CD34+ acute myeloid leukemia cells with low levels of reactive oxygen species show increased expression of stemness-genes and can be targeted by the BCL2 inhibitor Venetoclax


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Title: **CD34⁺ acute myeloid leukemia cells with low levels of reactive oxygen species show increased expression of stemness-genes and can be targeted by the BCL2 inhibitor Venetoclax**

Short title: **ROS-low CD34⁺ AML cells have stem cell features**

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Acute myeloid leukemia (AML) is a genetically heterogeneous disease characterized by the accumulation of immature myeloid blasts in the bone marrow. While conventional chemotherapy usually results in initial reduction of leukemic blasts in the majority of patients, disease relapse is frequent, especially in elderly patients. Disease relapse is likely driven by leukemia stem cells (LSCs) that are not affected by chemotherapy and therefore retain their disease-initiating properties. Further characterization of this LSC population is therefore highly relevant.

In hematopoietic cells, the stage of differentiation and metabolic properties are closely linked. Hematopoietic stem cells (HSCs) characteristically have low levels of mitochondrial oxidative metabolism and consequently low levels of reactive oxygen species (ROS), which is relevant for their function. A similar concept seems to apply for leukemic cells: Within the total mononuclear AML cell population (AML-MNCs), LSCs were shown to predominantly reside in the cell fraction with relatively low levels of ROS.

While previous studies highlighted that LSCs have lower ROS levels compared to the remaining mononuclear AML cells, the present study investigates how distinct ROS levels within the stem-cell-enriched CD34+ AML cell fraction correlate with cellular characteristics like morphology, gene expression, metabolic activity and drug responsiveness. We found that CD34+ AML cells with low ROS levels are smaller, have a significantly increased expression of genes associated with stemness (such as ABCB1, MEIS1, CD109) and negative regulation of signaling (such as SPRY1, DUSP10, PIK3IP1), and show an increased sensitivity to the BCL2 inhibitor Venetoclax, which was not linked to an increased expression of BCL2, but might be related to an increased expression of the MCL1 regulator NOXA and the BCL2L1 repressor ATF3.

We compared morphological characteristics of CD34+ AML cells with low or high ROS levels, which were defined as cell fractions with the 15% lowest and 15% highest signal intensity for a fluorescence-based ROS dye (Figure 1A). Among AML patients with various genetic backgrounds, ROS-low CD34+ cells were consistently smaller compared to their ROS-high counterparts (Figure 1 B-C). ROS-low and ROS-high sorted AML CD34+ cells maintained a distinctive ROS-state for up to one week in culture (Supplementary figure 1 A-B), indicating that ROS levels in CD34+ AML cells do not fluctuate highly and that cell sorting based on ROS levels separates two distinct cell entities. Notably, we confirmed the presence of leukemia-associated mutations in both the ROS-low and ROS-high fraction. In some samples, the co-mutational pattern was different between the two fractions, likely reflecting different leukemic subclones (Supplementary figure 1 C-D and supplementary table S1).
RNA sequencing was used to gain insight into transcriptional differences between ROS-low and ROS-high CD34+ AML cells (n=4). Due to high biological variance, principal component analysis (PCA) showed that samples cluster based on patient samples and not on ROS levels (Supplementary figure 2A). Differential expression analysis identified 58 commonly up-regulated and 148 commonly down-regulated high confidence genes in the ROS-low AML CD34+ cell fraction compared to ROS-high AML CD34+ cells (Figure 1D, supplementary figure 2 B-C). Gene ontology (GO) analysis revealed that genes upregulated in the ROS-low fraction were associated with negative regulation of signaling, whereas down-regulated genes were associated with increased cell differentiation (Figure 1E). Validation of RNA-seq data by RT-qPCR in additional AMLs (n=8) demonstrated that ROS-low CD34+ AML cells have significantly increased expression of stemness-associated genes (ABCB1, MEIS1, CD109, GFI1B), genes related to negative regulation of signaling (SPRY1, DUSP10, PIK3IP1, DDIT4) and apoptosis (NOXA, ATF3) (Figure 1F, supplementary figure 3). High expression of the drug-efflux transporter ABCB1 is associated with therapy resistance and poor prognosis in AML, and functional validation of ABCB1 activity by transporter activity assays showed almost exclusive activity in the ROS-low CD34+ AML cells (Figure 1G, supplementary figure 4). In line with the increased expression of genes related to stemness, we observed an increased percentage of CD34+CD38- cells in the ROS-low fraction (Figure 1H).

