Transcriptional activation of the miR-17-92 cluster is involved in the growth-promoting effects of MYB in human Ph-positive leukemia cells

Manuela Spagnuolo,[1‡] Giulia Regazzo,[1‡] Marco De Dominici,[2‡] Andrea Sacconi,[1] Andrea Pelosi,[1†] Etleva Korita,[1] Francesco Marchesi,[3] Francesco Pisani,[3] Alessandra Magenta,[4] Valentina Lulli,[5] Iole Cordone,[6] Andrea Mengarelli,[3] Sabrina Strano,[1] Giovanni Blandino,[1] Maria G. Rizzo[1*] and Bruno Calabretta[2*#]

[1]Department of Research, Advanced Diagnostics and Technological Innovation, Oncogenomic and Epigenetic Unit, Translational Research Area, IRCCS - Regina Elena National Cancer Institute, Rome, Italy; [2]Department of Cancer Biology and Sidney Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA, USA; [3]Department of Clinical and Experimental Oncology–Hematology and Stem Cell Transplant Unit, IRCCS - Regina Elena National Cancer Institute, Rome, Italy; [4]Istituto Dermopatico dell'Immacolata-IRCCS, FLMM, Laboratorio di Patologia Vascolare, Rome, Italy; [5]Department of Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy and [6]Department of Research, Advanced Diagnostics and Technological Innovation, Clinical Pathology Unit, IRCCS - Regina Elena National Cancer Institute, Rome, Italy

[†]Present address: Department of Immunology, IRCCS, Ospedale Pediatrico Bambino Gesù, Rome, Italy. [‡]MS, GR and MDeD contributed equally to this work. [#]MGR and BC contributed equally as last authors.

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Correspondence: maria.rizzo@ifo.gov.it or bruno.calabretta@jefferson.edu

Supplemental

Cell cultures and viral infection

Ph+ BV173, SUP-B15 and K562 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin–streptomycin and 2mM L-glutamine at 37°C, 5% CO_2.

The GFP-expressing, doxycycline (Doxy)-inducible pLVTSH ShMYB lentivirus was kindly provided by Dr. Thomas Gonda [1]. To produce VSV-G pseudo-typed lentiviral particles the ShRNA vector was transfected by calcium phosphate method with the psPAX2 and pMD2.G plasmids. The supernatant was collected 24 h after transfection and BV173 and K562 cells were transduced by three cycles of spinoculation at 800 x g for 45 minutes at 32°C. GFP-positive cells where isolated by FACS. For ShRNA induction, K562 cells were plated at 50000/mL and BV173 at 250000/mL in the presence of doxycycline (Doxy; RPI Corp, Mount Prospect, Illinois).

The transduced cell line BV173 shRNA-resistant MYB was obtained as previously described [2].

For miR-17-92 overexpression, cells were transduced with the retroviral plasmid MSCV-miR-17-92 (Addgene, plasmid #64100) or the empty retroviral vector (EV). VSV-G pseudo-typed retroviral particles were produced by co-transfection of 293T cells with the retroviral plasmid, the pHIT123 and the pVSVG plasmids. The supernatant was collected 24 h later and used to transduce BV173 cells selected by puromycin (3 µg/mL).

Cell proliferation, cell viability, cell cycle analysis and apoptosis assays

Cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltrazolium bromide (MTT) assay. Briefly, MTT solution (Sigma-Aldrich) was added to BV173 cells with/without Doxy (2,5 µg/mL). After 3 hours (37°C) isopropanol solution was added. The absorbance was measured at 570 nm (microplate reader; Thermo LabsystemsMultiskan EX).

Viability of untreated and Doxy-treated cells was assessed using ATPlite Luminescence Assay System (Perkin Elmer, Waltham, MA, USA) according to the manufacturer's instructions. Each

plate was evaluated on a microplate reader (Expire Technology, Perkin Elmer) and analyzed using the GraphPad Prism Software.

For cell cycle analysis, cells were permeabilized in a solution (0,1% Triton X-100, 0,1% sodium citrate) and stained with 50 μg/mL propidium iodide. DNA content was determined by flow cytometry using the BD LSR-Fortessa. Apoptosis was evaluated by Annexin V staining.

