasts), and Phase 3: Day 13 (comprising mainly of polychromatic and orthochromatic erythroblasts), were stained with cell surface markers CD235a (Glycophorin A) and CD233 (Band 3) which showed increased expression during differentiation, and CD49d (α4 integrin) showing a progressive decrease in expression during differentiation (Cii). **D**. ImageStream based analysis of nucleus and cell size using maturation stages resolved in nucleated erythroblasts (CD45$^-$TER119$^+$, DRAQ5$^+$) from Gate 1 to Gate 3 (Gate 1: Proerythroblasts/Basophilic Gate 2: Polychromatophilic; Gate 3: Orthochromatic).

Figure S3

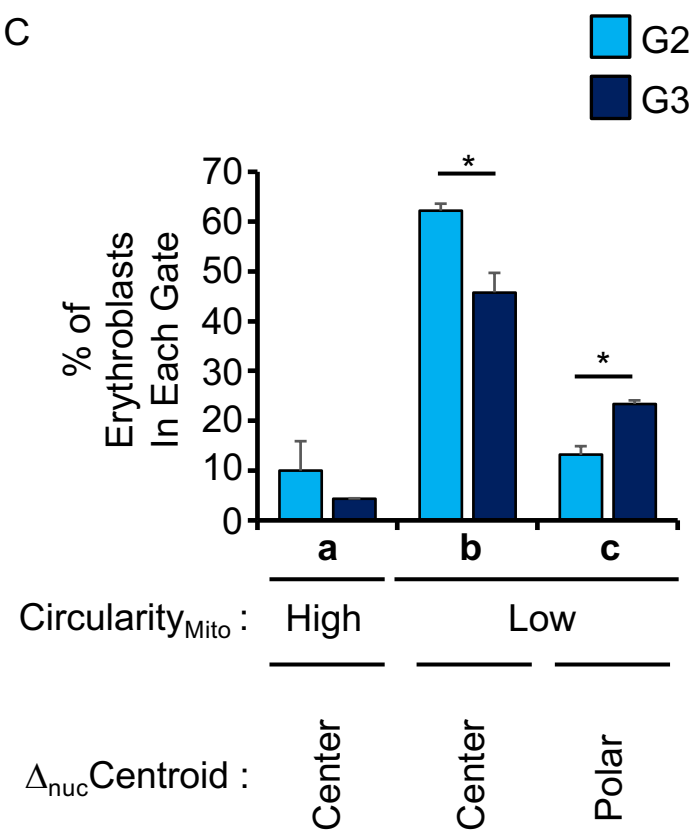
A



B



C



D

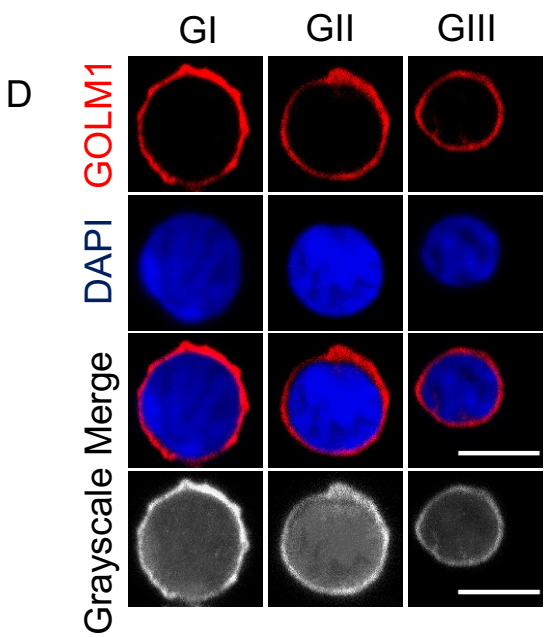
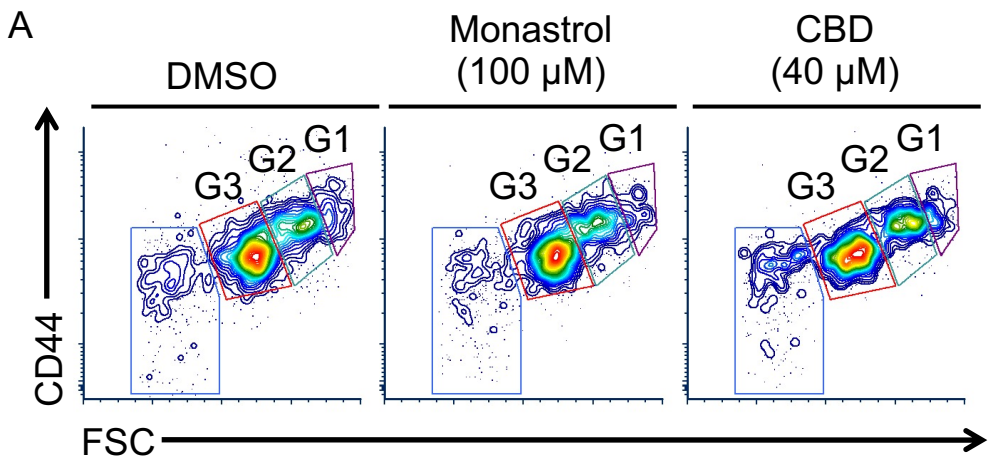
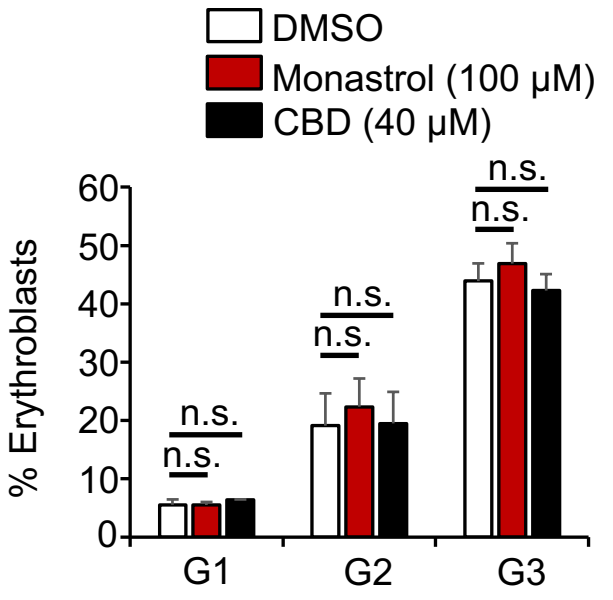