Notably, the observed transcriptional differences between ROS-low and ROS-high CD34+ cells were not AML-specific, since increased expression of most of these genes could also be found in the ROS-low fraction of normal CD34+ cells isolated from cord blood (CB) or mobilized peripheral blood mononuclear cells (PBSCs) (Supplementary figure 3, supplementary figure 5A). Further similarities between normal- and leukemic CD34+ ROS-low cells were the small size (Supplementary figure 5 B,C) and a higher frequency of CD34+CD38- cells (Supplementary figure 5D). To functionally confirm the relevance of increased stemness-related gene expression, Long-Term Culture-Initiating Cell (LTC-IC) assays were performed on 3 distinct cell fractions from CB and PBSCs: ROS-low/CD34+/CD38-, ROS-low/CD34+/CD38+ and ROS-high/CD34+ (Supplementary figure 5E). For both CB and PBSCs, a significantly higher LTC-IC frequency was found in the ROS-low cells compared to ROS-high cells (Supplementary figure 5F), with the highest frequency observed in ROS-low/CD34+/CD38- CB cells.
Even though ROS production is strongly correlated with mitochondrial activity, our RNA-seq data didn’t show differentially expressed genes related to mitochondrial energy metabolism in ROS-low vs. ROS-high CD34+ AML cells. Analysis of mitochondrial characteristics by electron microscopy revealed fewer mitochondrial structures in ROS-low CD34+ AML cells (Figure 2 A-B), which was in line with a lower mitochondrial DNA copy number (Figure 2C). However, ROS-low CD34+ AML cells had a smaller cytoplasm area (Figure 2D), and the ratio calculated from cytoplasm area and number of mitochondria was similar in both fractions (Figure 2E). This suggests that ROS-low cells contain a lower absolute number of mitochondria compared to ROS-high cells, but their mitochondrial content relative to their cell size is the same. Consistent with that, we observed comparable TOM20 protein expression in both fractions when the signal was normalized to β-ACTIN (Figure 2F). ROS-low AML CD34+ cells had significantly lower amounts of ATP (Figure 2G) and the fluorescence probe TMRM (tetramethylrhodamine) indicated lower levels of mitochondrial membrane potential (MMP) in this fraction (Figure 2H). To exclude that ROS-low and ROS-high cells might metabolize fluorescence dyes differentially, MMP was also measured using Rhodamine 123 in the presence of cyclosporine A, which blocks drug efflux transporters. These measurements yielded similar results (Figure 2I).

It has been shown that ROS-low AML cells from the MNC fraction are more sensitive to inhibition of the anti-apoptotic mitochondrial regulator BCL2 compared to ROS-high cells, which was linked to increased BCL2 expression at both RNA- and protein level. Furthermore, combination of the BCL2 inhibitor Venetoclax and azacitidine was shown to target LSCs in AML patients. Notably, our RNA-seq data did not reveal increased BCL2 expression in ROS-low CD34+ AML cells compared to ROS-high CD34+ AML cells, nor did we observe a consistent overexpression of BCL2 protein (Figure 3A). Despite this, we observed that the ROS-low CD34+ AML cells were significantly more sensitive to Venetoclax treatment compared to their ROS-high counterparts (Figure 3B, supplementary figure 6A), whereby ROS-low CD38+ and CD38- cells were equally efficiently targeted (Supplementary figure 6B). This seems to be Venetoclax specific, since treatment of ROS-low and ROS-high CD34+ AML cells with the MCL1 inhibitor S63845 only mildly affected the viability of both cell fractions (Figure 3C). While combined treatment with both Venetoclax and S63845 had no additional effect on the AML CD34+ ROS-low fraction, it eventually also eliminated ROS-high cells in a dose-dependent manner (Figure 3D). This supports the notion that the anti-apoptotic proteins BCL2 and MCL1 are both involved in regulating AML cell survival, but demonstrates that ROS-low cells have an increased BCL2 dependency. Considering the role of BCL2 as a mitochondrial regulator, we wondered if ROS-
low cells are generally more sensitive to a direct block of mitochondrial activity. However, treatment of ROS-low and ROS-high CD34+ AML cells with the mitochondrial uncoupler FCCP targeted both cell fractions (Supplementary figure 7).