For all functional assays, a sample of each cell culture was collected after each time to determine MYB knockdown by Western blotting.

Western Blot

Extracts from untreated and Doxy-treated cells were subjected to immunoblot analysis as described [3]. Proteins of interest were detected using: anti-c-MYB (05-175; Millipore, Darmstadt, Germany), anti-beta-Actin (Cell signaling, Lexington, KY), anti-E2F1 (KH95; Santa Cruz Biotechnology, Santa Cruz, CA) monoclonal antibodies; anti-BCL-2 (N-19; Santa Cruz Biotechnology, Santa Cruz, CA), anti-PARP (9542S; Cell Signaling, Milan, Italy), anti-p21 (C-19; Santa Cruz Biotechnology, Santa Cruz, CA) polyclonal antibodies.

Microarray and data analysis

RNA from untreated or Doxy-treated (7,5 μg/mL for K562-ShMYB and 2,5 μg/mL for BV173-ShMYB; 24 h) BV173- and K562-ShMYB cells was extracted using the miRNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Concentration and purity of total RNA were assessed using a Nanodrop TM 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA integrity number (RIN) was measured using Bioanalyzer RNA Nano Chip (Agilent Technologies). Only samples with a RIN $\geq$ 8.0 were selected for microarray hybridization.

Total RNA (200 ng) was labeled and hybridized to Human miRNA Microarrays V19 (Agilent) for 2006 human miRNAs, using the miRNA Complete Labeling and Hyb kit to generate fluorescently labeled miRNAs.

Scanning and image analysis were performed using Agilent DNA Microarray Scanner (P/N G2565BA) equipped with extended dynamic range (XDR) software according to the Agilent miRNA Microarray System. FE Version 10.7.3.1 was used for data extraction from raw microarray image files using the miRNA_107_Sep09 Feature Extraction protocol. Signals from miRNA's arrays were verified for quality control and extracted by Agilent Feature Extraction 10.7.3.1 software. All values < 1 were considered below detection and given a value of 1. The signal of each sample was scaled dividing by the median intensity and log2-trasformed. Bioinformatic analyses were performed by MATLAB (The MathWorks Inc.). Deregulation of miRNAs was assessed using a permutation test and a false discovery procedure was included for multiple comparisons [4]. Statistical significance was set to 5%. Unsupervised hierarchical clustering was performed to identify specific pattern of expression using the Euclidean distance metric. Predicted miRNA-target interaction was investigated by the web server tool miRWalk 2.0.

Transcriptional profiling and data analysis.

Total RNA was extracted and purified from BV173-ShMYB untreated or Doxy-treated (2,5 µg/mL; 24 h) or K562 cells transfected with MYB siRNAs (24 h after transfection) by using RNeasy Mini kit (Qiagen, Hilden, Germany) and used (1 µg) to generate biotin-labeled cRNA using an oligo T7 primer in a reverse transcription reaction followed by *in vitro* transcription reaction with biotin-labeled UTP and CTP. 10 µg of cRNA were fragmented and hybridized to GeneChip Human Gene 1.0 ST Array (Affymetrix). Hybridized arrays were stained according to the manufacturer's protocols on a Fluidics Station 450 and scanned on an Affymetrix scanner 3000. Signal values were determined using the Gene Chip Operating System 1.0 (GCOS, Affymetrix, Santa Clara, CA, USA).

Reverse transcription and quantitative Real-Time-PCR (qRT-PCR).

Total RNA was isolated using Trisure Reagent (Bioline, London, UK). Analysis of mature miRNAs was performed using TaqMan® MicroRNA Reverse Transcription Kit followed by qRT–PCR according to manufacturer's instructions (Applied Biosystems Inc., Foster City, CA, USA) on an ABI 7900 Real Time PCR System and SDS 2.2.2 software. Samples were normalized to RNU44 small RNA.

RNA quantification of *PBX2, FRZB*, *PTEN, BIM* and *THBS1* genes (primers are listed in **Table S1**) was performed using SYBR Green-based qRT-PCR as described [5]. *GAPDH* gene expression was used as endogenous control.

