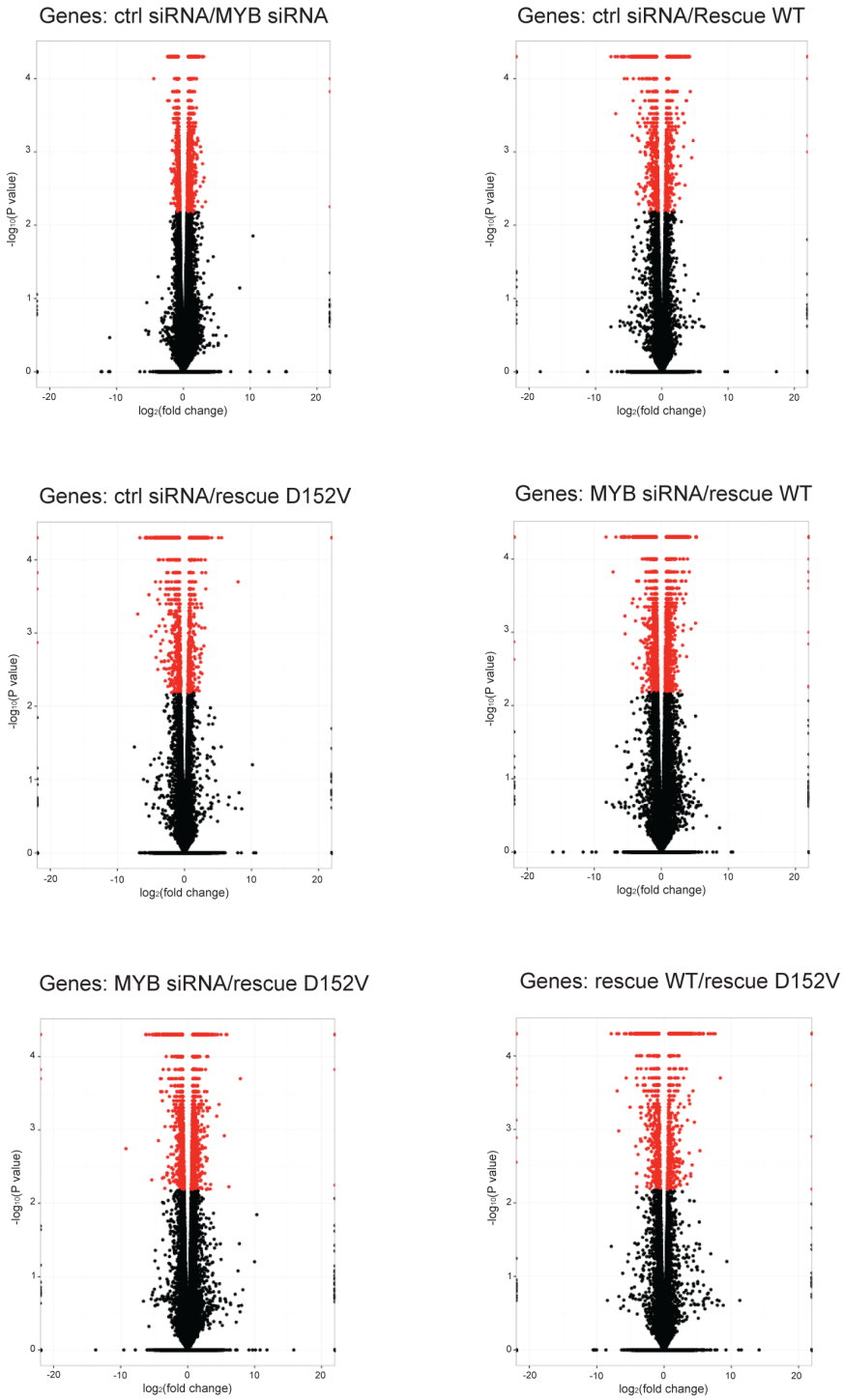
Supplementary Information

A c-Myb mutant causes deregulated differentiation due to impaired histone binding and abrogated pioneer factor function