In summary, our data highlight that CD34+ AML cells with low ROS levels are smaller, have stemness-related features and thereby most likely coincide with the LSC population. ROS-low CD34+ AML cells showed increased expression of genes previously linked to stress- and drug-resistance and poor prognosis in AML patients (e.g. CD109, MEIS1, ABCB1), highlighting that targeting this population is crucial for successful AML therapy. We show that ROS-low AML CD34+ cells are highly sensitive to BCL2 inhibition by Venetoclax, which could not be explained by higher BCL2 expression in this fraction, but potentially is related to increased expression of additional apoptosis regulators such as NOXA or ATF3, which are involved in inactivation of the anti-apoptotic proteins MCL1 and BCL2L1, respectively. Notably, MCL1 expression was shown to inversely correlate with sensitivity to BCL2 inhibition in AML, whereas recently impaired ATF3 expression was associated with resistance to the combination of Venetoclax and ibrutinib in mantel cell lymphoma.

**Acknowledgements**

We would like to acknowledge W. Abdulahad, T. Bijma, J. Teunis and G. Mesander for help with Flow Cytometry. We thank Dr. A. van Loon, Dr. J.J. Erwich and colleagues (Obstetrics departments from the Martini Hospital and UMCG) for collecting cord blood, and Dr. A.B. Mulder (Department of Laboratory Medicine, UMCG) and Dr. E. van den Berg (Department of Genetics, UMCG) for providing laboratory data.
References


Figure legends

Figure 1 ROS-low CD34⁺ AML cells express stemness-associated genes (A) Gating strategy for defining ROS-low and ROS-high cells. CD34⁺ isolated AML cells were stained with the fluorescence dye CellROX Deep Red and gated for cells with the 15% lowest or highest signal intensity. (B) Representative FACS plots (ROS-low: blue, ROS-high: red) and May-Grünwald Giemsa staining indicating the size of ROS-low and ROS-high CD34⁺ AML cells (n=4). (C) Median forward scatter (FSC) of ROS-low and ROS-high CD34⁺ AML cells (n=9). (D) Graphical summary of RNA-seq analysis performed with ROS-low and ROS-high CD34⁺ cells from AML patients (n=4). Overlap of the two indicated methods revealed 58 high confidence genes that are commonly upregulated in ROS-low cells (left panel), and 148 high confidence genes that are commonly downregulated (right panel). (E) Gene ontology enrichment analysis of genes upregulated (left panel) or downregulated (right panel) in ROS-low CD34⁺ AML cells. (F) RT-qPCR analysis of indicated genes that where assigned to the categories “Stemness”, “Negative regulation of signaling” and “Apoptosis”. Fold change of expression in ROS-low vs ROS-high cells is shown (n=6). (G) ABCB1 transporter activity in ROS-low and ROS-high CD34⁺ AML cells.
is shown (n=5). Rh123: Rhodamine 123; MFI: mean fluorescence intensity. (H) Summary of FACS analysis indicating percentages of CD38⁺ and CD38⁻ cell populations within ROS-low and ROS-high CD34⁺ AML cells (n=8). (C, F-H) Error bars indicate s.d.; *p<0.05; **p<0.01; ***p<0.001.