Plasmids, transfections, and luciferase assays

The human FRZB 3'UTR (NM_001463) containing the target sites for miR-17-92 was amplified by PCR from genomic DNA using specific primers and cloned into pGL3 Control vector (Promega) downstream of the luciferase gene. The mutant plasmid containing mutations of the miR-17-92 target sites was obtained by site specific mutagenesis (primers sequences in **Table S1)**. All the plasmids were verified by sequence analysis.

Human 293T cells were transiently co-transfected by Lipofectamine 2000 (Invitrogen), with 800 ng of firefly luciferase reporter plasmid containing wild-type or mutant FRZB 3'UTRs and of either the miRIDIAN hsa-miR-17 and hsa-miR-19a mimics or control (Ctr)-mimics RNA oligonucleotides (20 pmol, Dharmacon/ThermoScientificBio). Renilla luciferase vector (50 ng; pRL-TK vector) was co-transfected as an internal control to account for differences in transfection and harvest efficiencies. 48 h post-transfection cells were lysed and luciferase activity quantified using the Dual Luciferase Reporter kit (Promega Inc.), as reported [5].

A total of $2x10^6$ untreated or Doxy-treated BV173-ShMYB cells transiently co-transfected, by electroporation (Nucleofector 4D system, Lonza; SF cell line kit, Lonza; pulse program EN-138), with Renilla luciferase pRLTK (800 ng) and 5 μg of pGL3–prom1353 or a deletion mutant lacking

MBS#1 (ΔMBS#1-prom230) [6]. The pRL-TK Renilla luciferase vector was co-transfected as internal control to normalize differences in transfection and harvest efficiencies. Cells were harvested 48 h post-transfection, lysed and luciferase activity quantified using the Dual Luciferase Reporter Kit (Promega Inc.) as described [7]. Firefly luciferase activity of each sample was normalized to Renilla luciferase activity. Results are mean ± SEM of three independent experiments performed in duplicate.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed as described [7]. Briefly, cross-linked chromatin from BV173- and K562-ShMYB cells was immunoprecipitated using the anti-c-MYB antibody (ab45150, Abcam, Cambridge, UK). Segments of the MIR17HG miR-17-92 promoter which include putative MYB binding sites were amplified with primers designed by the Primer Express software (Applied Biosystem; **Table S1**). Quantitation of immunoprecipitated DNA was performed in triplicate on an Applied Biosystem 7900 Real Time PCR System SDS v2.2, using the SYBR green dye detection method. ChIP results are evaluated by ΔΔCt method. Values of each immunoprecipitated sample are expressed as percentage relative to their respective input and by subtracting the values obtained in the negative controls (no antibody).

In vivo studies assessing the effects of ectopic FRZB expression

The FRZB cDNA (NM_001463.3) was obtained by Dharmacon (Cat. # MHS6278), amplified with primers introducing XbaI and BamHI sites and inserted into the XbaI-BamHI-digested lentiviral vector pUltra-hot (Addgene plasmid # 24130), which expresses the cDNA of interest and the mCherry protein as a bi-cistronic transcript under the control of the ubiquitin C promoter. BV173-ShMYB cells were transduced with the 7TFP lentiviral vector [8] expressing the firefly luciferase under the control of a promoter containing seven LEF/TCF consensus binding sites and then selected with puromycin 3 μg/mL. Subsequently, BV173-ShMYB 7TFP cells were transduced with

the pUltra-Hot-FRZB plasmid or the empty vector (EV) and sorted for mCherry expression. These derivative cell lines were used for *in vivo* studies as described in Materials and Methods.

Statistical analysis

Results are expressed as mean ± SEM of at least three independent experiments.

Expression of MYB and FRZB in normal and Ph+ ALL from microarray dataset GSE13159 were analysed with GEO2R from the Gene Expression Omnibus database. Gene expression was obtained by the sum of all probes intensity for each gene. Significance was determined by using two-tailed Student's t-test.

