BCR-ABL1 mediated miR-150 downregulation through MYC contributed to myeloid differentiation block and drug resistance in chronic myeloid leukemia

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Supplementary Appendix

Supplementary methods

Patients and samples

Bone marrow (BM) samples collected at the time of diagnosis were cytogenetically analyzed for the Ph chromosome with a median presence of 95 % (range, 67-100 %). First-line imatinib treatment was administered in 34/41 patients, while 3/41 patients received nilotinib, and 4/41 patients received IFNα as the first-line treatment. Eleven patients (11/41) were switched during first-line TKI treatment to another TKI due to therapy failure. Mutations were detected in 3 of the patients who experienced imatinib failure (Supplementary Table S1). BM samples were collected at the time of resistance to imatinib from 13 patients, from 1 patient resistant to second-line nilotinib treatment, from 3 patients resistant to third line dasatinib treatment and from 1 patient resistant to 4^{th} line treatment with dasatinib. Total leukocytes were isolated from BM and peripheral blood samples by lysing erythrocytes (RBC Lysis Buffer #42031; BioLegend, San Diego, CA, USA) and were used for subsequent analyses.

Cell sorting and separation

Total leukocytes isolated from the samples of CML patients were sorted into distinct subpopulations by fluorescence-activated cell sorting (FACS; BD FACS Aria Ilu; Becton Dickenson, Franklin Lakes, NJ, USA) according to the expression of the cell surface markers CD34 (PE anti-human CD34 antibody 581 #343506; BioLegend) and CD38 (PE-Cy5 anti-human CD38 antibody HIT2 #303508; BioLegend). The sorted subpopulations included $CD34^+CD38^-$, $CD34^+CD38^+$, $CD34^-CD38^+$, $CD34^-CD38^+$low and $CD34^-CD38^-$ cells.

The phenotypic characterization of total leukocytes isolated from BM samples collected at the time of diagnosis (n=10) was performed via FACS (BD FACS Canto II, Becton Dickenson) in two separate antibody-fluorochrome panels according to the expression of the cell surface markers 1) CD34, CD38 and CD19 (FITC anti-human CD19 HIB19 #302206; BioLegend) or 2) CD34, CD38, CD11b (APC anti-human CD11b IC RF44 #301310; BioLegend) and CD14 (PE-Cy7 anti-human CD14 HCD14 #325606; BioLegend).

$CD34^+$ and $CD34^-$ populations were isolated from samples of peripheral blood from healthy donors and from the samples of CML-CP patients at diagnosis by magnetic-activated cell sorting (MACS) using a CD34 MicroBead kit (130-046-702; Miltenyi Biotec, Bergisch Gladbach, Germany) and an autoMACS Pro Separator (Miltenyi Biotec).

Leukemic cell lines

Cell lines were authenticated by the provider and tested for Mycoplasma contamination using the MycoAlert PLUS detection kit (Lonza Group AG, Basel, Switzerland; LT07-705). Mycoplasma contamination was tested no later than one month after the cells were thawed. The K562R cell line resistant to 1 μM imatinib was established by gradually exposing K562 cells to increasing concentrations of imatinib in the medium, beginning with 0.2 μM imatinib for 4 months, followed by 0.4 μM imatinib for 2 months, 0.6 μM imatinib for 1 month and,

finally, 1.0 µM imatinib for 2 months. The KCL-22R cell line resistant to 4 µM imatinib was established by gradually exposing KCL-22 cells to increasing concentrations of imatinib in the medium, beginning with 0.4 µM imatinib for 1.5 months, followed by 1.2 µM for 0.5 month, then, 4.0 µM. The presence of BCR-ABL1 kinase domain mutations in the established resistant cell lines was determined at the transcript level by next-generation deep-sequencing (NGS) on a GS Junior platform (454 technology; Roche Applied Science, Basel, Switzerland) using BCR-ABL1 assays developed and distributed within the framework of the IRON-II phase study (Interlaboratory RObustness of NGS)[41]. A previously established protocol was used for NGS analysis[42]. No BCR-ABL1 mutant transcripts were detected in the K562R cell line, whereas the E255K mutation was present in 100 % of BCR-ABL1 transcripts from the KCL-22R cell line. The K562 and KCL-22 cells were verified by PCR detection of specific mRNA fusion variants of BCR-ABL1 gene (b3a2 for K562 and b2a2 for KCL-22) and KCL-22 cells were further verified by mBAND/mFISH analysis. No cell lines used in this work are listed in the database of commonly misidentified cell lines.

Transient transfection and cell treatment

Leukemic cells (10^6) were transfected with 30 nM synthetic hsa-miR-150 (ID: PM10070; Thermo Fisher Scientific, Waltham, MA, USA), 50 nM siRNA BCR-ABL1 (siRNA Duplex HPLC SR-HP001-001-29349990; Eurogentec, Seraing, Belgium), 50 nM siRNA MYB (Stealth siRNA MYBHSS106819, cat. #127985B05, Thermo Fisher Scientific) and 1 µg of a pMaxGFP expressing vector (Lonza Group AG). The BCR-ABL1 siRNA was designed as previously described[43]. All leukemic cell lines were cultured in triplicate. Cells were transfected via electroporation using a nucleofector device (Amaxa NucleofectorTM II, Lonza) and the appropriate nucleofection kits according to the manufacturer's instructions. K562 and KCL-22 cells were cultivated in the presence of 1 µM imatinib and in the presence 1 or 5 µM bromodomain inhibitor JQ1 (Sigma-Aldrich, St. Louis, MO, USA).