Figure 2 ROS-low and ROS-high CD34⁺ AML cells show altered metabolic activity (A) Electron microscopy (EM) images of ROS-low and ROS-high sorted CD34⁺ AML cells. Samples from 2 AML patients are shown. Mitochondrial structures are outlined in red. (B) Number of mitochondrial structures in ROS-low and ROS-high cells quantified from EM images. Per sample, 25-72 cells were scored (n=4). Two-way ANOVA revealed a significant impact of ROS-levels on the number of mitochondrial structures (p<0.0001). (C) Mitochondrial copy number was calculated by comparing levels of mitochondrial and nuclear DNA measured by RT-qPCR. Relative fold change between ROS-low and ROS-high CD34⁺ AML cells is shown (n=6). (D) Cytoplasm surface area of ROS-low and ROS-high CD34⁺ AML cells on EM images was determined using ImageJ software. Per sample, 25-72 cells were scored (n=4). (E) The ratio calculated from average cytoplasm surface area and average number of mitochondrial structures for the ROS-low and ROS-high cell fraction is shown. (F) Western blots indicating TOM20 and β-ACTIN protein levels in ROS-low and ROS-high sorted CD34⁺ AML cells (left panel). Numbers indicate relative TOM20 levels normalized to β-ACTIN, and a summary of this quantification is shown in the right panel (n=5). (G) ATP levels of ROS-low and ROS-high sorted CD34⁺ AML cells are shown (n=9). (H) Measurement of mitochondrial membrane potential using the fluorescent dye Tetramethylrhodamine Methyl Ester (TMRM). Mean fluorescence intensity of ROS-low and ROS-high sorted CD34⁺ AML cells is shown (n=8). (I) Fluorescence intensity of staining with Rhodamine 123 (Rh123) in ROS-low and ROS-high sorted CD34⁺ AML cells is shown. The staining was performed in the presence of cyclosporine A to prevent dye efflux (n=5). (C-I) Error bars indicate s.d.; *p<0.05; **p<0.01; ***p<0.001; ns=not significant.

Figure 3 ROS-low CD34⁺ AML cells have an increased sensitivity to the BCL2 inhibitor Venetoclax. (A) Western blots indicating protein expression of BCL2; HDAC1 and β-ACTIN served as loading controls; Numbers indicate relative BCL2 levels normalized to HDAC1 or β-ACTIN. (i) Expression of indicated proteins is shown for the ROS-low and ROS-high cell fraction sorted from CD34⁺ AML cells. (ii) Expression of indicated proteins is shown from the ROS-low and ROS-high fractions of the total mononuclear cell population (MNC) or CD34⁺ AML cells. (B)
ROS-low and ROS-high sorted CD34⁺ AML cells were treated for 24h with 100nM of the BCL2 inhibitor Venetoclax. The reduction of viability compared to DMSO-treated cells (indicated by DAPI staining) is shown. (C) Similar as in (B), cells were treated for 24h with various concentrations of the MCL1 inhibitor S63845. (D) ROS-low and ROS-high sorted CD34⁺ AML cells were treated for 24h with a combination of 100 nM Venetoclax and increasing concentrations of the MCL1 inhibitor S63845. One-way ANOVA revealed a dose-dependent effect of MCL1-addition on the viability of CD34⁺ ROS-high- but not of ROS-low AML cells. (B-D) Error bars indicate s.d.; *p<0.05; **p<0.01; ***p<0.001; ns=not significant.
Figure 1

A gated on CD34* ROS low and high

B ROS-low and ROS-high

C median FSC

D High confidence genes upregulated in ROS-low

EdgeR: ≥1 log diff. FDR: ≤0.01
34 58 47

High confidence genes downregulated in ROS-low

EdgeR: ≥1 log diff. FDR: ≤0.01
172 148 25

E Negative regulation of phosphate metabolic process
Negative regulation of cell differentiation
Blood vessel morphogenesis
TNFs bind their physiological receptors
Signaling by receptor tyrosine kinases
Fat cell differentiation
Phosphatidylinositol 3-kinase signaling
Apelin signaling pathway
ATF2 pathway
MicroRNAs in cancer
Epithelial cell migration
Gliogenesis
Neuron migration

Granulocyte activation
Inflammatory response
Cytokine production
Osteoclast differentiation
Activation of immune response
Phagosome
Regulation of TNF production
ERK1 and ERK2 cascade
Adaptive immune system
Mononuclear cell migration
Toll endogenous pathway
Cell division
Response to interferon-gamma
Cell cycle
Leukocyte proliferation
Receptor-mediated endocytosis
Macrophage activation

F Stemness
Neg. Regulation signaling
Apoptosis

G ABCB1

H % of cell population
Figure 2

A

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1 μM

B

- **ROS-low**: mean 16.75, p<0.0001
- **ROS-high**: mean 23.15

C

- Relative mitochondrial copy number
- Avg. cytoplasm area μm²

D

- Ratio of cytoplasm area/membrane width

E

- Ratio of mitochondrial structures per cell
- ns

F

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G

- ATP (μM) 2500 cells
- TMRM MFI
- Rh 123 MFI (cyclosporine A)