QRT-PCR experiments were carried out in triplicate and data analyzed using the comparative $2^{-\Delta Ct}$ method. Statistical significance ($p \leq 0{,}05$) was determined using Student's t-test.

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Figure S1

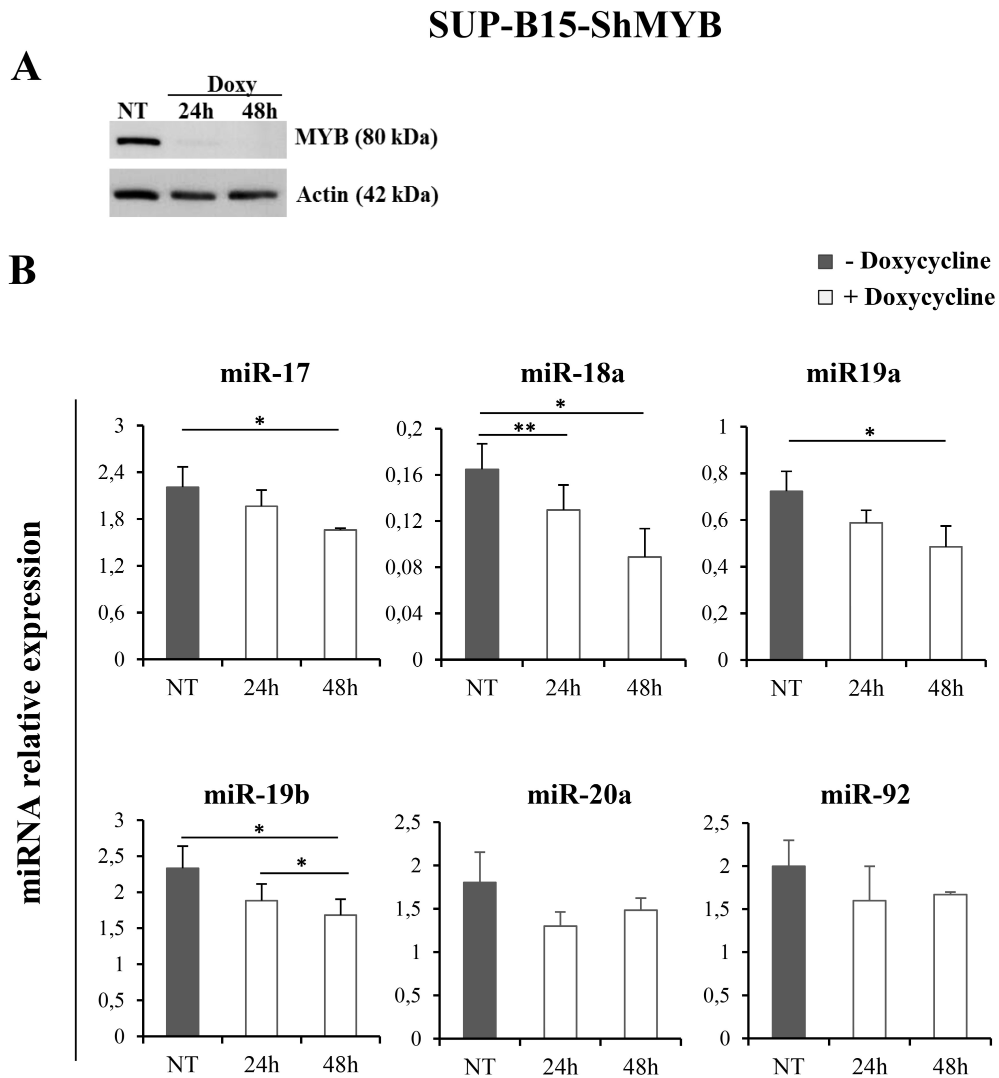

Figure S1. Expression levels of the miR-17-92 cluster in Ph+ ALL cells. (A) Western blot of a representative experiment showing specific knockdown of MYB in Doxy-treated cells; **(B)** qRT-PCR of the indicated members of the miR-17-92 cluster in NT or Doxy-treated (24-48 h) SUP-B15-ShMYB cells. Samples were normalized for RNU44 expression. QRT-PCR was performed in triplicate, including no-template controls. Relative expression was calculated using the comparative C_t method. Data are the average of three independent experiments, error bars indicate SEM. *p*-values (*= $p \leq 0{,}05$; **= $p \leq 0{,}01$) were determined using the Student's t-test.

