Supplementary Material

Chroscicki et al., Stroma-driven horizontal transfer of TCA proteins mediates metabolic plasticity and imatinib resistance in leukemia.

Correspondence: k.piwocka@nencki.edu.pl

Supplemental Methods

Cell lines and co-culture system

Human bone marrow mesenchymal stromal (HS-5; ATCC#CRL-11882) and human chronic myeloid leukemia (K562; ATCC#CCL-243) cell lines were used to model the stroma-leukemia interaction. Cells were routinely tested for mycoplasma contamination. The cultures were grown in IMDM culture medium (Biowest, #L0191) supplemented with 10% [v/v] fetal bovine serum (Biowest, FBS, #S1819) and 1% [v/v] penicillin (100 U/mL) and streptomycin (100 µg/mL) (Biowest, #L0022) in advised conditions as described (5). All the analyses were conducted after 24-hour-incubation of the co-culture. To physically separate donor HS-5 and recipient K562 cells, a transwell system (ThinCert, Greiner Bio-One), 1 µm pores, 2 × 106 pores/cm2 was used. Conditioned medium (CM) was collected after 24h of HS-5 cell culture, centrifuged to remove cells and cellular debris, and added to acceptor cells in 12-well culture plates. HS-5-DLST-GFP cell line was generated using lentiviral vector encoding the DLST gene (Origene, #RC201220L4V) in the presence of 8 µg/mL polybrene (Santa Cruz Biotechnology, #sc-134220). 72 hours post-transduction cells were selected with 0.5 µg/mL puromycin for 8 days. The population highly expressing the DLST protein (GFP-positive) was sorted on Cytek Aurora CS Cell Sorter. K562 rho0 (ρ0) cells were generated by adding 10 µM ddC (zalcitabine, ZellBio GmbH, #T0120) to the culture medium. After 72h, culture medium was replaced with a fresh portion of 10 µM ddC and incubated for another 48h. Such prepared cells deprived of mtDNA were used for further experiments. Imatinib was added to a final concentration of 1 µM during the 24h of co-culture.

Isolation of CD34+ CML-BP patient cells

Patients' material was obtained obtained from the Department of Hematology, Transplantation and Internal Medicine, Medical University of Warsaw, following informed written consent, in accordance with the Declaration of Helsinki and the guidelines for good clinical practice. All protocols were approved by the local Ethical Committee (Ethical and Bioethical Committee UKSW, Approval No. KEiB-19/2017, Approval No.WAW2/059/2019 and WAW2/51/2016). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation and CD34+ cells were separated using EasySep human CD34+ selection cocktail (StemCell Technologies, Inc.). CD34+ cells were short-term cultured in StemProTM-34 SFM medium with nutrient supplement (40x), 1% penicilin-streptomycin, 1% L-glutamine, 50 ng/ml of stem cell factor (SCF), 50 ng/ml of FLT3 ligand, 50 ng/ml of thrombopoietin (TPO), 10 ng/ml of interleukin 3 (IL-3), 10 ng/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) and 5 ng/ml of interleukin 6 (IL-6).

Exchange of membrane vesicles between cells

Donor cells (HS-5) were labeled with DiD Cell-Labeling Solution (catalog no. V22887, ThermoFisher Scientific) as described (5). Working solution containing 1.5 µl of DiD dye/1 mL of cell culture medium was added to cells for 15 min at 37°C. Then, cells were washed with PBS and seeded in fresh cell culture medium for an additional 24h. Acceptor K562 cells were labeled with 20 µM eBioscience Cell Proliferation Dye eFluor 450 (Invitrogen by Thermo Fisher Scientific, cat. no. 65-0842), in cell culture medium according to the manufacturer's instructions. Then cells were washed and seeded into co-culture with HS-5 donor cells to reach a 1:1 ratio.