H

- ATP (μM) 2500 cells
- TMRM MFI
- Rh 123 MFI (cyclosporine A)

I

- ATP (μM) 2500 cells
- TMRM MFI
- Rh 123 MFI (cyclosporine A)
Supplementary Information
to
“CD34⁺ acute myeloid leukemia cells with low levels of reactive oxygen species show increased expression of stemness-genes and can be targeted by the BCL2 inhibitor Venetoclax”


Supplementary figure 1. ROS-low and ROS-high cells represent two distinct cell entities but both carry leukemia associated mutations (A) Experimental design to monitor cellular ROS-states over time under different culture conditions. (B) FACS plots indicating ROS levels of AML cells (sorted as in A) at the time of sort and after 3 or 6 days of culture in either serum-rich medium
(upper panel) or serum-free medium (lower panel). (C) PCRs to detect FLT3-ITD or wildtype in total CD34+ AML cell fractions, ROS-low or ROS-high sorted cells, respectively. All cell fractions of AML1, AML8 and AML10 carry both the FLT3 wildtype and FLT3-ITD allele. In AML9, the FLT3-ITD allele is only found in the ROS-low fraction. (D) Sequencing results verifying that the NPM1 mutation is present in all indicated cell fractions of AML9.
Supplementary figure 2. **ROS-low CD34⁺ AML cells show increased expression of genes that negatively regulated cell- proliferation and differentiation (A)** Principle component analysis (PCA) of RNA-sequencing data of indicated fractions of CD34⁺ enriched AML cells. PC1 (x-axis) represents 47.4% and PC2 (y-axis) represents 23.9% of the total variation in the data. (B,C) Analysis of RNA-seq data performed with ROS-low CD34⁺ and ROS-high CD34⁺ cells from AML patients (n=4). (B) Heatmap of high-confidence genes that are downregulated in ROS-low cells compared to ROS-high cells. (C) Heatmap of high-confidence genes that are upregulated in ROS-low cells compared to ROS-high cells.
Supplementary figure 3. ROS-low CD34⁺ express gene signatures associated with stemness, negative regulation of signaling and apoptosis RT-qPCR data. Relative mRNA expression of indicated genes is shown for the ROS-low and ROS-high fraction of CD34⁺ AML cells, CD34⁺ cord blood (CB) cells or CD34⁺ peripheral blood stem cells (PBSCs). Error bars indicate s.d. of RT-qPCR triplicates.
Supplementary figure 4. **ROS-low and ROS-high CD34⁺ cells show altered drug efflux transporter activity** ABCB1 transporter activity assay. FACS plots indicating signal intensities for the dye Rhodamine 123 (Rh123) are shown. Staining was performed either in the presence (+ cyclosporine A) or absence (-cyclosporine A) of the efflux transporter inhibitor cyclosporine A.
Supplementary figure 5. **Normal ROS-low CD34**⁺** have similar features compared to leukemic ROS-low CD34**⁺** **(A)** mRNA expression fold change of indicated genes in the ROS-low vs the ROS-high cell fraction of normal (both CB and PBSCs) CD34⁺ stem- and progenitor cells (n=4). Genes are assigned to the categories “stemness”, “negative regulation of signaling” and “apoptosis”. Error bars indicate s.d. **(B)** May-Grünwald Giemsa (MGG) staining of ROS-low and ROS-high sorted PBSCs. **(C) (i)** Representative FACS plots indicating the size of ROS-low (blue) and ROS-high (red) CD34⁺ cells isolated from CB or PBSCs. **(ii)** Median forward scatter (FSC) of ROS-low and ROS-high cells from four CB donors and five PBSC donors. **(D)** Summary of FACS analysis indicating the percentages of CD38⁺ and CD38⁻ cell populations within ROS-low and ROS-high CD34⁺ CB cells (n=4) or PBSCs (n=5). **(E)** Sorting strategy for LTC-IC assays. Three populations were sorted: ROS-high CD34⁺, ROS-low CD34⁺CD38⁻dim and ROS-low CD34⁺CD38⁺.
Supplementary figure 6. CD34+ AML cells with low ROS levels show an increased sensitivity to BCL2 inhibition but not to MCL1 inhibition (A) CD34+ AML cells (n=8) were sorted into ROS-low and ROS-high cell fractions and treated for 24h with 100nM Venetoclax and/or indicated concentrations of the MCL1 inhibitor S63845. Reduction of cell viability compared to control cells as indicated by DAPI staining is shown. Error bars indicate s.d. of duplicates. (B) Summary of FACS analysis indicating the percentages of CD34+CD38- and CD34+CD38+ cells of control and treated cells.
Supplementary figure 7. **FCCP mediated depolarization of the mitochondrial membrane potential targets both ROS-low and ROS-high CD34+ AML cells** ROS-low and ROS-high CD34+ AML cells were treated with the indicated concentrations of FCCP (carbonyl cyanide-4(trifluoromethoxy)phenylhydrazone) for 24h. The increased percentage of DAPI positive cells compared to control cells after 24h is shown.
Table S1. Characteristics of AML patient samples used in the present study
Clinical characteristics of the studied AML samples. If sequencing data was available, identified mutations and the variant allele frequency are indicated. All material was obtained from newly diagnosed AML patients. AML: acute myeloid leukemia; N.D: not determined.