Figure S2

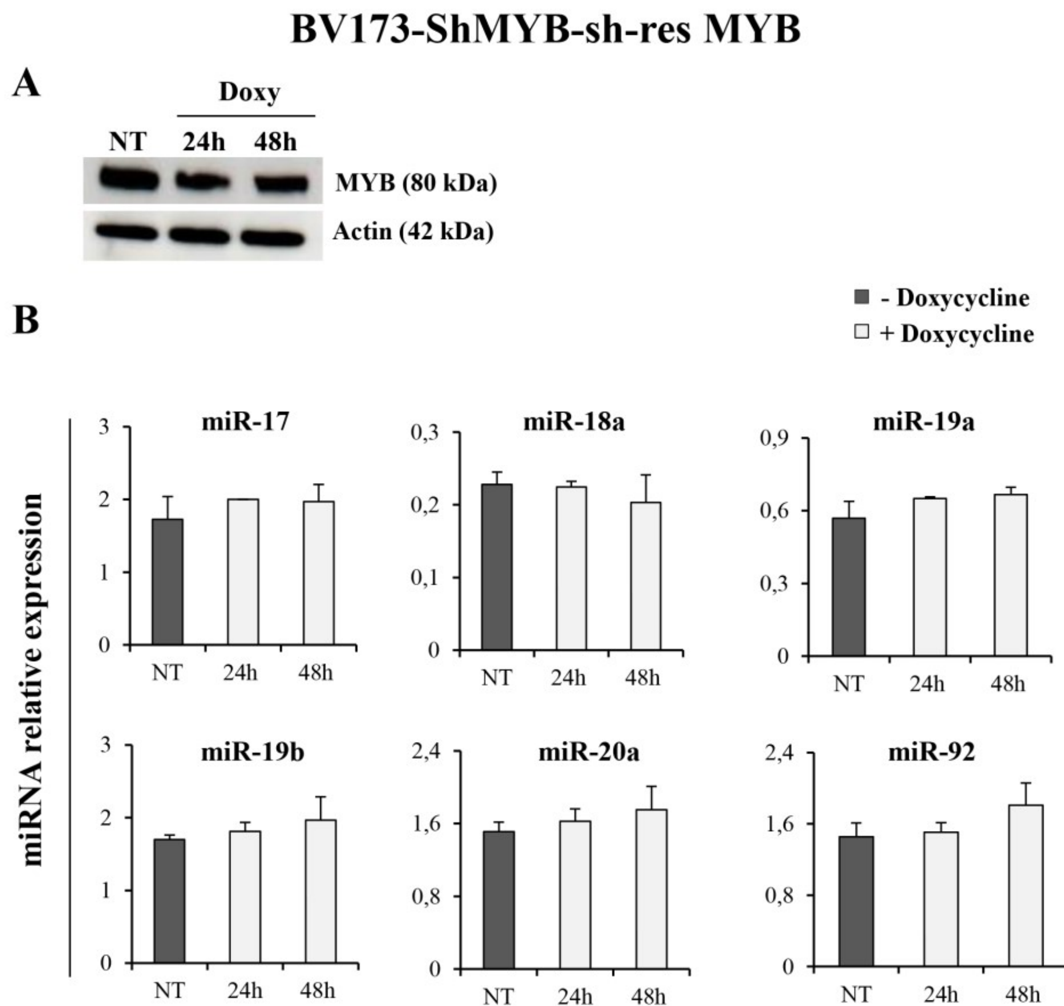

Figure S2. Expression levels of miR-17-92 cluster in shRNA-resistant MYB BV173 cells. (A) Western blot of a representative experiment showing specific knockdown of MYB in Doxy-treated cells; **(B)** qRT-PCR of the indicated members of miR-17-92 cluster in NT or Doxy-treated (24-48 h) BV173-ShMYB-sh-res MYB cells. Samples were normalized for RNU44 expression. Relative expression was calculated using the comparative Ct method. Data are the average of three independent experiments, error bars indicate SEM.