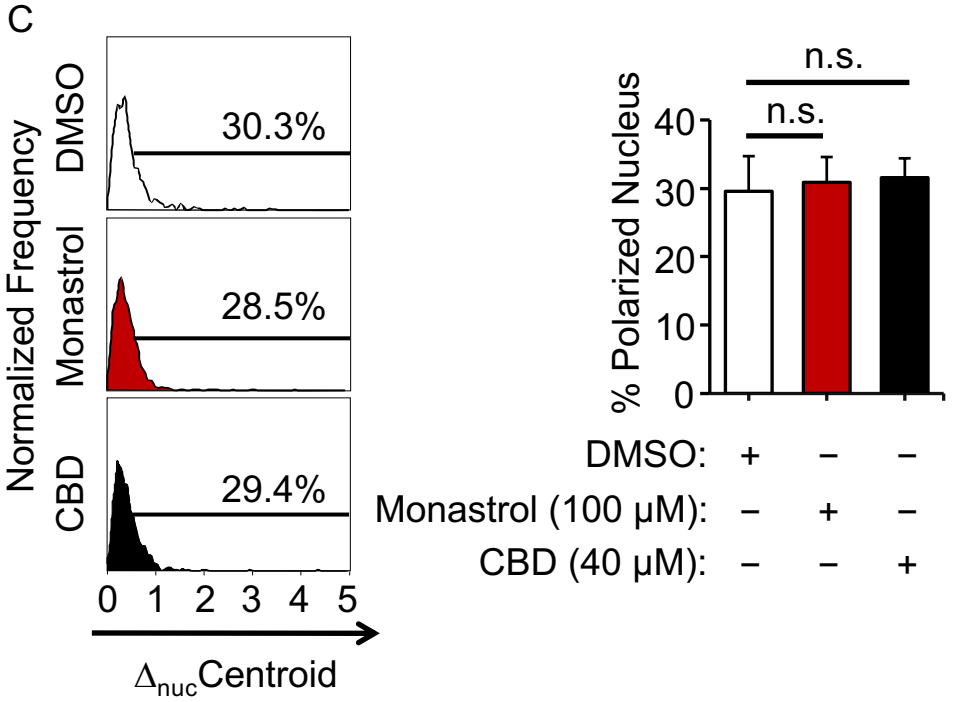
Figure S3: Mitochondrial localization precedes nuclear polarization during erythroblast maturation

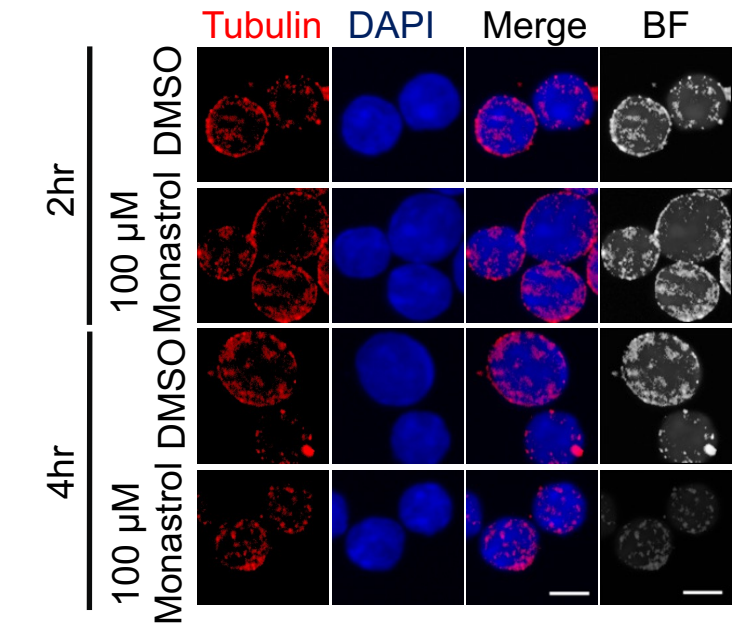
A. Representative ImageStream flow cytometry analyses of erythroblasts stained with MitoTracker, DRAQ5, and TER119-PB, showing mitochondrial circularity ($Circularity_{Mito}$), and centered ($\Delta_{nuc}Centroid < 0.5$) or polarized nucleus ($0.5 < \Delta_{nuc}Centroid < 1$). **B.** Representative ImageStream scatter-plots of $Circularity_{Mito}/\Delta_{nuc}Centroid$ of Gate 2 (top) or Gate 3 (bottom) erythroblasts. Gates a, b, c as indicated in A. **C**. The percentage of cells with high $Circularity_{Mito}$ and low $\Delta_{nuc}Centroid$ (centered nucleus) (a), low $Circularity_{Mito}$ and centered (b) or polarized (c) nucleus among total Gate 2 versus Gate 3 erythroblasts. Results as mean $\pm$ SD, $^{*}P < 0.05$, student's t-test. **D.** Representative confocal images of the golgi localization in maturing erythroblasts stained with GOLM1 (the golgi marker, red) and DAPI (nucleus, blue); bar = 5 μm.