Exchange of mitochondria between cells

To analyze mitochondria transfer, HS-5 cells were transduced with rLV.EF1.AcGFP1-mito-9 lentiviral vector (TaKaRa) for stable mitochondria labeling. Afterward, HS-5-GFP and K562 cells were seeded in co-culture with acceptor cells in 12-well cell culture plates to reach a 1:1 ratio. Flow cytometric analysis of stromal GFP-tracked mitochondria uptake by K562 cells was performed using a BD LSRFortessa cytometer, followed by data analysis using Diva and FlowJo software.

Oxygen consumption

Cellular respiration was measured polarographically using Oxygraph-2k (OROBOROS®INSTRUMENTS GmbH, Austria) at 37 °C. 1 mln of cells was resuspended in PBS containing respiratory substrates (1 mM pyruvate, 5 mM glutamine, 25 mM glucose). Oligomycin (0.1 µg/ml) and CCCP (1 µM) were added sequentially. The respiration rate was normalized to the amount of protein (number of cells) in the assay.

Lactate production
Cell extracts were prepared in the same manner as for the ATP content analysis. Lactate content was measured in the neutralized (2M K2CO3) extracts fluorometrically using the standard enzymatic assay with lactate dehydrogenase in a buffer containing: 0.4 M hydrazine sulfate, 0.5 M glycine, 5 mM EDTA; pH 9.5 and NAD 25mg/mL. All data were normalized according to the protein content and presented as lactate concentration in nanomoles per milligram of total protein. All reagents were obtained from Sigma Aldrich.

Seahorse Analysis
20 µL/well of 10% poly-L-lysine (PLL; Sigma, cat.P8920-100ML) solution was applied on the Agilent Seahorse culture plate (cat. 103793-100). After 20 min incubation at 37oC, the wells were washed and left to dry overnight at 37oC. K562 cells were collected from cultures, spinned and resuspended in Seahorse XF DMEM bicarbonate-free culture medium (pH 7.4; 103575-100, Agilent Technologies, Inc.). 0.2 mln cells/well were seeded onto a PLL-coated Seahorse culture plate. Next, the plate was centrifuged to attach the cells to the PLL surface and incubated at 37oC, 0% CO2, atmospheric oxygen for 40 minutes. Oxygen consumption rate (OCR) was measured with the Agilent Seahorse XF Analyzer. Standard Mitochondrial Stress Test was performed according to the manufacturer's instructions. The modulators of respiration were added as follows: oligomycin 1.5 µM, FCCP 1 µM, rotenone and antimycin A 0.5 µM (Sigma Aldrich). Simultaneously, extracellular acidification rate (ECAR) was measured to assess the characteristics of the glycolysis process.

CENCAT metabolic profiling
Human primary CD34+ cells were transferred onto the 96-well U-bottom plate (0.2 mln/well), followed by the original CENCAT protocol (18). Briefly, after 30 minutes of methionine depletion in methionine-free RPMI 1640 medium (Sigma Aldrich, #R7513) supplemented with 65 mg/L L-cystine dihydrochloride (MedChemExpress,#HY-W009203), 10% FBS, L-glutamine, cells were treated with metabolic inhibitors to shut down glycolysis (100 mM 2-deoxy-D-glucose; MedChemExpress, #HY-13966), mitochondrial metabolism (1 µM oligomycin A; MedChemExpress, #HY-16589) or both (combination of 2-deoxy-D-glucose and oligomycin A). Afterwards, cells were treated with a noncanonical amino acid analog of methionine - L-homopropargylglycine (HPG, 100µM; MedChemExpress, #HY-140345). After incubation and washing cells were stained with fixable viability dye (Invitrogen, #L34976), fixed and permeabilized. Finally, the cells were labelled through copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) in the CuAAC reaction mix (0.5 mM CuSO4, 10 mM sodium ascorbate, 2mM THPTA) with 0.5 µM AF647 Azide Plus (VectorLabs, #CCT-1482). The samples were analyzed on BD LSRFortessa cytometer, followed by data analysis using Diva and FlowJo software. Calculations of MFI values were made following the formula from original protocol (18) : Glucose dependence (%): 100*((Ctr-DG)/(Ctr-DGO)); Mitochondrial dependence (%): 100*((Ctr-O)/(Ctr-DGO)); Glycolitic capacity (%): 100-(100*((Ctr-O)/(Ctr-DGO)); FAO&AAO capacity (%): 100-(100*((Ctr-DG)/(Ctr-DGO)).