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Material and Methods

Normal Hematopoietic cells and AML patient material
Umbilical cord blood (CB) was derived from healthy full-term pregnancies after informed consent from the Obstetrics departments of the Martini Hospital and University Medical Center Groningen (UMCG), the Netherlands. Mononuclear cells (MNCS) were isolated by density gradient centrifugation using Lymphoprep (Alere Technologies AS, Oslo, Norway) and CD34+ cells were
selected using the MACS CD34 microbead kit on autoMACS (Miltenyi Biotec, Leiden, The Netherlands). A purity of >96% CD34+ cells after isolation was confirmed by flow cytometry. AML blasts from peripheral blood or bone marrow from patients were studied after informed consent was obtained in accordance with the Declaration of Helsinki. The protocol was approved by the Ethical Review Board of the University Medical Center Groningen, Groningen, The Netherlands. CB CD34+ cells or AML MNCs were cryopreserved in medium containing 10% fetal calf serum (FCS; Sigma, F7524) and 10% DMSO.

**Preparation of primary material for subsequent analysis**

Frozen material was defrosted one day prior to further analysis. CB cells were cultured overnight (o/n) in Stemline II (Sigma, Zwijndrecht, the Netherlands) supplemented with SCF, FLT-3 ligand and N-Plate (Amgen) (100 ng/ml each). AML MNCs were enriched for CD34+ cells as described above, and cultured o/n on a confluent layer of murine MS5 cells in Gartner's medium consisting of αMEM (containing glutamine, Lonza) supplemented with 12.5 % heat-inactivated FCS (Sigma), 12.5 % heat-inactivated horse serum (Sigma), 100 U/mL penicillin and streptomycin (PAA Laboratories), 57.2 μM β-mercaptoethanol (Merck Sharp & Dohme BV), 1 mM hydrocortisone (Sigma) and 20 ng/mL G-SCF, N-Plate and IL-3. MS5 cells were cultured and maintained as described previously.1

**Cell separation based on ROS levels**

Normal – or leukemic CD34+ cells, prepared as described above, were resuspended in PBS/3%FCS/Fc-block and stained with 5 μM CellROX Deep Red reagent (Life Technologies) and antibodies for CD34/CD38 selection (CD34-PE, CD38-FITC, both BD Biosciences) for 30 min at 37 °C. Afterwards, cells were washed with PBS/3%FCS and resuspended in PBS/3%FCS containing 10 μM DAPI. Labelled cells were further analyzed using LSR II (BD Biosciences) and FlowJo software (Treestar), or sorted on MoFLo XDP or Astrios (DakoCytomation, Carpinteria, CA, USA). For cell sorting, viable CD34+ cells with the 15 % lowest – and 15 % highest signal intensity for the ROS-dye were collected.