siRNA BCR-ABL1 design

Custom siRNA Duplexes-siRNA Duplex HPLC purified (10 nmol) were designed by a commercial supplier (Eurogentec). The design of siRNA Duplex was as follows:

b3a2_1, 5-GCAGAGUUCAAAAGCCCUUdTdT-3
b3a2_3, 5-AGCAGAGUUCAAAAGCCCUdTdT-3

b3a2_1, 5-AAGGGCUUUUGAACUCUGCdTdT-3
b3a2_3, 5-AGGGCUUUUGAACUCUGCUdTdT-3

The stealth siRNA MYB design

UAUAGUGUCUCUGAAUGGCUGCGGC

RNA isolation and RT-qPCR

Total cellular RNA enriched for small RNAs was isolated from primary cells using the MirVana miRNA Isolation Kit according to the manufacturer's instructions (Thermo Fisher Scientific). Total cellular RNA was isolated from leukemic cell lines using the TRIzol Reagent (#15596026; Thermo Fisher Scientific) according to the standard operating procedure established in our laboratory. RNA quality and quantity were analyzed on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). To measure GUSB, BCR-ABL1, MYB, MYC and PU.1 expression levels, 200 ng of RNA was transcribed using 200 U/µl of M-MLV Reverse Transcriptase–l (#28025013; Thermo Fisher Scientific) using random hexamers. To measure RNU48, miR-150 and miR-155 levels, reverse transcription was performed using 50 U/µl MultiScribe Reverse Transcriptase (#4319983; Thermo Fisher Scientific) with RT-specific primers (5x). To measure the expression of pri-miR150, pre-miR150 and HPRT1, reverse transcription was performed using the iScript cDNA Synthesis Kit (#170-8891; BioRad Laboratories, Hercules, CA, USA). The conditions used for the reverse transcription reactions were set according manufacturer instructions. TaqMan Gene Expression Assays Hs00193527_m1 20x for MYB and Hs2786711_m1 20x Mix for PU.1 (SPI1) (Thermo Fisher Scientific) and the TaqMan MicroRNA Assays RT000473 20x for hsa-miR-150, RT002623 20x for hsa-miR-155 and RT001006 20x for the housekeeping gene RNU48 (Thermo Fisher Scientific) were used to perform relative quantification using TaqMan Universal Master Mix II (#4427788; Thermo Fisher Scientific) on the StepOnePlus system (Thermo Fisher Scientific). Transcript quantification of BCR-ABL1 is standardized in our laboratory using the European Treatment and Outcome Study (EUTOS) for CML project, and data are reported according to the International Scale (IS)[44, 45.] Primers and probes for BCR-ABL1 and the housekeeping gene *GUSB* have been validated and applied according to Europe against Cancer recommendations[46]. Pri- and pre-miR-150 expression was quantified using specific primers (appropriate sequences were kindly provided by Prof. Jianjun Chen, Section of Hematology/Oncology, Department of Medicine, University of Chicago, Chicago, IL, USA) using SYBR Green-based PCR[22]). RT-PCR conditions were set according manufacturer instructions as follows: 95°C for 10 min; and 45 cycles of 95°C for 15 sec and 60°C for 1 min. The converted CT values of specific (s) and control (c) amplicons calculated using the $2^{-(CTs-CTc)}$ equation were compared using Student's *t*-test.

Protein isolation and immunoblotting

The cells (10^6) were lysed for 15 min in RIPA buffer (50 mM Tris-Cl, pH 8.0, 137 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate, and protease inhibitor cocktail 1:1000 dilution P8340; Sigma-Aldrich) and sonicated using an M500 digital sonifier (Emerson, St. Louis, MO, USA). The denatured cell lysates (10 µg of protein per sample) were resolved on 4 %-12 % gradient Bis-Tris gels (NuPage; Thermo Fisher Scientific). The gels were dry-blotted on an iBlot Gel Transfer System according to the manufacturer's instructions (Thermo Fisher Scientific). The primary antibody (diluted 1:500) anti-v-MYB/c-MYB [EP769Y] (Abcam, Cambridge, UK) was used. Anti-β-Actin [AC-15] (Santa Cruz Biotechnology, Dallas, TX, USA) at 1:1000 or anti-β-actin horseradish peroxidase-conjugated (HRP) antibody [I-19] at 1:10000 (Santa Cruz Biotechnology) was used to determine sample loading. The secondary HRP-conjugated antibody anti-rabbit (#711-030-152; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used at 1:3000 dilution. Bands were visualized using the ECL Plus Western Blotting Detection System (GE Healthcare, Chicago, IL, USA) on CL-

XPosure films (Thermo Fisher Scientific). Bands were quantified using ImageJ (National Institute of Mental Health, Bethesda, MD, USA).

Chromatin immunoprecipitation

Formaldehyde cross-linked chromatin from $1x10^7$ K562, KCL-22 and HL-60 cells and from separated $CD34^+$ and $CD34^-$ primary cells was prepared as previously described[47] using Anti-MYC (ab56 [9E11]; Abcam) or control IgG (NI01; Calbiochem®, Merck KGaA, Darmstadt, Germany). The enrichment quantification was performed as previously described[48].

miRNA RNA sequencing

Total RNA (100 ng) from FACS-purified $CD34^+CD38^-$ and $CD34^+CD38^+$ cell fraction of non-identifiable BM samples from healthy donors (n=3) and untreated CML patients in chronic (n=3) and blastic (n=3) phases was used in RNA sequencing for assessing differences in miR-150 and miR-155 expression. Sequences of miR-150 and miR-155 were applied from miRbase. miRNA libraries were prepared using a modified version of the TruSeq Small RNA Library preparation kit (Illumina, San Diego, CA, USA). According to the manufacturer's protocol, adapters were diluted 1:2 for sample input under 150 ng. Samples with input over 150 ng used 1X adapters. Post adapter annealing and cDNA synthesis samples were subject to 15 cycles of PCR per manufacturer's protocol. The 3' and 5' adapters were replaced by adaptors from TriLink Biotechnologies (San Diego, CA, USA). All other reagents and incubation times were as prescribed in the TruSeq protocol. The final library size selection was performed by gel selection on a 3 % agarose gel. Fragments between 140-160 bp were cut and extracted from the agarose using the Qiagen gel extraction kit (cat. number 28704, Qiagen, Valencia, CA, USA). Post purification samples were evaluated for size, quality, and quantity on the Perkin Elmer Gx. Using GX quantification, samples were pooled in equimolar amounts. The sample pool was further quantified using the Kapa Library Quantification Kit (cat. number KK4873). The sample pool was diluted to 2 nM, denatured with 0.1 N sodium hydroxide and loaded on a MiSeq sequencing system for 50 cycles of sequencing using a MiSeq ReagentKit v2 (catalog number MS-102-200). Final library QC was performed using the LabChip XT (Caliper Life Sciences, Hopkinton, MA, USA). Following standard data processing and demultiplexing, adaptor trimming was performed using Trimmomatic. The number of reads that overlapped with each miR-150 and miR-155 sequences from miRBase v21 after alignment with human genome GRCh38 was calculated using miRDeep quantifier module[49,50]. We normalized the read counts for all samples to counts per million (CPM) by dividing the total read counts of a miRNA by the total read counts of the sample and multiplying this number by 10^6. We used the normalized read counts per miRNA to look at the expression profile of the miRNAs.