Trans-SILAC MS
Cell labeling with heavy isotopologues of lysine and arginine was performed as previously described (5). Briefly, the SILAC medium was supplemented with 10% of dialyzed FBS, 1% Pen/Strep, 0.274 mM L-lysine, and 1.15 mM L-arginine and filtered (0.22 µm pores). Donor cells were labeled with heavy isotopologues of lysine and arginine: L-lysine:2HCL (13C6, 99%; 15N2, 99%) and L-arginine:HCL (13C6, 99%; 15N4, 99%; Cambridge Isotope Laboratories). Cells were maintained for 9 days to enable complete labeling of the proteome. On day 8, donor cells were labeled with DiD dye as described above.

LC-MS/MS analysis
Mass spectrometry analysis was performed using an EASY nLC 1000 coupled to a Q-Exactive Plus mass spectrometer (ThermoFisher Scientific). Peptides were separated by a 180-min linear gradient of 95% solution A (0.1% formic acid in water) to 35% solution B (acetonitrile and 0.1% formic acid). Three washing runs preceded the measurement of each sample to avoid cross-contamination. The mass spectrometer was operated in the data-dependent MS-MS2 mode. Data was acquired in the m/z range of 300-1750 at a nominal resolution of 70,000.
Data were analyzed using the Max-Quant 1.5.3.12 platform, with the reference human proteome database from UniProt. False discovery rates of protein and peptide-spectrum matches (PSM) levels were estimated using the target-decoy approach at 0.01% (protein FDR) and 0.01% (PSM FDR), respectively. The minimal peptide length

Oxidation (M) and Acetyl (Protein N-term) were included as variable modifications. Only proteins that were represented by at least two unique peptides in two biological replicates are shown and were further considered. The data analysis was performed using MaxQuant software and the MaxLFQ algorithm (Supplementary Table 1). Lists of proteins were analyzed using the Panther application for GeneOntology software, STRING-confidence view, and Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/index.html). Additionally, lists of proteins were grouped according to their molecular weights based on the UniProt database. The mass spectrometry data from this publication have been deposited to the ProteomeXchange Consortium via the PRIDE [https://www.ebi.ac.uk/pride] partner repository with the dataset identifier PXD013504.

Metabolome MS

The normalized number of K562 cells (1.1 mln cells) were lysed with 100 µl of ice cold (-20 °C) methanol (LC–MS hyper grade, Merck). The samples were incubated in -20 °C for 2 h during which the samples were thoroughly vortexed every 20 min. After this time, the samples were centrifuged (30 min, 18,000 × g, -10 °C) and 80 µl of the supernatant was lyophilized with the use of a concentrator (Eppendorf). The lyophilizate was dissolved immediately before injection in a mixture of ddH2O and methanol (50:50 ratio) with addition of 0.1% formic acid, centrifuged (10 min, 18,000 × g, -10 °C) and 10 µl of sample was loaded for LC-MS. Untargeted metabolomics was analyzed using ultra-high-resolution Fourier-Transform Ion Cyclotron Resonance Mass Spectrometry with an electrospray ion source (ESI-FT-ICR-MS, SolariX 2xR 7 T, Bruker) in direct injection. The injection was made at a flow rate of 300 µl/h in both positive and negative polarity (with 3 technical replications of each sample). The ions accumulation time was set to 0.03 s., with the dry gas flow set to 4.0 L/min and drying temperature 200 °C. The capillary in the ion source was set to 3500 V (negative polarity) and 4500 V (positive polarity). The collected mass spectra (64 accumulated scans per spectrum) were analyzed using the T-Rex 2D algorithm (MRMS single spectra) in the MetaboScape 5.0 software (Bruker) and the identified compounds were assigned to specific signaling pathways using MetaboAnalyst 5.0 and the KEGG database.