**Long-term Culture-Initiating Cell (LTC-IC) assay**

ROS-low and ROS-high sorted CD34+ CB cells or PBSCs were plated in limiting dilutions in the range of 9 to 1000 cells per well on MS5 stromal cells in 96-well plates in LTC medium (Glutamine containing αMEM supplemented with heat-inactivated 12.5% FCS, heat-inactivated 12.5% horse serum (Sigma), 100 U/mL penicillin/streptomycin (PAA Laboratories), 57.2 μM β-mercaptoethanol
After 5 weeks, methylcellulose (MethoCult H4230, Stemcell Technologies) supplemented with 19% (v/v) IMDM, 100 U/mL penicillin/streptomycin, 20 ng/mL IL-3, 20 ng/mL IL-6, 20 ng/mL G-CSF, 20 ng/mL SCF and 1 U/mL EPO was added to the wells. Two weeks later, wells containing colony forming cells (CFCs) were scored as positive. LTC-IC frequency was calculated using the L-Calc software.

Immunoblotting
Preparation of cell lysates and immunoblotting procedure was performed as described previously. Primary antibodies for immunoblotting were: BCL2 (2872, Cell Signaling), TOM20 (D8T4N, 42406, Cell Signaling), HDAC1 (10E2, 5356, Cell Signaling), β-ACTIN (C4, SC-47778, Santa Cruz).

ABCB1 transporter activity assay
Sorted CD34+ AML cells were divided in two tubes and resuspended in either 1 ml of αMEM or in 1 ml of αMEM + 4 μM cyclosporine A (CSA) (Sigma, #C3663). Rhodamine 123 (Dojindo Laboratories, via Tebu-bio) was added to a final concentration of 200 ng/ml and cells were incubated for 20 min at 37°C. Afterwards, cells were washed 2x with ice cold αMEM, resuspended in αMEM with or without addition of CSA, and incubated for 1h at 37°C. Subsequently, cells were washed 2x with ice cold PBS/FCS, resuspended in PBS/FCS/DAPI and analyzed on LSR II. The transporter activity was defined as the difference in Rhodamine 123 signal intensity between the fractions with and without the efflux pump inhibitor CSA (Transporter activity = MFI (-CSA)/MFI (+CSA)), as described previously.

RNA Extraction and Illumina high-throughput sequencing
RNA was isolated by separation of the aqueous phase by TRIzol Reagent (Thermo Fisher) according to the manufactures protocol. The aqueous phase was mixed with 70% ethanol (1:1) and isolation was continued using the RNeasy micro kit (Qiagen) including on-column DNaseI treatment. RNA libraries were prepared using the KAPA RNA HyperPrep Kit with RiboErase (HMR) according to the manufactures protocol (KR1351 – v1.16, Roche Sequencing Solutions). In brief: 25ng -1ug input RNA was depleted from ribosomal RNA by oligo hybridization, RNaseH treatment and DNase digestion. rRNA-depleted RNA was fragmented to ~200 bp fragments and first strand synthesis was performed using random primers. The second-strand was synthesized using dUTP for strand specificity. After adapter ligation, library amplification was performed and the number of cycles was dependent on the amount of starting material. A bioanalyser with a high
sensitivity DNA Chip (Agilent) was used to check fragment size. Samples were sequenced on an Illumina NextSeq 500 system with 2×43 bp paired-end sequencing (PE43).

**RNA-Sequencing analysis**
STAR aligner with UCSC gene annotation first indexed the hg19 reference genome. The resulting RNA-seq reads were mapped to the hg19 genome using STAR with two-pass mode, and the gene-level read counts were enumerated at the same time. EdgeR (v3.24.3) was used to examine differentially expressed genes and genes with ≥1log difference and adj p-value of <0.01 were considered significant. EdgeR analysis was overlapped with genes that have > 1.5 fold difference in all samples in a pairwise comparison. Raw RNA-Sequencing data are available at http://www.ncbi.nlm.nih.gov/geo, with accession code: GSE131422. GO-analysis was performed with metascape (www.metascape.org).