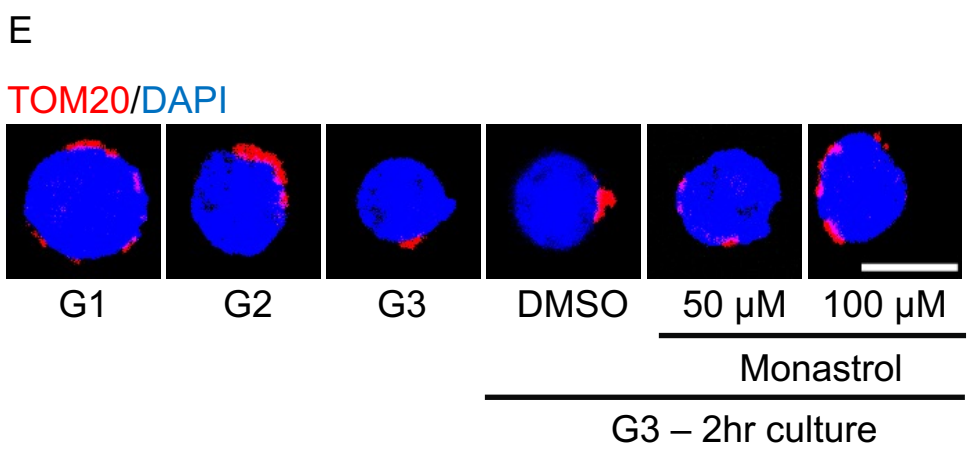
Figure S4

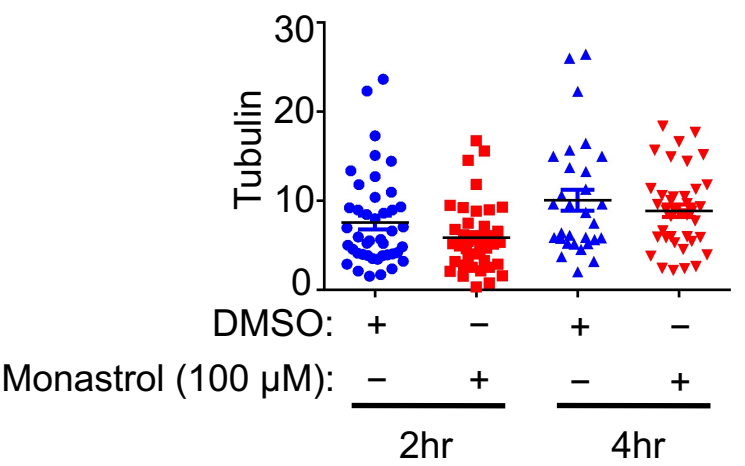

A
DMSO
Monastrol (100 μM)
CBD (40 μM)
G1
G2
G3
CD44
FSC

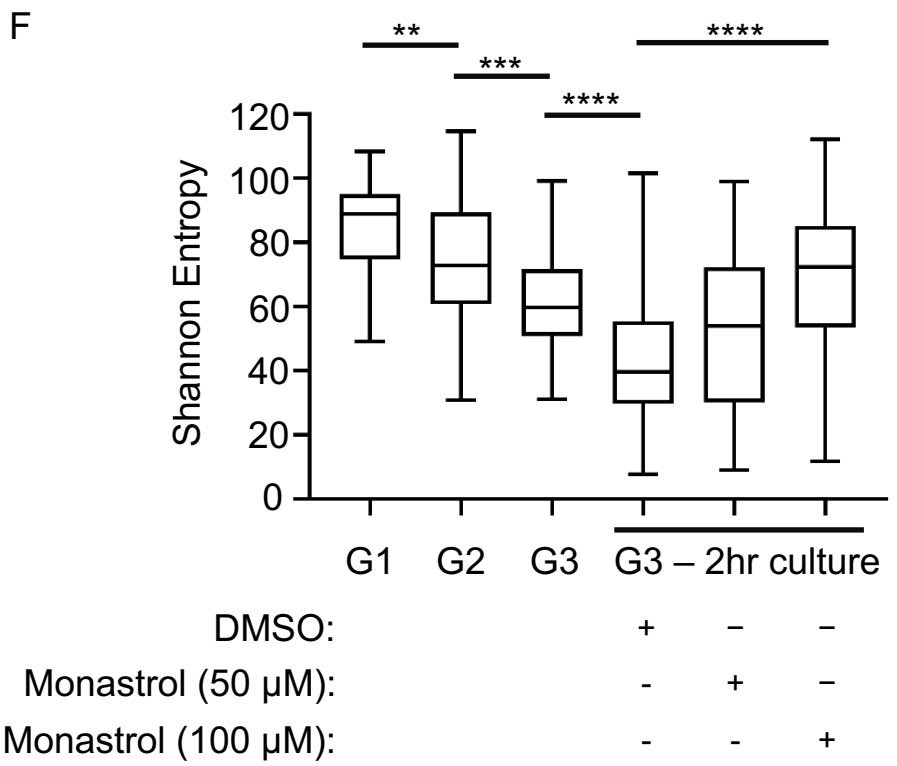

B
DMSO
Monastrol (100 μM)
CBD (40 μM)
% Erythroblasts
n.s.
G1
G2
G3


C
Normalized Frequency
DMSO
Monastrol
CBD
30.3%
28.5%
29.4%
Δnuc Centroid
% Polarized Nucleus
n.s.
DMSO:
Monastrol (100 μM):
CBD (40 μM):


D
Tubulin
DAPI
Merge
BF
2hr
4hr
DMSO
100 μM Monastrol


E
TOM20/DAPI
G1
G2
G3
DMSO
50 μM
100 μM
Monastrol
G3 – 2hr culture


Tubulin
DMSO:
Monastrol (100 μM):
2hr
4hr


F
Shannon Entropy
G1
G2
G3
G3 – 2hr culture
DMSO:
Monastrol (50 μM):
Monastrol (100 μM):