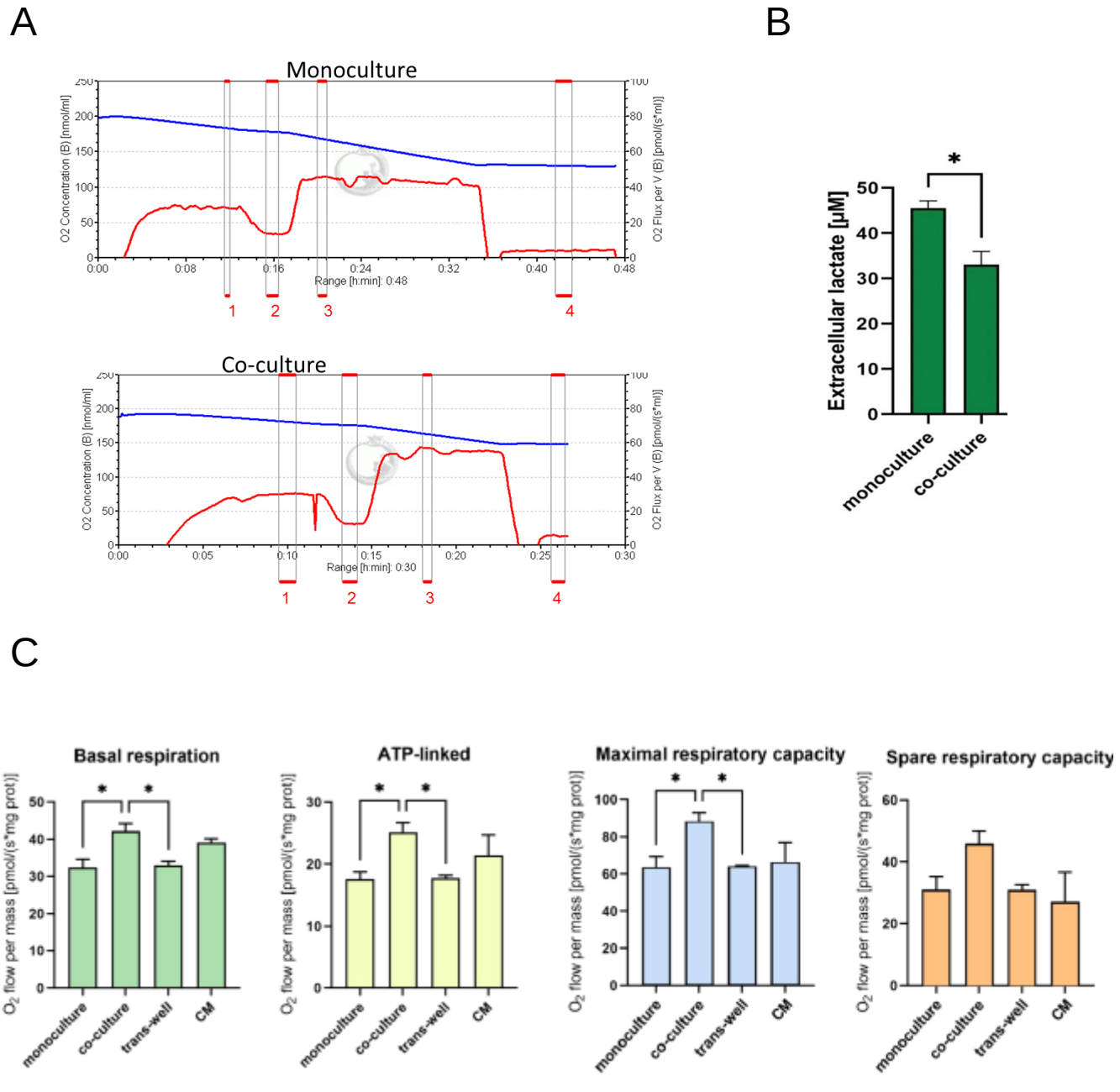
Total protein normalization

Cell pellets were solubilized in 200 µL of 0.5 M NaOH. For the total protein assay, the Bradford method was applied, using the premixed dye (Bio-Rad Protein Assay). The measurements were performed on 96-well plates using the microplate reader (Infinite M1000, Tecan, Switzerland). The protein amounts were calculated in reference to the standard curve prepared along with the samples based on the standard solution of bovine serum albumin (BSA).

Statistical analysis

All of the experiments were performed in at least three independent biological repetitions. Trans-SILAC was done in two biological replicates. All of the data are presented as mean ± SD. Data were analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). For Oroboros data analysis, unpaired parametric t-test was used. For further experiments, single comparisons were performed using ordinary one-way ANOVA test where normal distribution was checked with the Shapiro-Wilk test, and variance homogeneity with Brown-Forsythe test. For post hoc