**Mitochondrial copy number assay**
Total DNA was isolated from >1 x10⁵ cells using QIAamp DNA mini kit (Qiagen). DNA was amplified real-time in SYBR Green Supermix (Bio-Rad) using the CFX connect Thermocycler (Bio-Rad). Nuclear genes (GAPDH and B2M) and mitochondrial genes (12S and tRNA) were amplified using the following primers:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2M</td>
<td>5’- TGCTGTCTCCATGGTGTATCT -3’</td>
<td>5’- TCTCTGCTCCACCTCTGAAT -3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’- TACTGGTGTCTCCACCCACCTCTAAGT -3’</td>
<td>5’- CAGGATGCATTGCTGACAATC -3’</td>
</tr>
<tr>
<td>12sRNA</td>
<td>5’- AGAACACTACGAGCCACGC -3’</td>
<td>5’- ACTTGGCTTTACTTTGTAGCC -3’</td>
</tr>
<tr>
<td>tRNA-Leu</td>
<td>5’- CACCCAAGAACAGGGTTTGT -3’</td>
<td>5’- TGGCCATGGGTATGTTGTTA -3’</td>
</tr>
</tbody>
</table>

The obtained CT values were corrected for the corresponding calculated primer reaction efficiencies. Based on the corrected CT values, the mitochondrial DNA copy number was calculated relative to nuclear DNA copy number.⁴

**ATP assay**
Luminescent ATP detection Assay kit (Abcam, ab113849) was used according to the manufactures protocol to measure levels of ATP. ATP levels were measured using a Bio-Rad benchmark III Bio-Rad microtiter spectrophotometer.
**Mutational analysis and targeted sequencing**

Presence of the FLT-ITD mutation was analyzed by PCR using following primers: FLT3-ITD_fw: CGGCACAGCCCAGTAAAGATA; FLT3-ITD_rev: GCCCAAGGACAGATGTGATG; The NPM1 region was amplified using following primers: NPM1_W288fs_fw: TCGGGAGATGAAGTTGGAAG and NPM1_W288fs_rev: ACGGTAAGGAAAGTTCTCAC, and presence of the mutation was verified by Sanger-Sequencing (Eurofins).

**Ultrastructural analysis**

The experimental procedure for ultrastructural analysis of hematopoietic cells has been described previously. In brief, 1-2 million ROS-low and ROS-high sorted CD34+ AML cells were pelleted and subsequently fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1M cacodyladate buffer for 24h at 4 °C. After fixation, the cells were washed in in 0.1M cacodyladate buffer. Cells were stained with Evans blue and subsequently embedded in low melting point agarose as described previously. Agarose pieces containing the cell pellet were dehydrated, osmicated, and embedded in Epon according to routine procedures. Semi-thin sections (0.5mm) stained with toluidine blue were inspected using light microscopy to identify the position of cells. Ultra-thin sections (60-80 nm) were cut and stained with 4% uranyl acetate in water, followed by Reynolds lead citrate. Images were taken with the FEI/Philips CM100 (Eindhoven, the Netherlands).

**Measurement mitochondrial structures and cytoplasm area**

Electron microscopy images of ROS-low and ROS-high sorted CD34+ AML cells were taken as described above. On these images, mitochondrial structures were counted from 25-70 cells per sample. Similar, the cytoplasm area of 25-70 cells per sample was outlined and measured using ImageJ. To report the relation between cell size and mitochondrial content, the ratio of the average cytoplasm area and the average number of mitochondrial structures per sample was calculated.

**Analysis of cell viability after drug treatment**

ROS-low and ROS-high CD34+ AML cells were sorted as described above and resuspended in αMEM (containing glutamine) supplemented with 20 % heat-inactivated FCS, 100 U/mL penicillin and streptomycin, and 20 ng/mL G-SCF, N-Plate and IL-3. Afterwards, cells were treated with either DMSO (served as control), 100 nM of the BCL2 inhibitor Venetoclax (Selleckchem), or various concentrations of the MCL1 inhibitor S63845 (APExBIO). After 24h, the cell viability was determined by DAPI staining with subsequent FACS analysis. For Figure 3 B-D, the effect of drug treatment was presented as the percentage of cell viability reduction compared to DMSO-treated control cells.
Statistics

If not indicated in the figure legends otherwise, the paired two-sided student’s t-test was used to calculate statistical differences between the ROS-low and ROS-high cell fractions. A p-value of <0.05 was considered statistically significant.

References