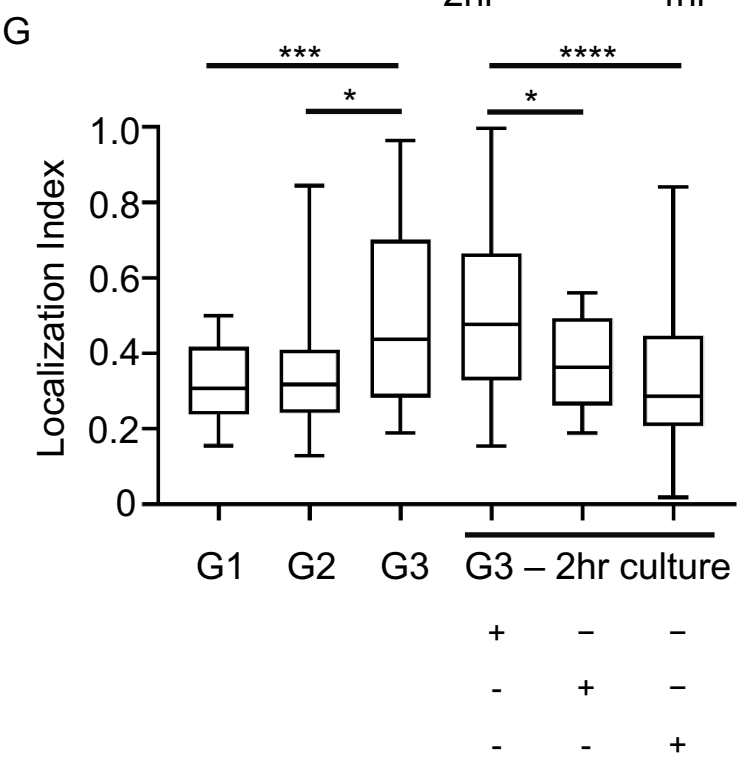
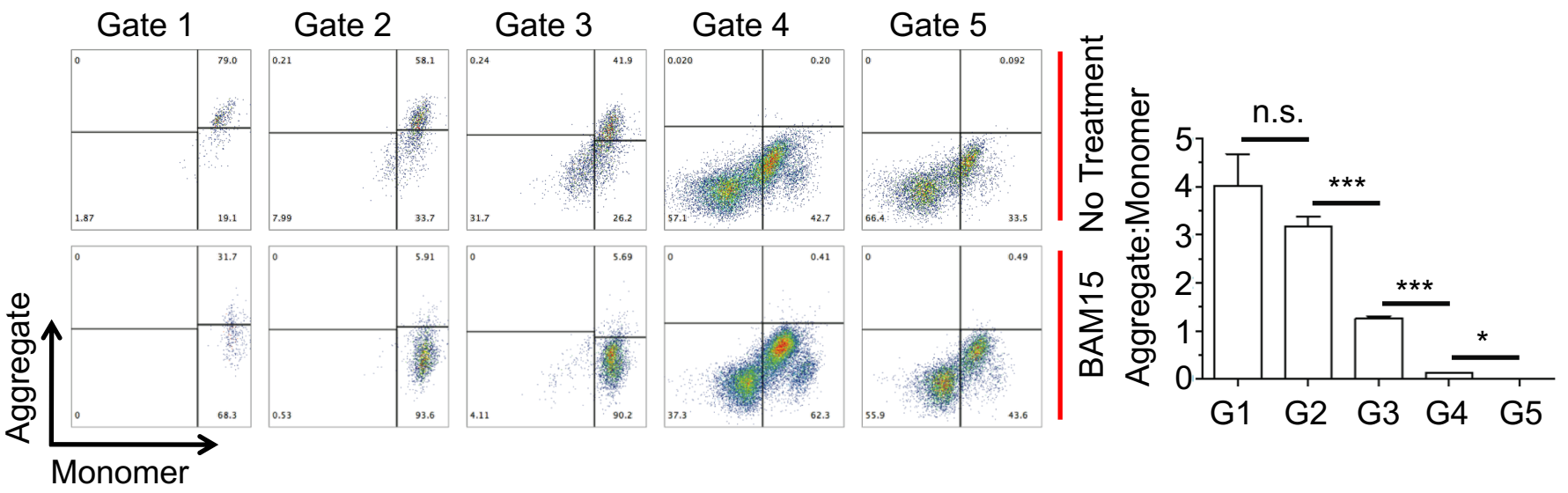

G
Localization Index
G1
G2
G3
G3 – 2hr culture

Figure S4: Inhibition of mitochondrial localization represses erythroid enucleation process

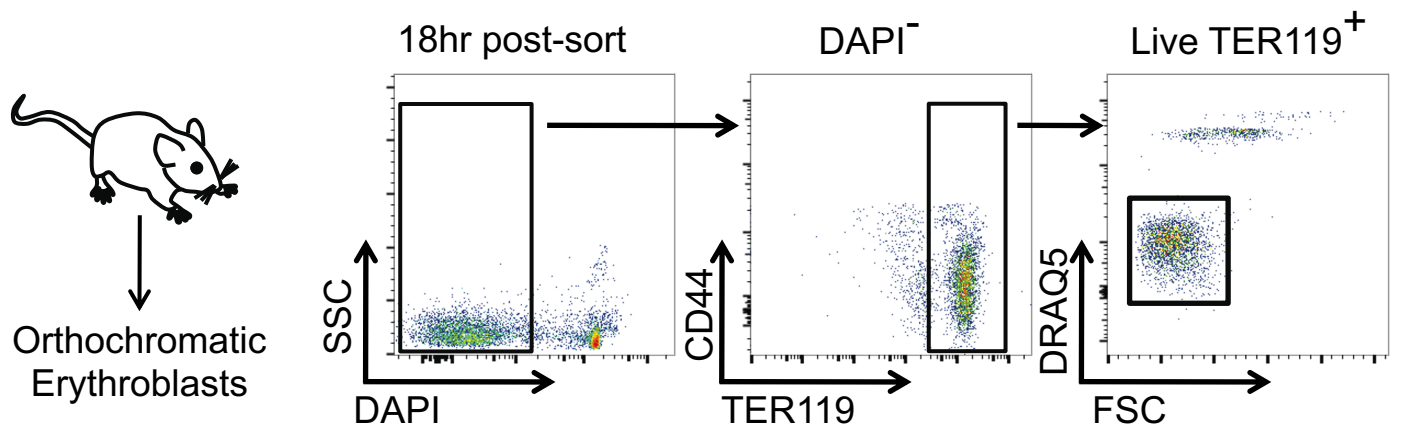
A. Representative FACS profile after 4 hrs incubation of sorted total erythroblasts from Gates 1 to 3 in the presence of DMSO, 100 μM monastrol, or 40 μM CBD. **B.** The percentage of erythroblasts in each gate relative to total (G1+G2+G3). **C.** Representative histogram of Δ_{nuc}Centroid of FACS sorted orthochromatic erythroblasts **(left)** incubated with DMSO, monastrol (100 uM) or CBD (40 uM) for 4 hrs in StemSpan medium with 2% FBS. Percentage of cells with polarized nucleus (Δ_{nuc}Centroid > 0.5) in total orthochromatic erythroblasts quantified (**right**). Results as mean ± SD, ns. $P > 0.05$ (n=3). **D.** Quantification of Tubulin in murine orthochromatic erythroblasts after 2 hrs and 4 hrs treatment with DMSO control or Monastrol. One representative of two independent experiments is shown; bar = 5 μm. **E.** Representative confocal images of TOM20 (mitochondria, red) and DAPI (nucleus, blue) during erythroid maturation and G3 erythroblasts incubated for 2 hrs with DMSO, or monastrol (50 μM or 100 μM), bar = 5 μm. **F-G.** The values of Shannon entropy (**F**) and Localization index (**G**) of freshly sorted cells in **E**. The value of Shannon entropy increases with higher extent of random distribution of fluorescence peak intensity. The values of Localization Index (LI) ranges between 0 and 1. Higher LI value represent fewer number of fluorescence signal peaks, each occupying greater proportion of total fluorescence signaling. Results shown as mean ± S.D. Controls (G1-G3) are the same as in Supplemental **Fig. 1D**. Floating box middle bar, bottom and top represent median, 25^{th} and 75^{th} percentile; Whiskers represent minimal and maximal value (**F, G**). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, student's t-test.