Figure S3

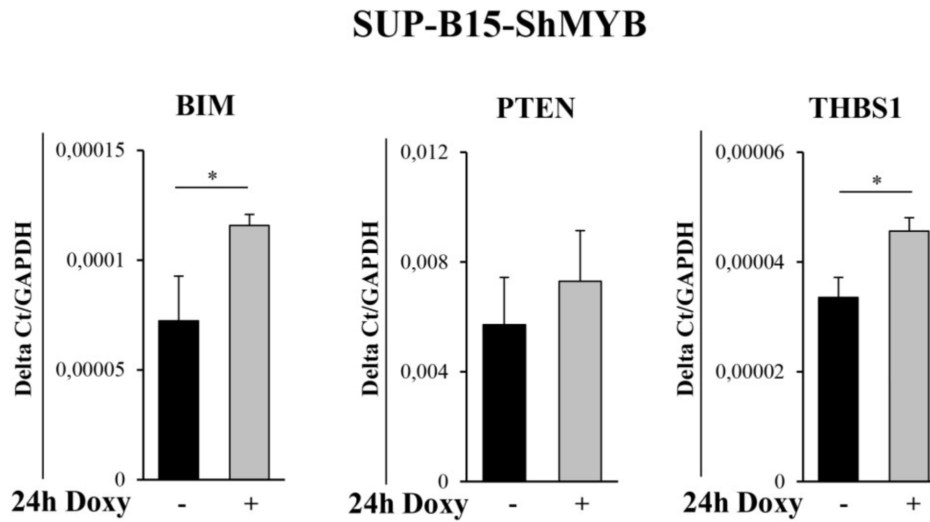

Figure S3. mRNA levels of BIM, PTEN and THBS1 genes in SUP-B15 cells. SYBR Green-based qRT-PCR of BIM, PTEN and THBS1 in untreated and Doxy-treated (24 h) SUP-B15-ShMYB cells. Results represent mean of three experiments. Error bars indicate SEM. *p*-value (*= $p \leq 0{,}05$) were determined using the Student's t-test.

Figure S4

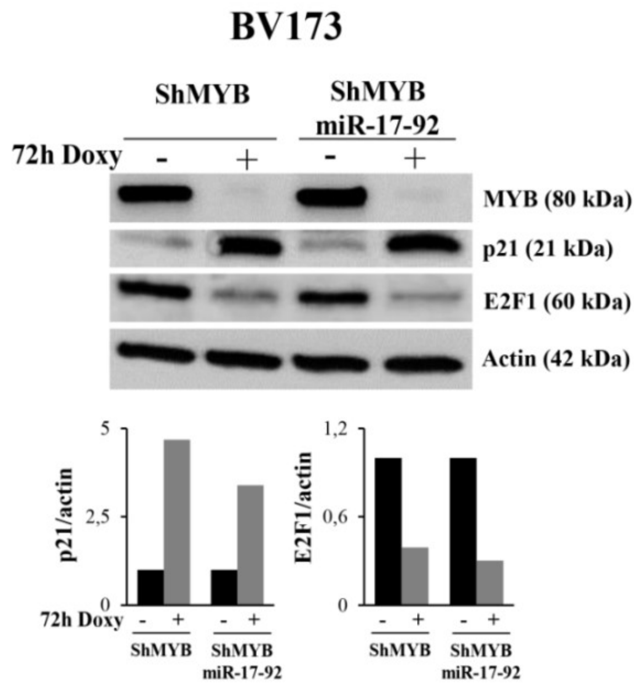

Figure S4. Evaluation of protein levels of miR-17-92 p21 and E2F1 targets in BV173 cells. Western blots of p21 and E2F1 in BV173-ShMYB and BV173-ShMYB-miR-17-92 cells after 72 h of MYB silencing. Densitometric analysis by imageJ software. Actin was used as loading control within the same sample and expressed as fold changes compared to control.