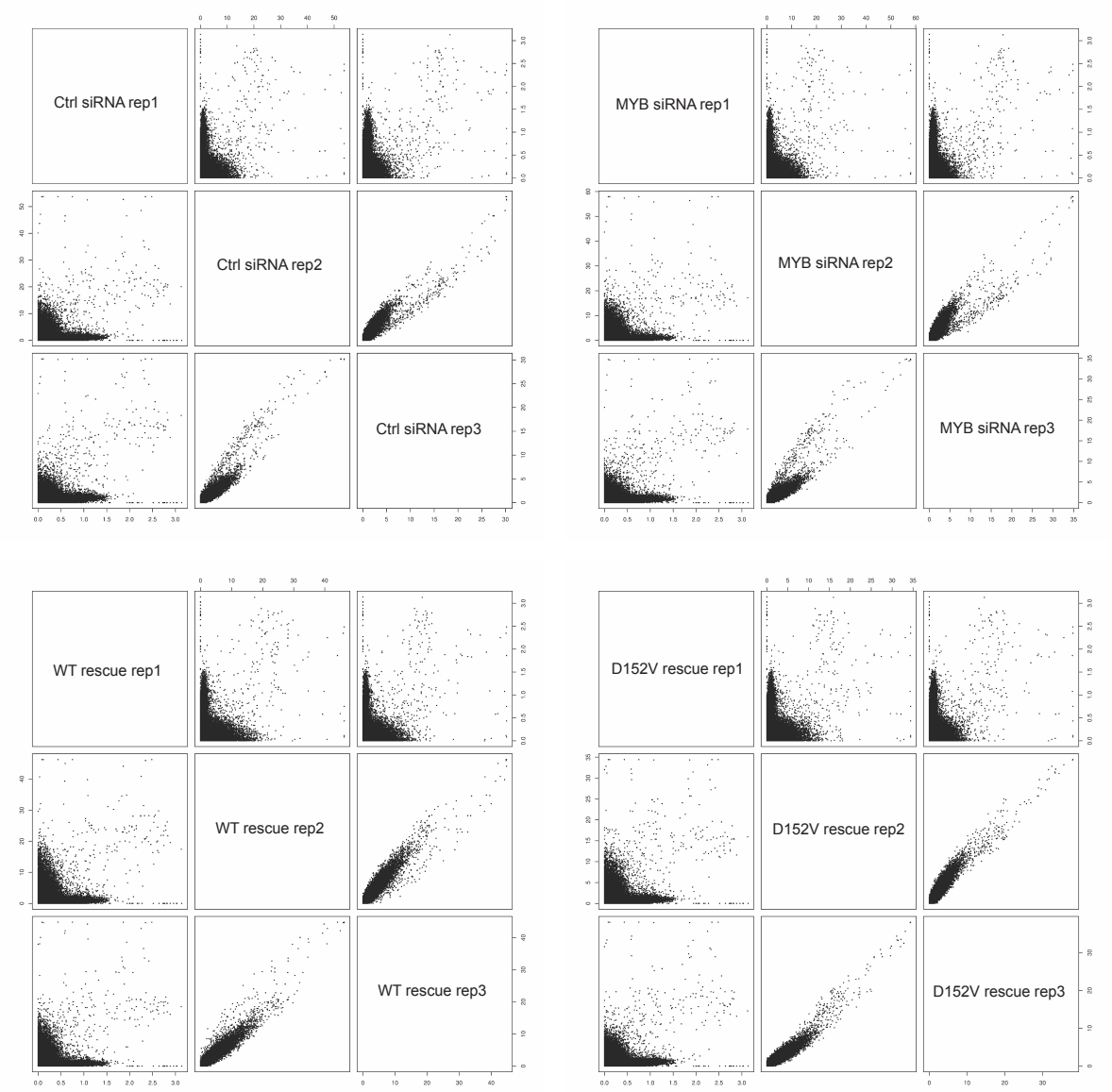
Bettina Maria **Fuglerud**, Roza Berhanu **Lemma**, Pimthanya **Wanichawan**, Arvind YM **Sundaram**, Ragnhild **Eskeland** and Odd Stokke **Gabrielsen**