Figure S5

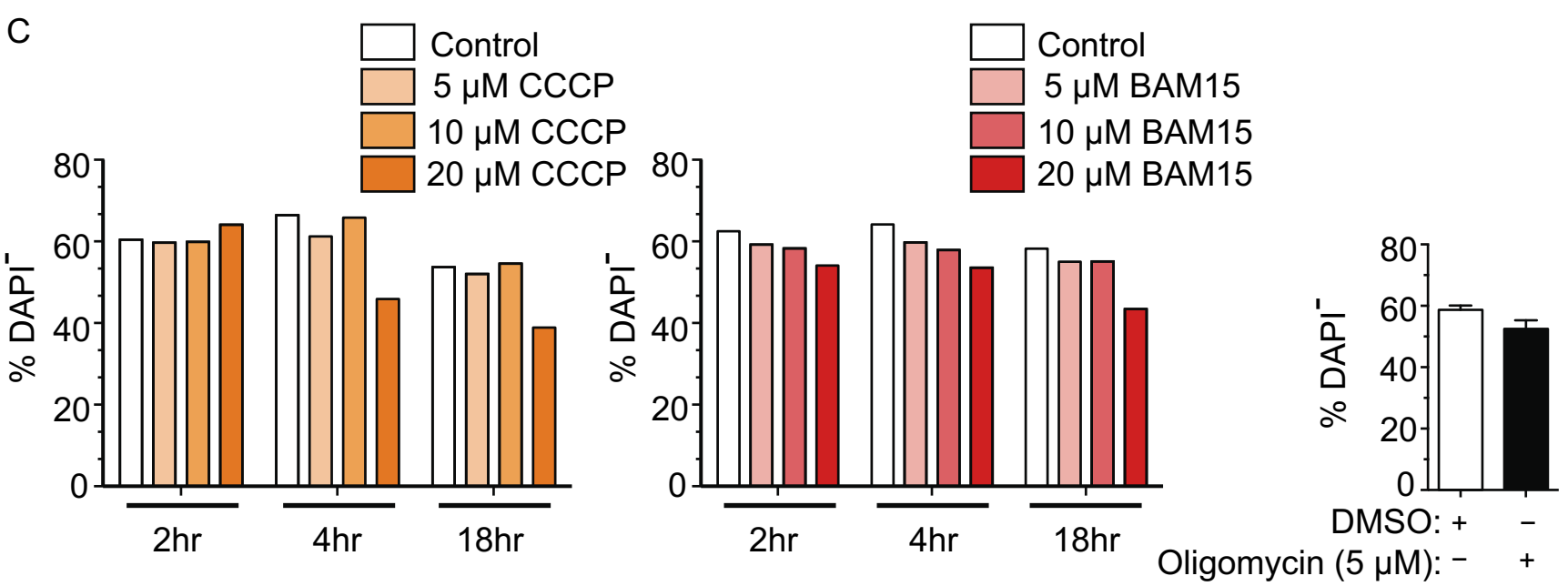
A



B



C



D

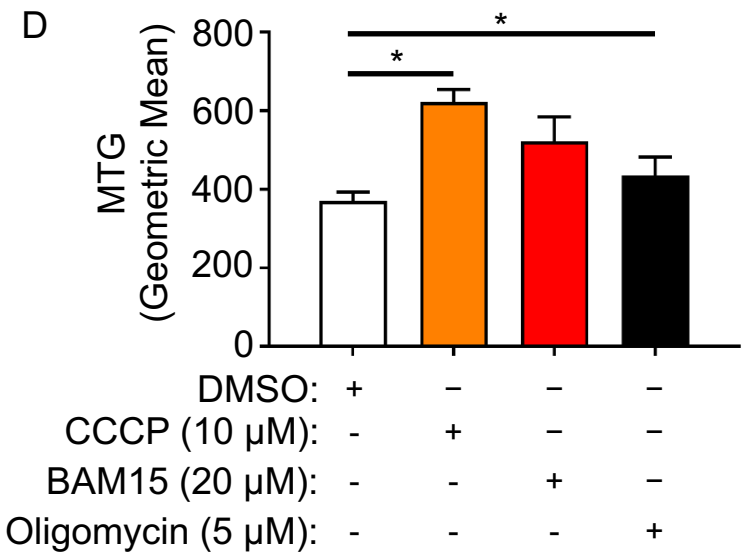
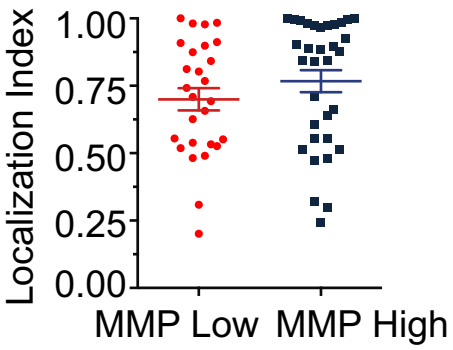


Figure S5: Progressive loss of mitochondrial membrane potential during murine erythroid maturation