Cell cycle and cell viability analyses

Cells (1-2 x 10^6) were washed with Annexin V binding buffer (#422201; BioLegend). The cell pellets were stained with 1 µM Annexin V – APC (#640920; BioLegend) for 15 min in the dark. Cells were fixed with 0.5 ml of 2 % formaldehyde (F8775; Sigma) for 10 min at room temperature and then permeabilized by incubation with 1 ml of 70 % ethanol for 30 min in

the dark. The cells were repeatedly washed with 1 % BSA in PBS and then stained with 1 µM FxCycle™ Violet Stain (F10347; Thermo Fisher Scientific). Cell cycle phases and cell viability were determined by FACS (BD FACS Canto II, Becton Dickenson).

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Supplementary figure legends

Supplementary Figure S1. Gene expression levels of leukemic pathway genes in primary CML cells and their mutual correlations. (a) MYC, miR-150, MYB, miR-155 PU.1 and BCR-ABL1 levels in $CD34^+CD38^-$, $CD34^+CD38^+$, $CD34^-CD38^+$, $CD34^-CD38^+$low and $CD34^-CD38^-$ cell subpopulations sorted from BM samples of CML-CP patients at the time of diagnosis (n=28) and at the time of resistance to TKIs (n=18). The blue lines indicate the median values. **(b)** Fractions (in %) of CD11b-, CD14- or CD19-positive cells among leukemic BM subpopulations defined by CD34 and CD38 expression obtained from CML-CP patients at the time of diagnosis. **(c)** Left: Correlations of the gene expression levels across all FACS sorted subpopulations: $CD34^+CD38^-$, $CD34^+CD38^+$, $CD34^-CD38^+$, $CD34^-CD38^+$low and $CD34^-CD38^-$ cells isolated from the BM of CML-CP patients at the time of diagnosis (n=28) and at the time of resistance to TKIs (n=18). The positive and negative correlations between the expression

levels of the studied molecules are marked in yellow or blue, respectively, and significant correlations are indicating by *. Right: P-values and correlation coefficients (r) values characterizing the possible relationships between the expression levels of specific molecules are shown in the tables. P- and R-values were rounded to two significant digits.

Supplementary Figure S2. Expression of genes involved in a putative oncogenic pathway in leukemic cell lines. MYC, miR-150, MYB, miR-155, PU.1 and BCR-ABL1 expression among selected CML (K562, KCL-22, MEG-01) and AML (KG-1, HL-60) cell lines. Unpaired two-tailed Student´s t-test was used to determine p-values. The error bars represent the standard deviations. The expression data for MYC, miR-150, MYB, miR-155 and PU.1 represent the expression fold change (FC; $2^{-\Delta\Delta Ct}$) of leukemic cell lines and $CD34^+$ PBMC in relation to $CD34^-$ PBMC cells sample normalized to 1. FACS analysis revealed that BCR-$ABL1^+$ cells did not express CD34. BCR-ABL1 expression is indicated as % in the International Scale (IS). BCR-ABL1 expression in $CD34^+CD38^-$ and $CD34^-CD38^+$ subpopulations of CML BM samples collected at the time of diagnosis is shown for comparison and better assessment. * P<0.05, ** P<0.01 and *** P<0.001.

Supplementary Figure S3. miR-150 levels in leukemic cell lines 48 h after miR-150 transfection compared with miR-150 levels in $CD34^-$ PBMCs. Please note that BCR-$ABL1^+$ cell lines are CD34 negative.

Supplementary Figure S4. The effects of miR-150 overexpression and BCR-ABL1 silencing on the cell cycle and apoptosis in CML cell lines. (a) Relative representation of the G1/G0, S and G2/M cell populations in K562 cells 96 h after miR-150 transfection and/or BCR-ABL1 activity inhibition with 1 μM imatinib. **(b)** The relative size of the apoptotic cell populations in the K562 cultures 48 and 96 h after miR-150 transfection and/or BCR-ABL1 activity inhibition with 1 μM imatinib. **(c)** Relative representation of the G1/G0, S and G2/M cell populations in KCL-22 cells 48 h after BCR-ABL1 activity inhibition with 1 μM imatinib. **(d)** Relative size of the apoptotic cell populations in the KCL-22 culture 48 and 96 h after BCR-ABL1 activity inhibition with 1 μM imatinib. * P<0.05, ** P<0.01 and *** P<0.001.

Supplementary Figure S5. Protein binding at miR-150 upstream DNA (ChIP-Seq from UCSC). Filled red squares mark regulatory loci containing protein-binding motifs. Red empty squares indicate putative MYC-binding sites.

Supplementary Figure S6. MYC (a), BCR-ABL1 (b) and miR-150 (c) levels in KCL-22 and K562 cells upon JQ1. The expression data represent the expression fold change (FC; $2^{-\Delta\Delta Ct}$) in relation to untreated, control (Ctrl) sample normalized to 1. * P<0.05, ** P<0.01, and *** P<0.001. The error bars represent the standard deviations.