Supplementary Figure S1



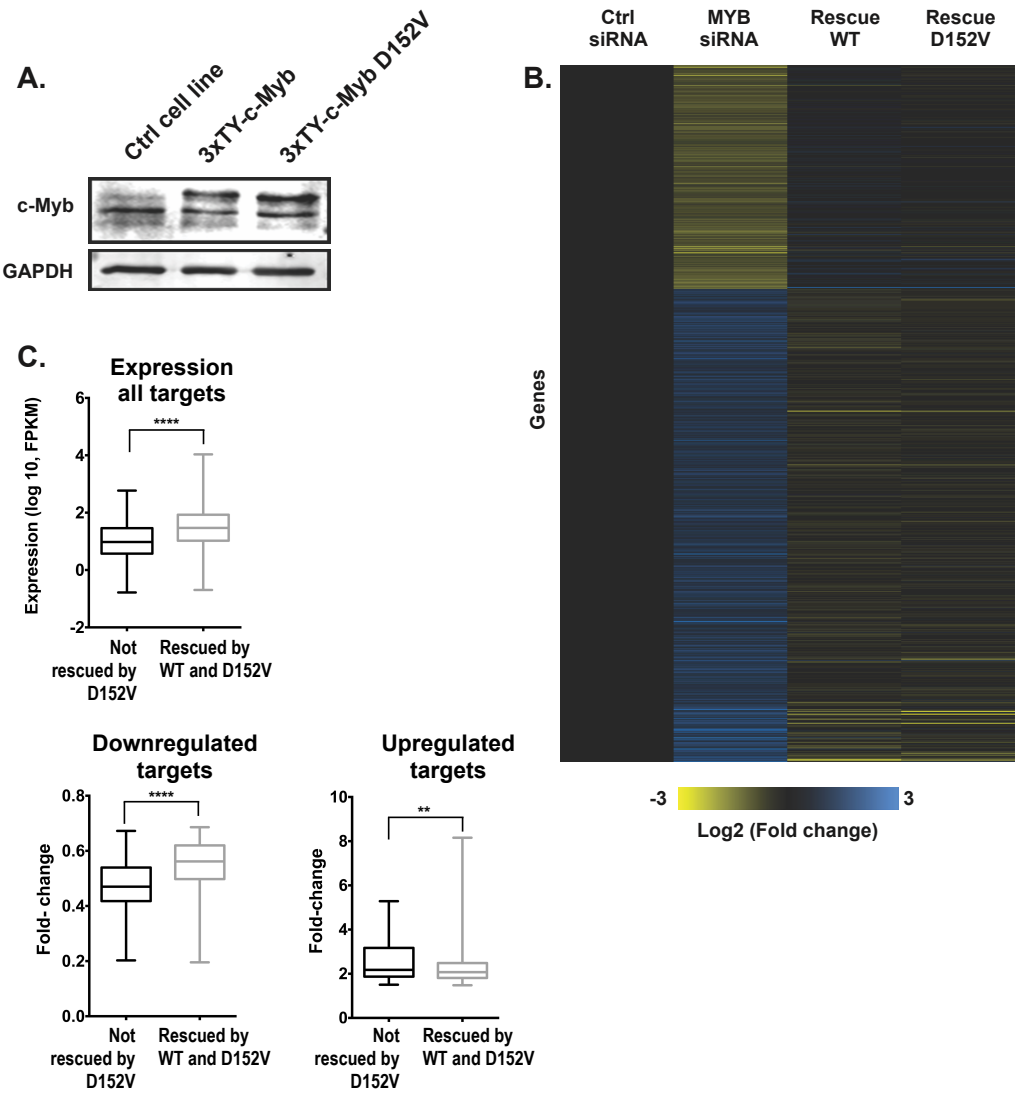
Supplementary Figure S1. Differentially expressed genes for each condition visualized through volcano plots (each gene is represented by one point). The X-axis represents fold change, whereas the Y-axis represents statistical significance. Red points indicate genes that pass the q<0.01 significance threshold (CummeRbund). Only these were included for further analyses.