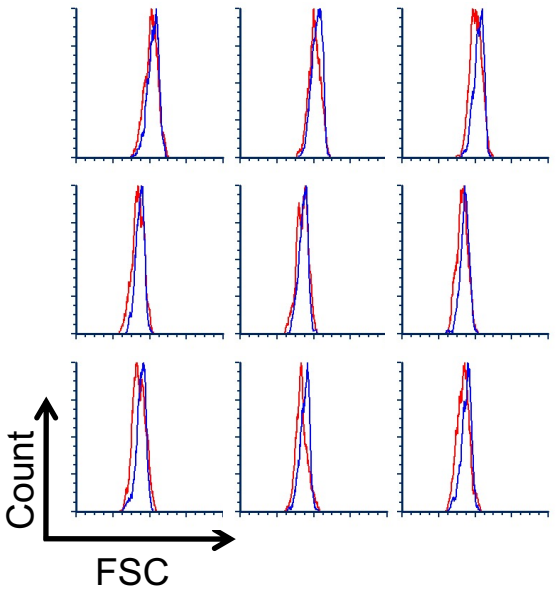
A. TER119^{+} cells were resolved from Gates 1 to 5 based on CD44 and FSC parameters. JC-1 staining within each gate of untreated cells with JC-1 monomers representing cells with depolarized mitochondria and JC-1 aggregates representing cells with polarized mitochondria (Flow plots on top). Bottom flow plots display cells treated with BAM15 to induce depolarization and loss of JC-1 aggregates; quantification (right panel). **B.** Gating strategy for analysis of *ex vivo* enucleation assay. DAPI positive cells representing unhealthy/dead cells and TER119 low cells representing pyrenocytes were excluded. For the metabolite exclusion assay, total TER119^{+} cells were included for DRAQ5 analysis since lack of pyruvate led to variabilities in viability. **C.** Viability of FACS-sorted orthochromatic erythroblasts based on DAPI exclusion after treatment with BAM15, CCCP, and oligomycin. **D.** Quantification of mitochondrial mass using MitoTracker Green (MTG; geometric mean) in response to inhibitors at indicated concentrations after 18 hrs treatment. Mean ± S.D.; $*P < 0.05$, $***P < 0.001$, One-way ANOVA followed by Tukey's multiple comparisons test (**A, C, D; n=3**).

Figure S6

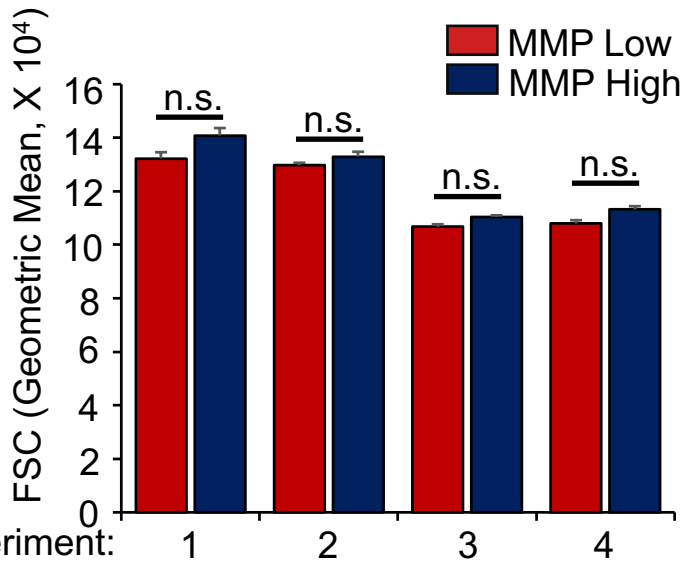
A



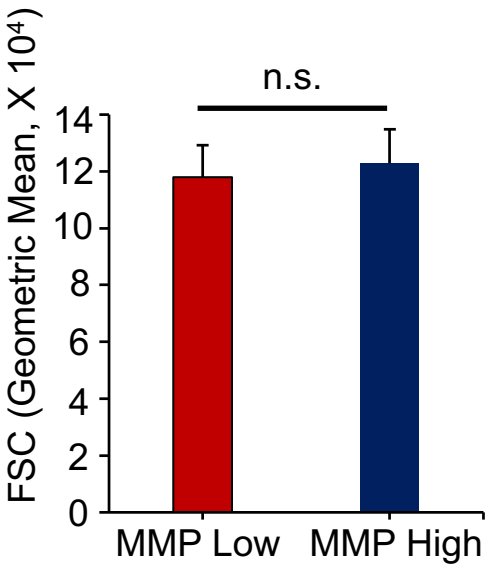
Bi



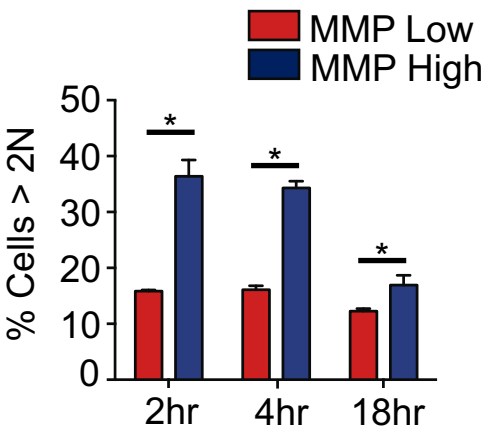
Bii



Biii



C



D

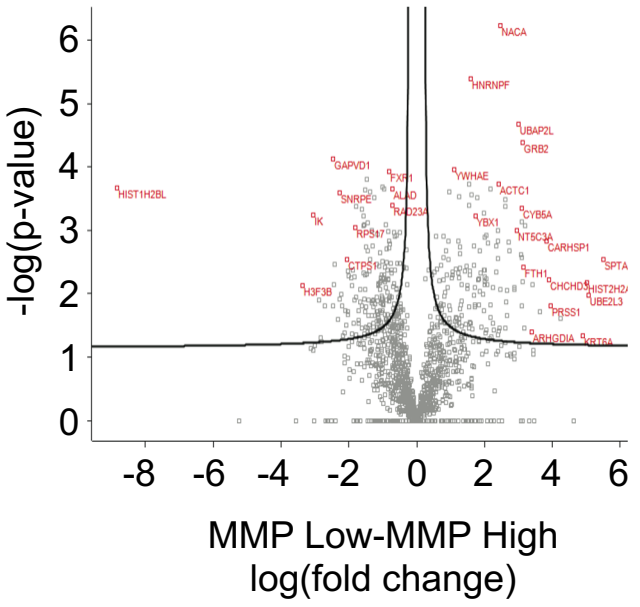
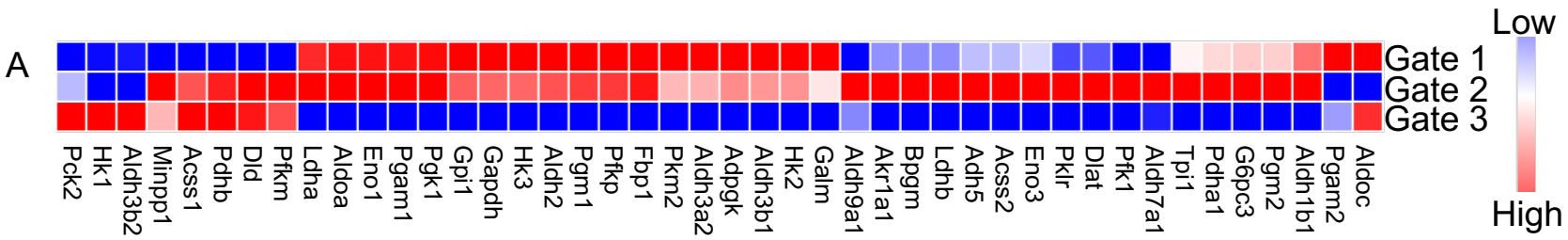
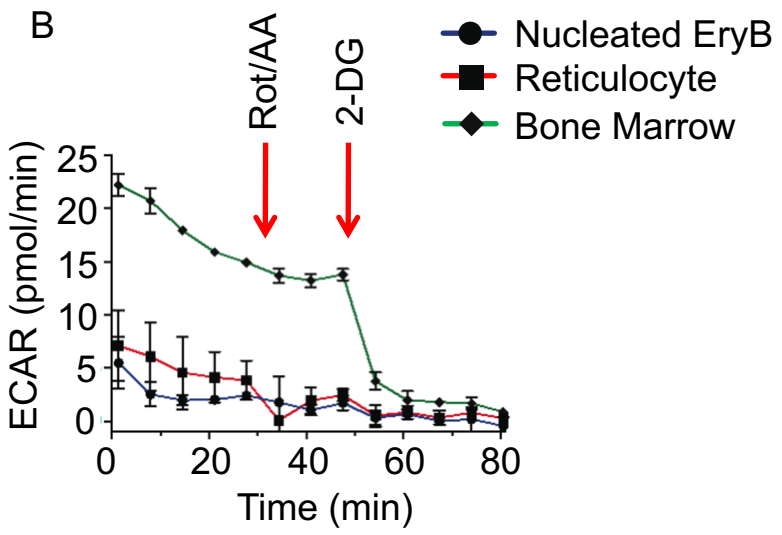