analysis Tukey test was performed. Two-way ANOVA performed in R was used to test the impact of imatinib and culture conditions on metabolic profiles. Values of $p < 0.05$ were considered statistically significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$.

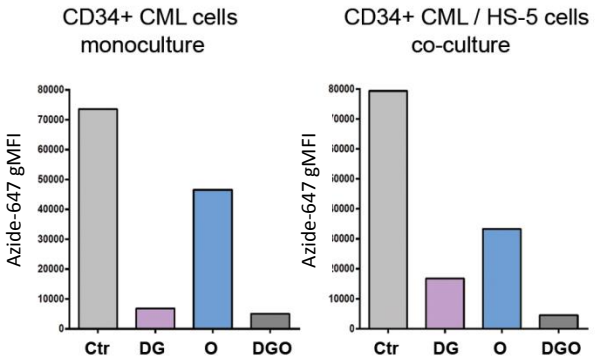
Supplementary Figures



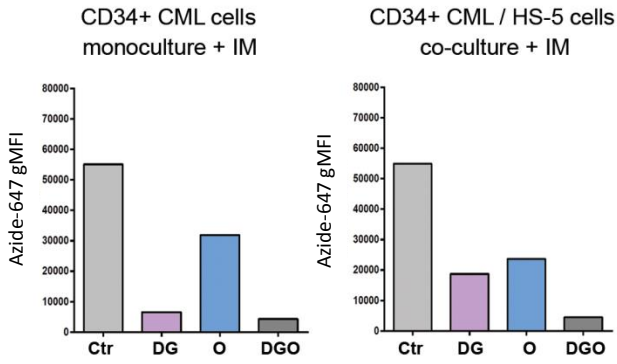
Supplementary Figure 1

A. The oxygen consumption rates measured by respirometry method (Oroboros) in leukemia cells grown in mono- or co-culture, treated with Oligomycin, CCCP, rotenone and antimycin A. The red line shows oxygen consumption. 1 - basal respiration, 2 - ATP-synthase independent respiration, 3 - maximal respiration, 4 - non-mitochondrial oxygen consumption. Representative oxygraphs are shown. **B**. Lactate production in leukemic cells cultured for 24 hours in monoculture or co-culture. **C** Metabolic profiles assessed by the respirometry analysis in monoculture, co-culture, co-culture in the trans-well system or leukemic cells incubated with conditioned medium (CM) from the stromal cells.