TABLE S1

Primers sequences		
primer name	**primer sequence 5' - 3'**	**use**
ADA forward	5'-CTCCTCCTTTTTGTCTTCCT-3'	ChIP
ADA reverse	5'-GAAACTCAGTCTCCTTTGTTCCCC-3'	ChIP
MBS#1 forward	5'-GGAGCAGGCCCCTAATTACC-3'	ChIP
MBS#1 reverse	5'-CCTGGGACTCGACGTGTAC-3'	ChIP
MBS#2 forward	5'-CCCCGCCTCAACCATTCTAA-3'	ChIP
MBS#2 reverse	5'-ACGTTACTTTTGGTCGGCAT-3'	ChIP
MBS#3 forward	5'-ATAAGACCGAAACACATGGGT-3'	ChIP
MBS#3 reverse	5'-TCAGTTCAACGTCAGAGCAGT-3'	ChIP
MBS#4 forward	5'-TTCCTAAACTTTTCATGGACACG-3'	ChIP
MBS#4 reverse	5'-TGCTTGCACTGGTCAAAGATATA-3'	ChIP
c-MYB forward	5'-TCAGGAAACTTCTTCTGCTCACA-3'	qRT PCR
c-MYB reverse	5'- AGGTTCCCAGGTACTGCT -3'	qRT PCR
GAPDH forward	5'-TCCCTGAGCTGAACGGGAAG-3'	qRT PCR
GAPDH reverse	5'-GGAGGAGTGGGTGTCGCTGT-3'	qRT PCR
BIM forward	5'-GCCCAGAGATATGGATCGCC-3'	qRT PCR
BIM reverse	5'-AAGAGGCATCCTCCTTGCAT-3'	qRT PCR
PTEN forward	5'-AGACATTATGACACCGCCAA-3'	qRT PCR
PTEN reverse	5'-GCTGTGGTGGGTTATGGTCT-3'	qRT PCR
THBS1 forward	5'-CTGGACCCCAAAGGGACATC-3'	qRT PCR
THBS1 reverse	5'-ACCTACAGCGAGTCCAGGAT-3'	qRT PCR
PBX2 forward	5'-CTGCAGCAGATAATGACCATCAC-3'	qRT PCR
PBX2 reverse	5'-GCAGTTTAGGGCGTGTTTCTTG-3'	qRT PCR
FRZB forward	5'-AGCCCTGTAAGTCTGTGTGC-3'	qRT PCR
FRZB reverse	5'-ACGAGTGGCGGTACTTGATG-3'	qRT PCR
FRZB forward	5'-CTGTTGTGTGTGTGTCTGTATTTGTGTAC-3'	FRZB 3'UTR Cloning
FRZB reverse	5'-GGATCTCACCACAGCTTGGGTAATGAG-3'	FRZB 3'UTR Cloning
FRZB forward	5'-GCAAACTGGCCTGAAGCTTATCATTTGGA-3'	Seed-miR-17 mutagenesis
FRZB reverse	5'-TCCAAATGATAAGCTTCAGGCCAGTTTGC-3'	Seed-miR-17 mutagenesis
FRZB forward	5'-TTTGAACCAATGGTACCTTTCTCATTACCC-3	Seed-miR-19a mutagenesis
FRZB reverse	5'-GGGTAATGAGAAAGGTACCATTGGTTCAAA-3'	Seed-miR-19a mutagenesis