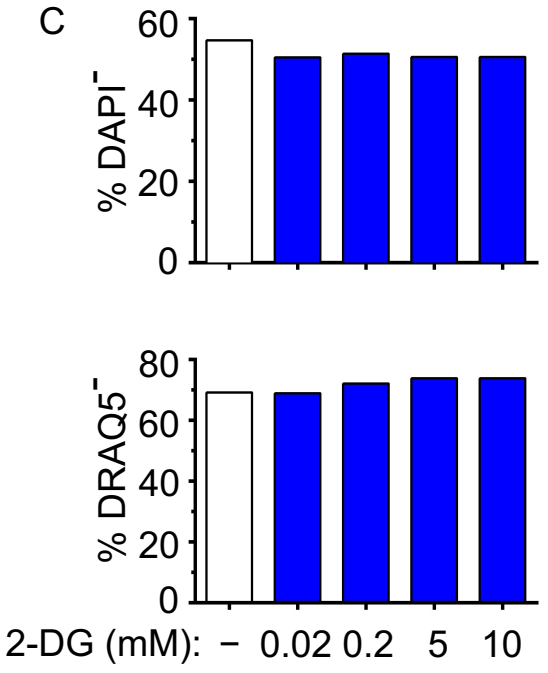
Figure S6: Distinct properties of MMP-low *vs*. MMP-high murine late maturing (orthochromatic) erythroblasts

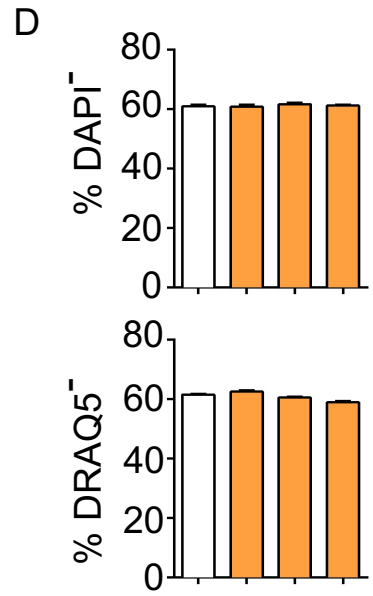
A. Quantification of mitochondrial localization of FACS-sorted MMP-low and -high fractions of orthochromatic erythroblasts relative to early erythroblasts. **B.(i)** Representative FSC histograms of MMP-low and -high Gate 3 erythroblasts from individual nine mice. **ii., iii**., Quantification of cell size based on FSC parameters between MMP-low and -high fractions. Mean values in four separate experiments (**ii)**. Mean values of combined four experiments (**iii) (**n=9 individual mice), n.s. $P > 0.05$. **C.** Ploidy analysis based on DRAQ5 staining at various time points of orthochromatic fractions. **D.** Volcano plot displaying differentially expressed proteins in red between MMP-low and -high orthochromatic erythroblast proteome determined by mass spectrometry. Results shown as mean $\pm$ S.D. (**B,C**), or S.E.M. (**A**); (n=3); $*P < 0.05$, student's t-test (**A-C**).