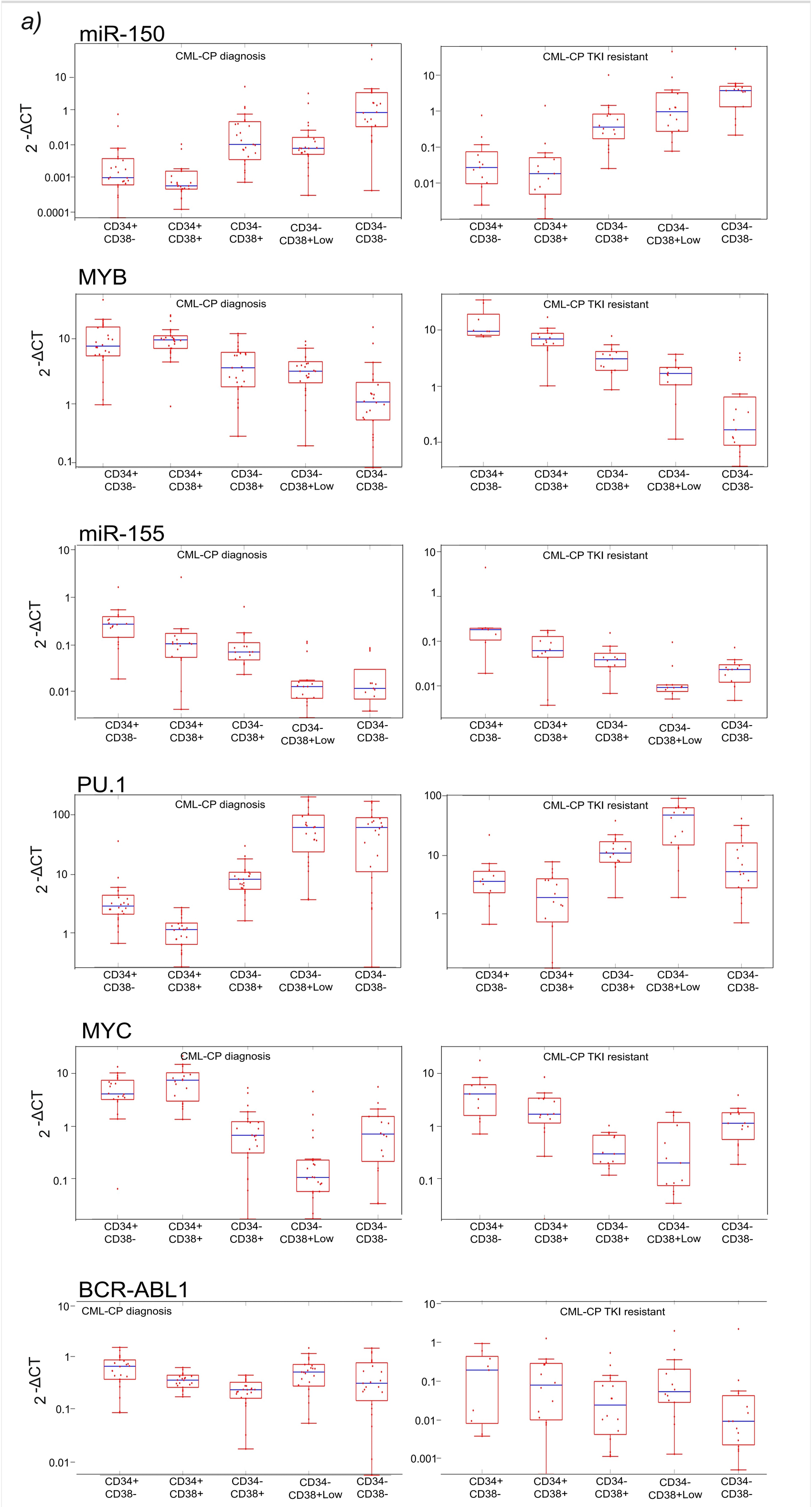
Supplementary Figure S7. MYB protein expression in **(a)** KCL-22 cells and **(b)** K562 cells 48 and 96 h after MYB siRNA transfection. The numbers at the top of the western blot indicate normalized MYB protein expression relative to the untreated control sample. Protein expression was normalized to that of control proteins (β-actin). **(c)** BCR-ABL1, MYC, miR-150, miR-155 and PU.1 levels in KCL-22 and K562 cells 96 h after MYB siRNA transfection.

Supplementary Table S1. Characteristics of the patients.