TABLE S2

15 miRNAs concordantly modulated in Ph+ leukemic cells upon c-MYB silencing

miRNAs	K562 Fold change (log2)	K562 P-value	BV173 Fold change (log2)	BV173 P-value
hsa-miR-7-5p	-1,038	0,0008	-1,606	0,0269
hsa-miR-17-5p	-0,137	0,0292	-0,821	0,007
hsa-miR-18a-5p	-0,517	0,006	-1,343	0,0079
hsa-miR-18b-5p	-0,415	0,0005	-1,213	0,0034
hsa-miR-30d-5p	0,482	0,0201	0,463	0,0014
hsa-miR-185-5p	-0,313	0,005	-0,966	0,0046
hsa-miR-320e	0,373	0,0089	0,261	0,0482
hsa-miR-324-5p	-0,586	0,0015	-1,099	0,0004
hsa-miR-361-3p	0,624	0,0276	0,58	0,0189
hsa-miR-641	-0,266	0,0098	-0,429	0,047
hsa-miR-1306-5p	-0,259	0,008	-0,933	0,0188
hsa-miR-1973	-1,122	0,0256	-0,642	0,0512
hsa-miR-3135b	-0,48	0,0425	-0,693	0,01
hsa-miR-4284	-1,314	0,0003	-2,235	0,0007
hsa-miR-5703	6,471	0,004	3,771	0,0574

Red arrows indicate the analyzed miRNAs

TABLE S3

20 common miRNAs modulated in opposite directions in Ph+ leukemic cells after c-MYB silencing				
MiRNAs	**K562 Fold change (log2)**	**K562 P-value**	**BV173 Fold change (log2)**	**BV173 P-value**
hsa-let-7a-5p	0,177	0,0193	-0,548	0,0435
hsa-miR-9-3p	0,131	0,0321	-0,678	0,0594
hsa-miR-15b-3p	0,355	0,0417	-1,454	0,0225
hsa-miR-15b-5p	0,298	0,0065	-0,744	0,038
hsa-miR-16-2-3p	0,721	0,0004	-0,563	0,0545
hsa-miR-16-5p	0,37	0,0097	-0,368	0,0297
➡ **hsa-miR-19b-3p**	0,158	0,0236	-0,52	0,0045
➡ **hsa-miR-20a-3p**	0,473	0,0153	-0,582	0,0511
➡ **hsa-miR-20a-5p**	0,192	0,0139	-0,721	0,0325
hsa-miR-20b-5p	0,248	0,0244	-0,773	0,0214
hsa-miR-30e-3p	0,488	0,036	-0,409	0,0274
hsa-miR-135a-3p	-5,798	0,0126	2,345	0,0108
hsa-miR-301a-3p	0,205	0,0316	-0,414	0,0159
hsa-miR-301b	0,483	0,0157	-0,155	0,0413
hsa-miR-378a-3p	0,451	0,0083	-0,063	0,022
hsa-miR-378i	0,398	0,0203	-0,103	0,0201
hsa-miR-513a-5p	-1,097	0,0271	0,857	0,03
hsa-miR-652-3p	0,262	0,0387	-0,814	0,025
hsa-miR-1260a	0,498	0,03	-0,807	0,0002
hsa-miR-5100	0,375	0,02	-0,495	0,004

Red arrows indicate miR-17-92 cluster members

TABLE S4

Overview of microarray data of all members of miR-17-92 cluster

MiRNAs	K562 Fold Change (log2)	K562 P-value	BV173 Fold Change (log2)	BV173 P-value
hsa-miR-17-5p	-0,136	0,029	-0,821	0,007
hsa-miR-18a-5p	-0,517	0,006	-1,342	0,007
hsa-miR-19a-3p	ND	ND	-0,818	0,002
hsa-miR-19a-5p	ND	ND	-4,659	0,006
hsa-miR-20a-3p	0,473	0,015	-0,582	0,051
hsa-miR-20a-5p	0,191	0,013	-0,721	0,032
hsa-miR-19b-3p	0,157	0,023	-0,519	0,004
hsa-miR-92a-1-5p	1,037	0,004	ND	ND

TABLE S5

MYB binding sites and Matrix score

MYB binding site	Strand:	Core similarity:	Matrix similarity:
MBS #1	+	**1**	**0,975**
MBS #2	+	**1**	**0,891**
MBS #3	+	**0,866**	**0,886**
MBS #4	-	**0,99**	**0,961**
MBS #5	+	**0,817**	**0,898**
MBS #6	-	0,807	0,793
MBS #7	+	0,754	0,773
MBS #8	+	1	0,785
MBS #9	-	0,814	0,752
MBS #10	-	0,781	0,816
MBS #11	+	0,782	0,812
MBS #12	+	0,814	0,753

In bold: MBS choosen for ChIP analysis