A



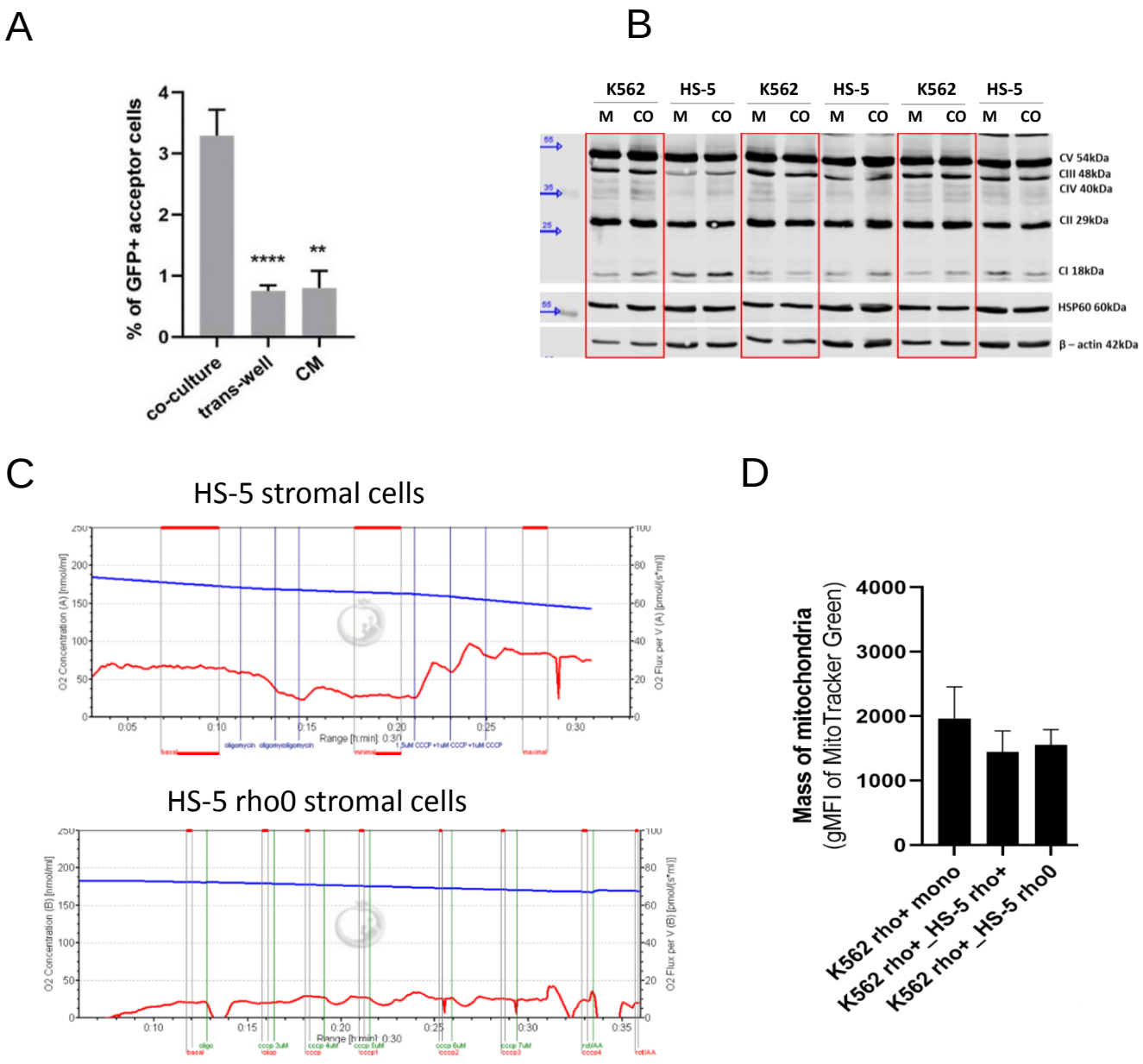
B



C

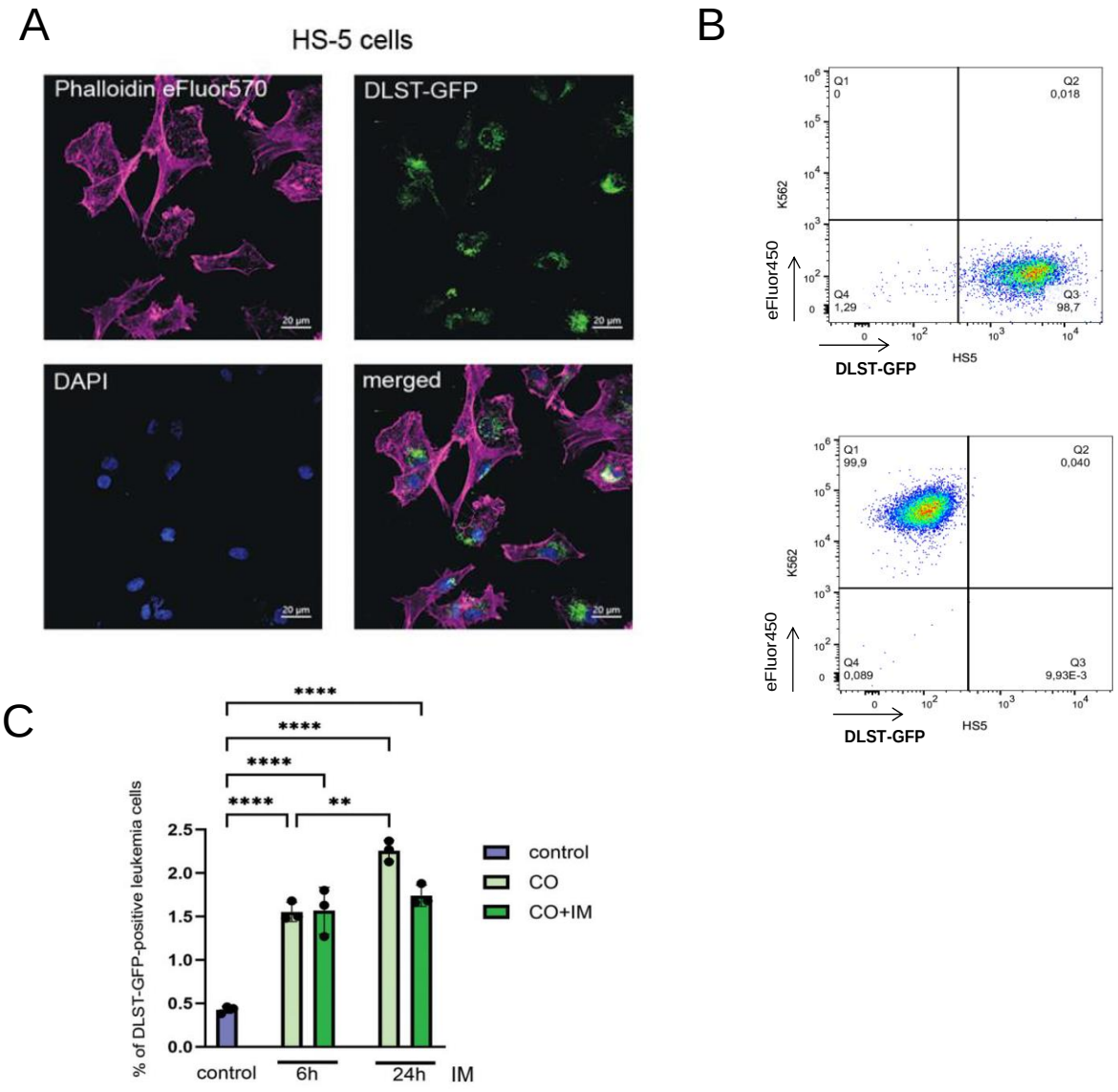
Glucose dependence (%):	100*((Ctr-**DG**)/(Ctr-**DGO**))
Mitochondrial dependence (%):	100*((Ctr-**O**)/(Ctr-**DGO**))
Glycolitic capacity (%):	100-(100*((Ctr-**O**)/(Ctr-**DGO**))
FAO&AAO capacity (%):	100-(100*((Ctr-**DG**)/(Ctr-**DGO**))