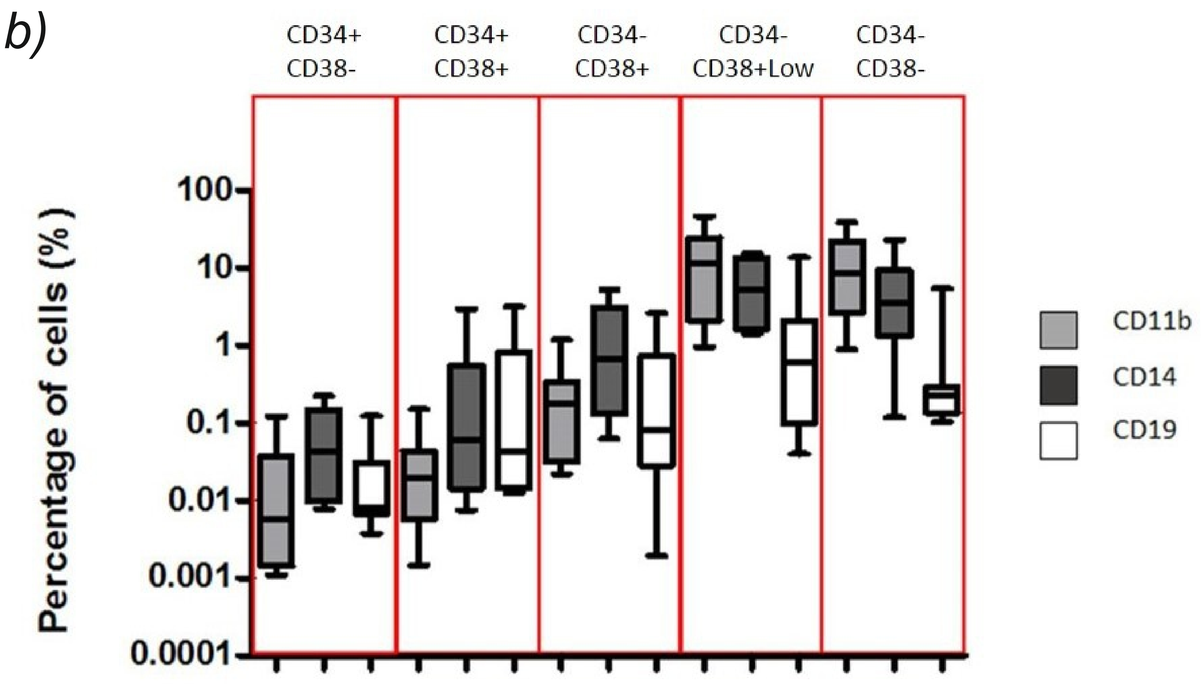
	Cohort of patients
Number	41
Median of age (range)	55 (30-72)
Sex	Female n=16 Male n=25
EUTOS score	Low n=31 High n=7 N/A n=3
Sokal score	Low n=15 Intermediate n=15 High n=8 N/A n=3
Hasford score	Low n=15 Intermediate n=18 High n=5 N/A n=3
First line treatment	n=34 imatinib n=4 IFNα n=3 nilotinib
Response to 1st line treatment at 12 months	Optimal n=20 Warning n=3 Failure n=14 n=4 data not available
MMR achievement on 1st line treatment Median months therapy start (range)	n=23 8.9 (4-25.1) 20 patients achieved MMR at 12 months
Therapy switch due to 1st line treatment failure; response lost or progression (median months after start of 1st line treatment; range)	n=11 19.0 (2-80)
Mutations (median time of detection on 1st line TKI treatment; range)	n=3 (12.6; 10-45) S417Y; M244V; E453V
Second line treatment	n=6 dasatinib n=3 nilotinib n=2 imatinib
MMR achievement on 2nd line treatment Median months therapy start (range)	n=5 6.65 (2.5-33.2)
Third line treatment	n=2 nilotinib n=1 imatinib
MMR achievement on the 3rd line treatment Median months therapy start (range)	n=2 2.65 (2.5-2.8)
Fourth line treatment	n=1 dasatinib
MMR achievement on 4th line treatment Median months therapy start (range)	n=0
Death related to CML	n=1 during 5th line treatment
Death un-related to CML	n=3 during 1st line treatment

Response to imatinib treatment was evaluated according to the ELN recommendation[19]

Supplementary Figure S1



b)



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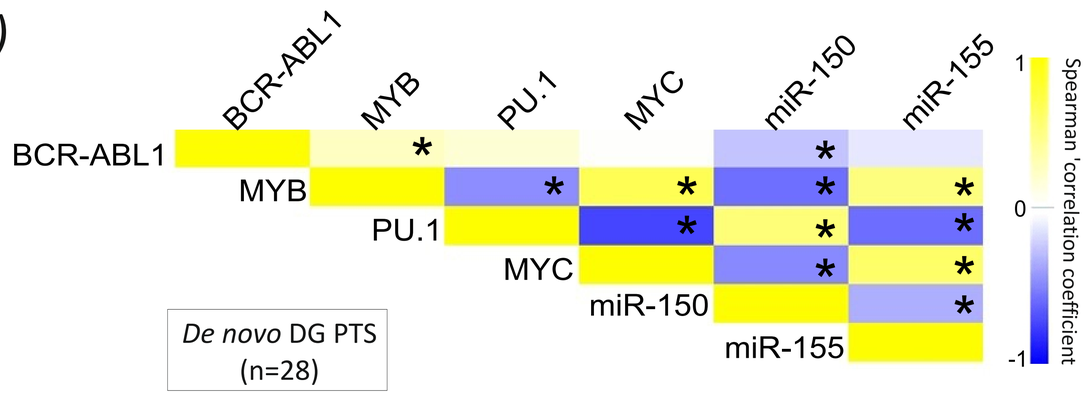
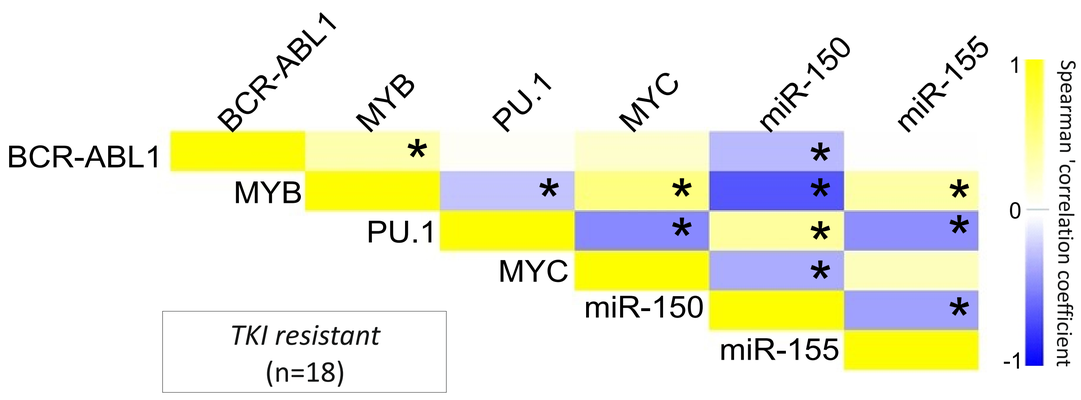
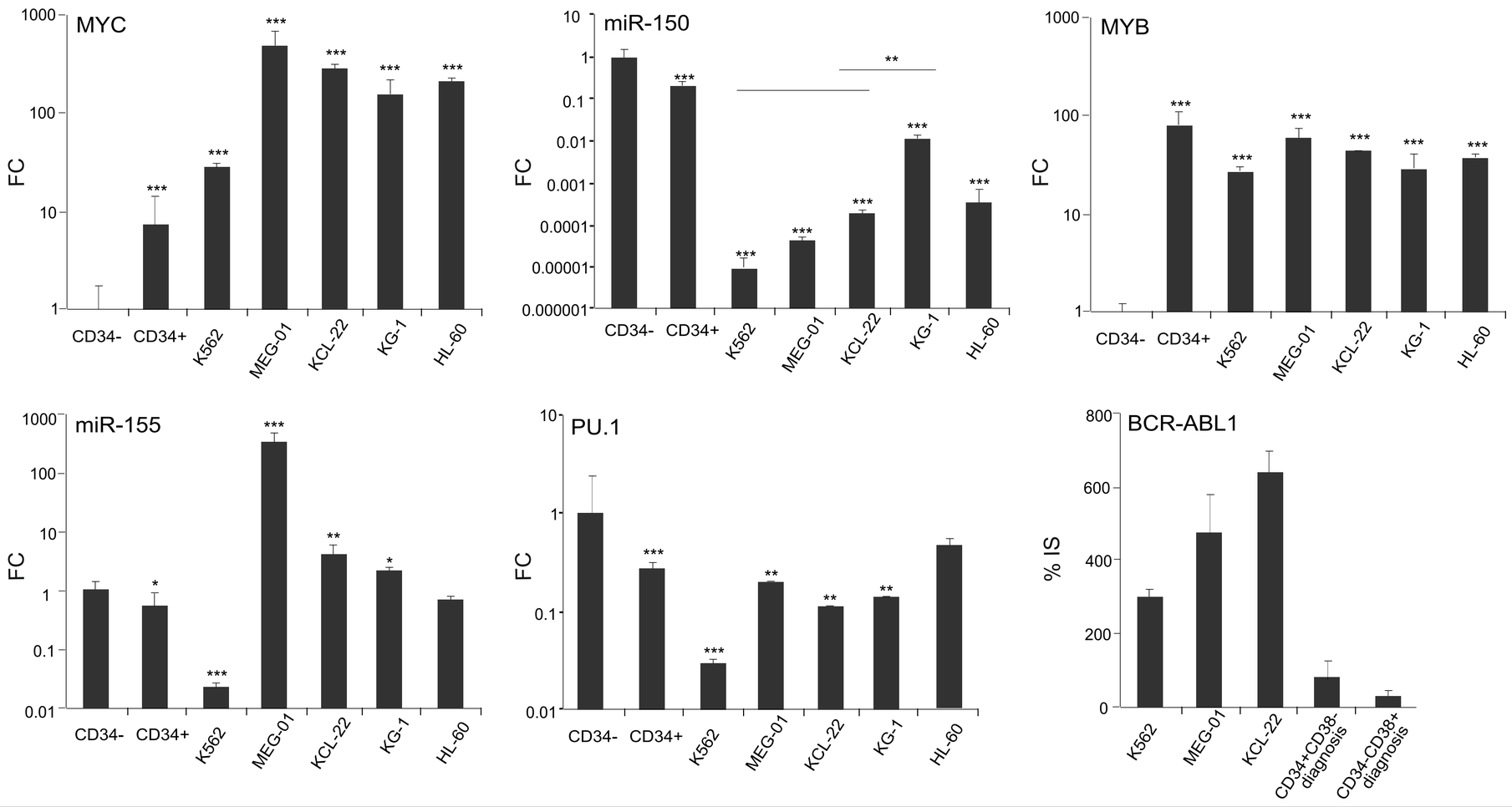