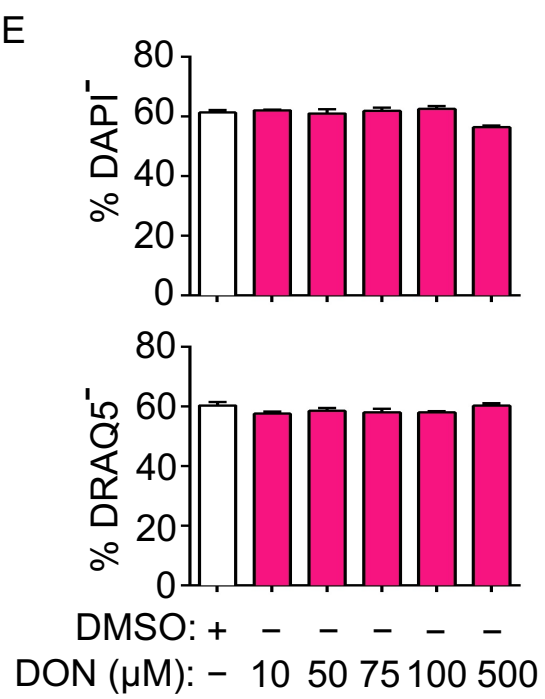
Figure S7

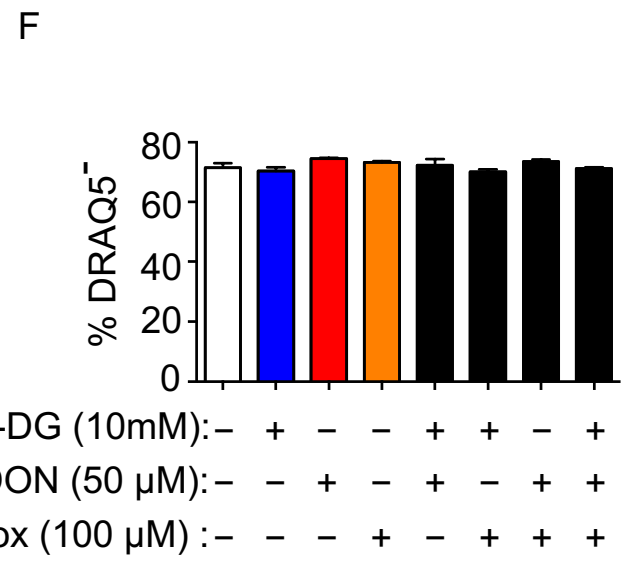

A
Gate 1
Gate 2
Gate 3
Low
High
Pck2
Hk1
Aldh3b2
Minpp1
Acss1
Pdhb
Dld
Pfkm
Ldha
Aldoa
Eno1
Pgam1
Pgk1
Gpi1
Gapdh
Hk3
Aldh2
Pgm1
Pfkp
Fbp1
Pkm2
Aldh3a2
Adpgk
Aldh3b1
Hk2
Galm
Aldh9a1
Akr1a1
Bpgm
Ldhb
Adh5
Acss2
Eno3
Pklr
Dlat
Pfk1
Aldh7a1
Tpi1
Pdha1
G6pc3
Pgm2
Aldh1b1
Pgam2
Aldoc

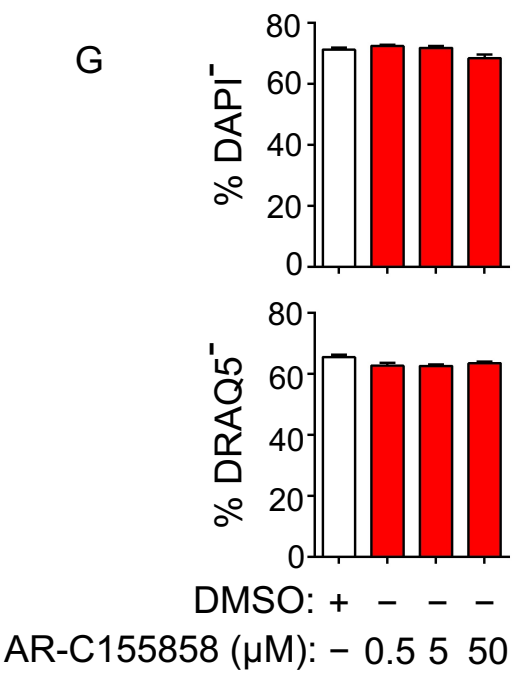

B
Rot/AA
2-DG
Nucleated EryB
Reticulocyte
Bone Marrow
ECAR (pmol/min)
Time (min)


C
% DAPI-
% DRAQ5-
2-DG (mM): − 0.02 0.2 5 10


D
% DAPI-
% DRAQ5-
DMSO: + − − −
Etox (μM): − 10 100 500


E
% DAPI-
% DRAQ5-
DMSO: + − − − − −
DON (μM): − 10 50 75 100 500


F
% DRAQ5-
2-DG (10mM): − + − − + + − +
DON (50 μM): − − + − + − + +
Etox (100 μM) : − − − + − + + +


G
% DAPI-
% DRAQ5-
DMSO: + − − −
AR-C155858 (μM): − 0.5 5 50

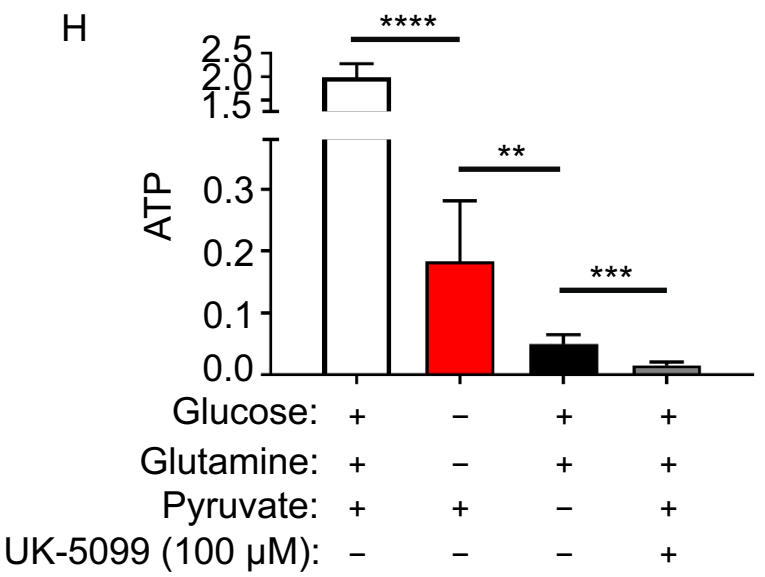

H
ATP
****
**
***
Glucose: + − + +
Glutamine: + − + +
Pyruvate: + + − +
UK-5099 (100 μM): − − − +

Figure S7: Murine erythroblast enucleation is dependent on pyruvate but not glucose metabolism

A. Heatmap of RNA-seq data [1] displaying the relative expression of glycolytic enzymes during erythroblast maturation. **B.** Glycolytic rate of indicated cell type based on proton efflux rate (PER) as determined by the Seahorse XF-96. **C-F.** *Ex vivo* enucleation assay testing the effect of 2-deoxyglucose (2-DG) (**C**), etomoxir (ETOX) (**D**), or 6-diazo-5-oxo-l-norleucine (DON) (**E**) or the combination (**F**) on viability and enucleation 18 hrs post-plating with indicated concentration of inhibitor**. G.** Viability and enucleation after inhibition of pyruvate flux through blocking MCT1 (AR- C155858). **H.** Graph displaying ATP levels of orthochromatic erythroblasts in the presence or absence of glucose (10 mM), glutamine (4 mM), pyruvate (4 mM), or UK-5099 (100 µM). Results shown as mean ± S.E.M. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, student's t-test.

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