Supplementary Figure 2
Metabolic profiles assessed by the CENCAT method in CD45+CD34+ primary CML cells grown in monoculture or co-culture with stromal HS-5 cells, without treatment (**A**) or after treatment with imatinib (**B**). The geomean MFI values of fluorescent Azide-647 measured by BD Fortessa flow cytometer in control (Ctr) sample, or samples treated with 2-Deoxy-Glucose (DG), Oligomycin (O) or combination (DGO) are shown. (**C**).



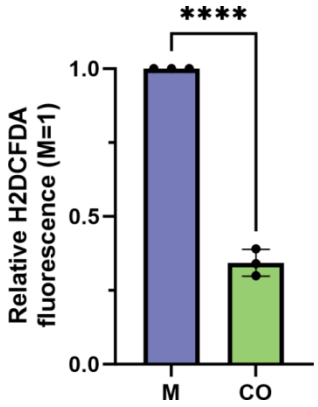
Supplementary Figure 3

A. A transfer of GFP+mitochondria from stromal to leukemic cells assessed by flow cytometry. The percentage of GFP+acceptor cells shows leukemic cells which received stromal mitochondria. B. The levels of respiratory subunit proteins estimated by Western Blotting in either HS-5 or K562 cells growing in mono- (M) or co-culture (CO). Data from three sets of experiments are presented. **B**. Oxygraphs showing oxygen consumption in control HS-5 cells and rho0 HS-5 cells. Cells were treated with Oligomycin, CCCP, and rotenone. Red line shows oxygen consumption. **C**. Mitochondrial mass estimated by flow cytometry in K562 cells stained with MitoTracker Green. Leukemic control cells were grown in monoculture (K562 rho+ mono) or co-culture with either HS-5 with metabolically active mitochondria (K562 rho+_HS-5 rho+) or dysfunctional rho0 stromal cells (K562 rho+_HS-5 rho0). Geomean values (gMFI) are shown. Data from four (n=4) independent experiments are shown.



Supplementary Figure 4

A. Expression of DLST-GFP protein in HS-5 cells additionally stained with Phalloidin eFluor670 (purple) and DAPI to stain nuclei (blue). Single fluorescence images or merges are presented. Images of HS-5 cells expressing DLST protein tagged with GFP. Single fluorescence images and merge signals are presented. **B**. Flow cytometry analysis of the expression of DLST-GFP protein in HS-5 and K562 cells tracked with a fluorescent dye (eFluor450). Upper panel - HS-5 cells (DLST donors) express DLST-GFP protein (96.7%).Lower panel - K562 cells (acceptors) are GFP-negative and eFluor450-positive (99.9%). Representative dot plots are shown. Analysis was performed using BD LSR Fortessa flow cytometer. **C**. The percentage of DLST-GFP -positive leukemic recipients indicating transfer from stromal donors to leukemic acceptor cells estimated by flow cytometry in control cells or co-culture (CO) (after 6 and 24 hours) without or with imatinib treatment (+IM). The data from n=3 is represented as means ± SD. Unpaired parametric t-test was used, p-values <0.05 were considered as statistically significant; *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001.



Supplementary Figure 5

ROS levels measured in leukemic cells growing in monoculture (M) or co-culture (CO) by the H2DCFDA assay. Cells were incubated with cell-pearmeable ROS probe and fluorescence was detected by flow cytometry. The values in monoculture are normalized to 1, the data from n=3 is shown represented as means ± SD. Unpaired parametric t-test was used, p-values <0.05 were considered as statistically significant; *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001.