Diagnosis	MYB		PU.1		MYC		miR-150		miR-155	
	P	r	P	r	P	r	P	r	P	r
BCR-ABL1	0.01	0.24	0.06	0.17	0.9	0.01	0.01	-0.24	0.4	-0.01
MYB			p<0.0001	-0.45	p<0.0001	0.67	p<0.0001	-0.57	p<0.0001	0.51
PU.1					p<0.0001	-0.73	p<0.0001	0.53	p<0.0001	-0.57
MYC							p<0.0001	-0.46	p<0.0001	0.59
miR-150									0.003	-0.32

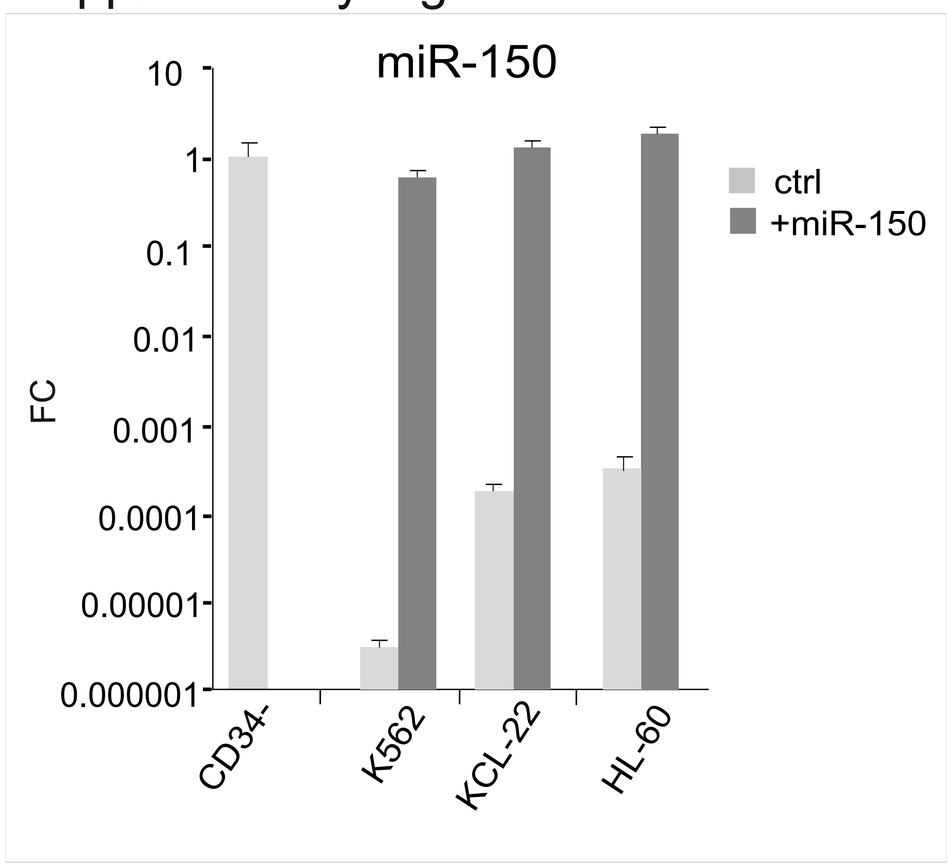


resistant	MYB		PU.1		MYC		miR-150		miR-155	
	P	r	P	r	P	r	P	r	P	r
BCR-ABL1	0.01	0.32	0.77	0.04	0.12	0.2	0.03	-0.27	0.98	0.01
MYB			0.04	-0.24	p<0.0001	0.5	p<0.0001	-0.66	0.01	0.35
PU.1					p<0.0001	-0.46	0.001	0.38	0.001	-0.44
MYC							0.01	-0.33	0.06	0.27
miR-150									0.01	-0.37

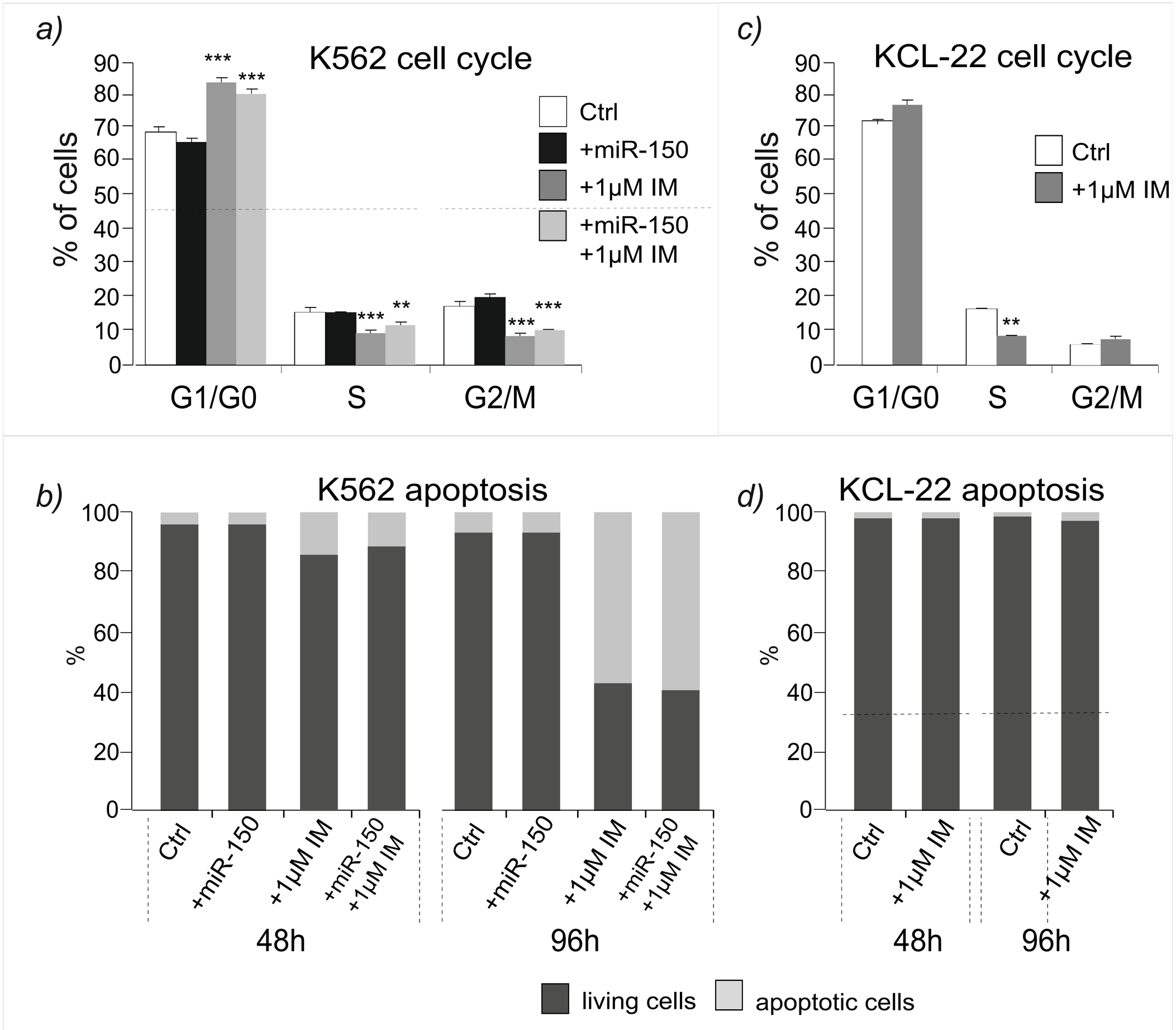
Supplementary Figure S2



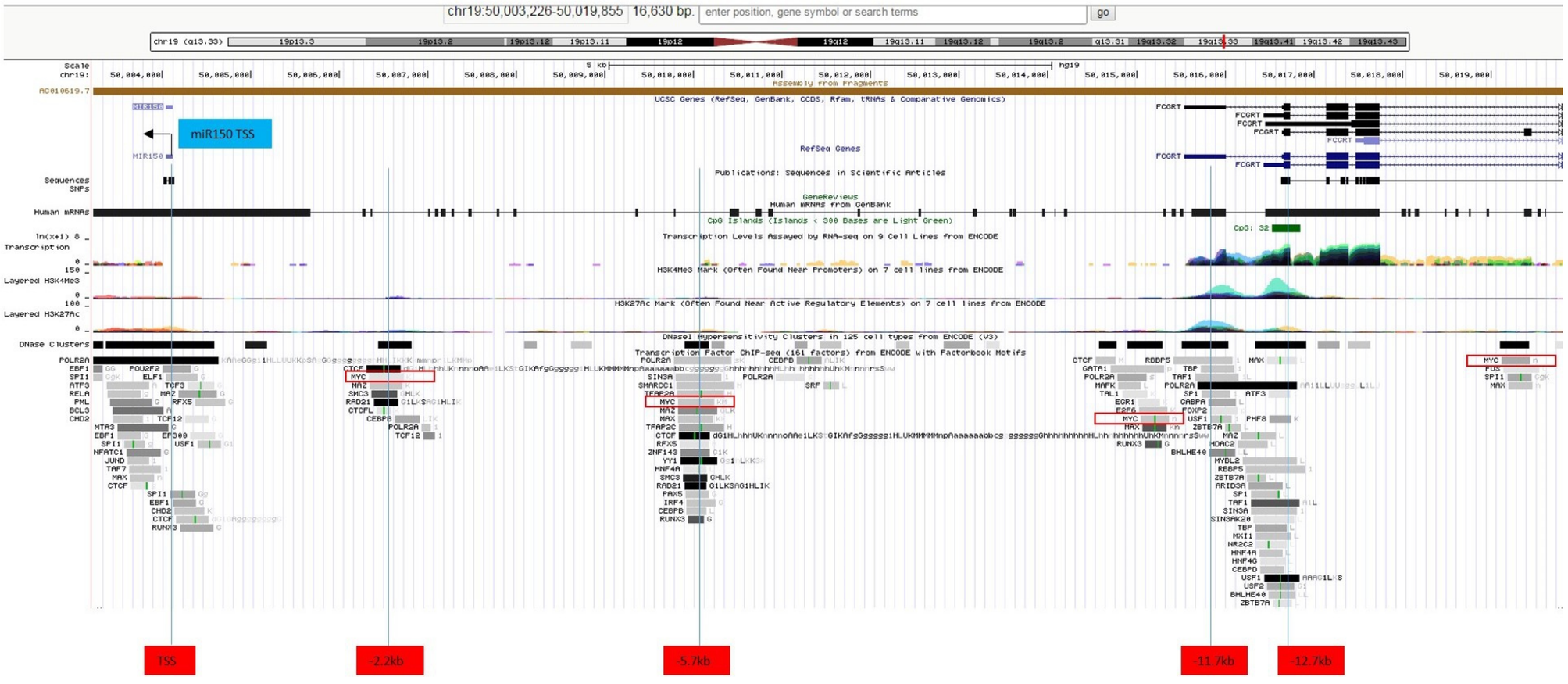
Supplementary Figure S3



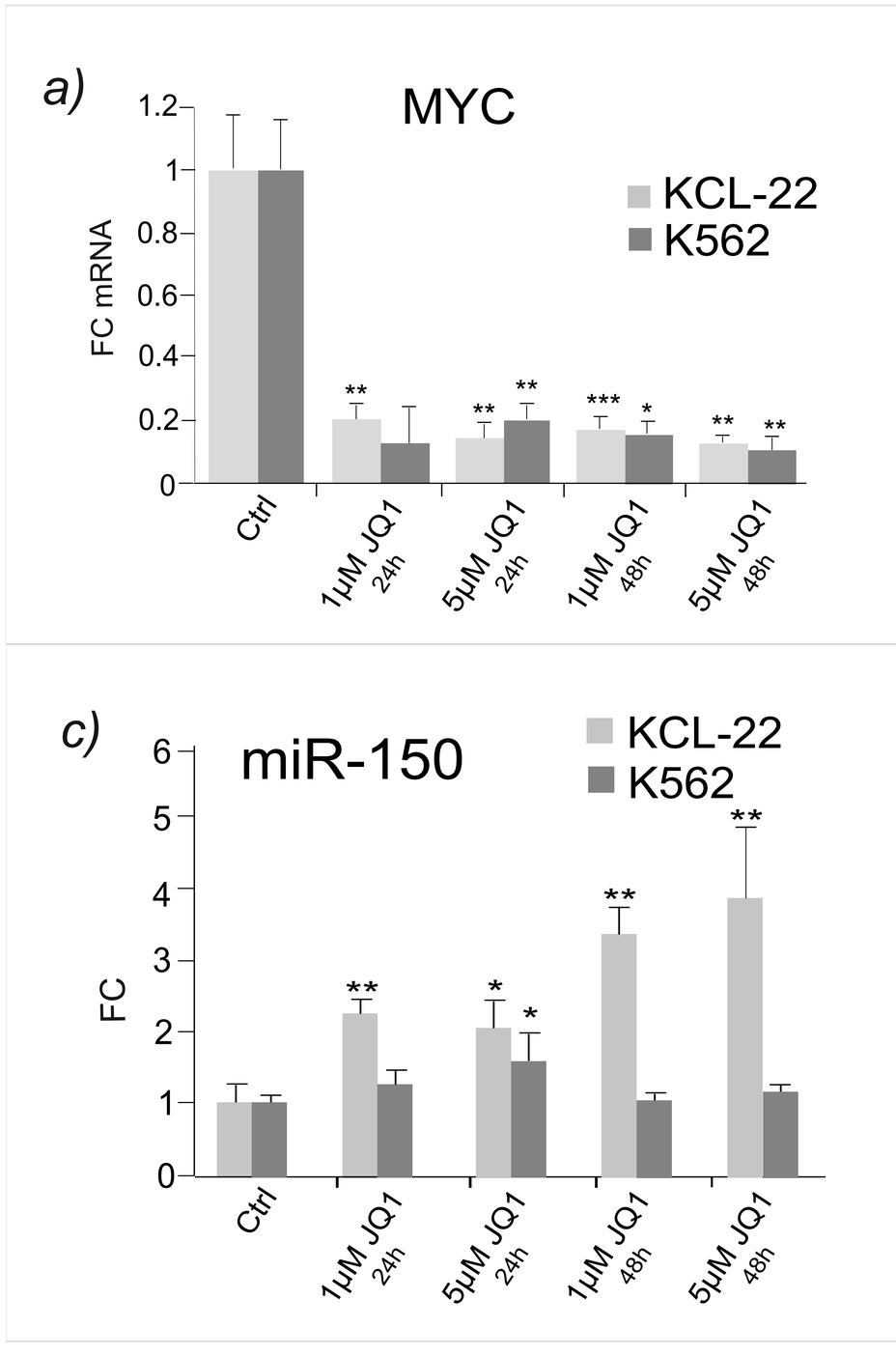
Supplementary Figure S4



Supplementary Figure S5



Supplementary Figure S6



Supplementary Figure S7