Supplementary Figure S2



Supplementary Figure S2. ATAC-seq signals (log2 reads) between the three biological replicates for each condition. The signals correlated well between two of the biological replicates (rep2 and rep3).

Supplementary Figure S3



Supplementary Figure S3. RNA-seq following c-Myb knockdown and rescue in K562 cells. **A)** K562 cells stably expressing TY-tagged proteins. The control cell line was generated by selecting for cells containing pEF1neo-3xTY, while the 3xTY-c-Myb and 3xTY-c-Myb D152V cell lines were selected for pEF1neo-3xTY-hcM and pEF1neo-3xTY-hcM D152V, respectively. The Western blot was analysed using anti-c-Myb and anti-GAPDH primary antibodies. The TY-tagged c-Myb proteins are seen as slightly larger bands compared to endogenous c-Myb. **B)** Heat map showing the differential gene expression pattern after c-Myb knockdown for the genes rescued by TY-c-Myb and TY-c-Myb D152V. Yellow colour represents decreased expression while blue colour represents increased expression relative to the control (black). **C)** Box plots of the FPKM values (log10) of the two groups of genes (not rescued by D152V n=103; rescued by WT and D152V n=766) and the fold change after c-Myb knockdown of the downregulated (not rescued by D152V n=59; rescued by WT and D152V n=245) and upregulated genes (not rescued by D152V n=44; rescued by WT and D152V n=520). The box indicates the lower (25%) and upper (75%) quantile and the horisontal line indicates the median. Whiskers extend to maximum and minimum of each distribution. Asterisks indicate p-values from an unpaired t test.

Supplementary Figure S4

A.

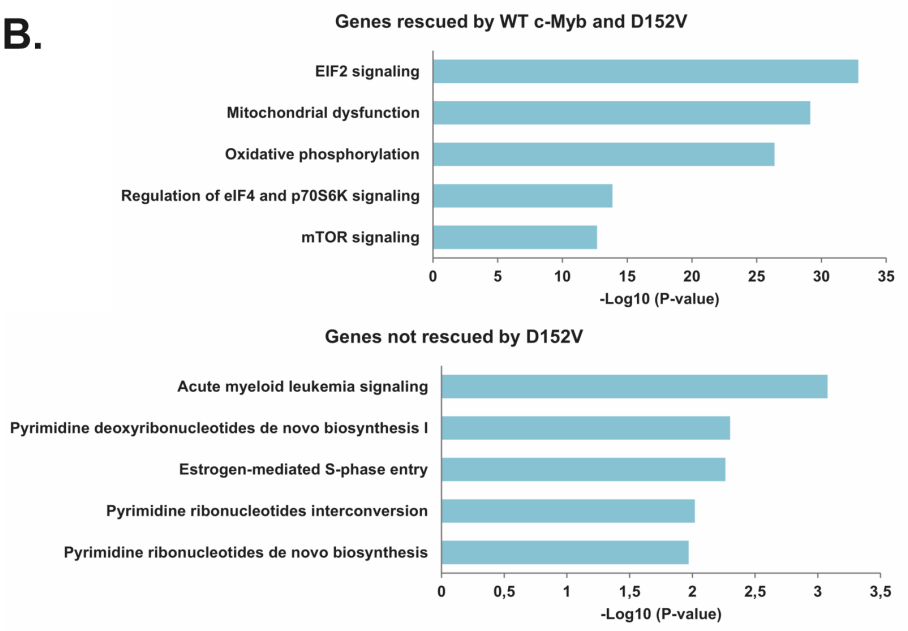
Genes rescued by WT c-Myb and D152V

Molecular and cellular functions	P-value range
Cell death and survival	3,16E-02 - 1,91E-18
RNA post-transcriptional modification	7,25E-03 - 3,31E-08
Gene expression	1,90E-02 - 1,23E-07
Protein synthesis	3,11E-02 - 1,23E-07
Cell cycle	3,17E-02 - 5,84E-06

Genes not rescued by D152V

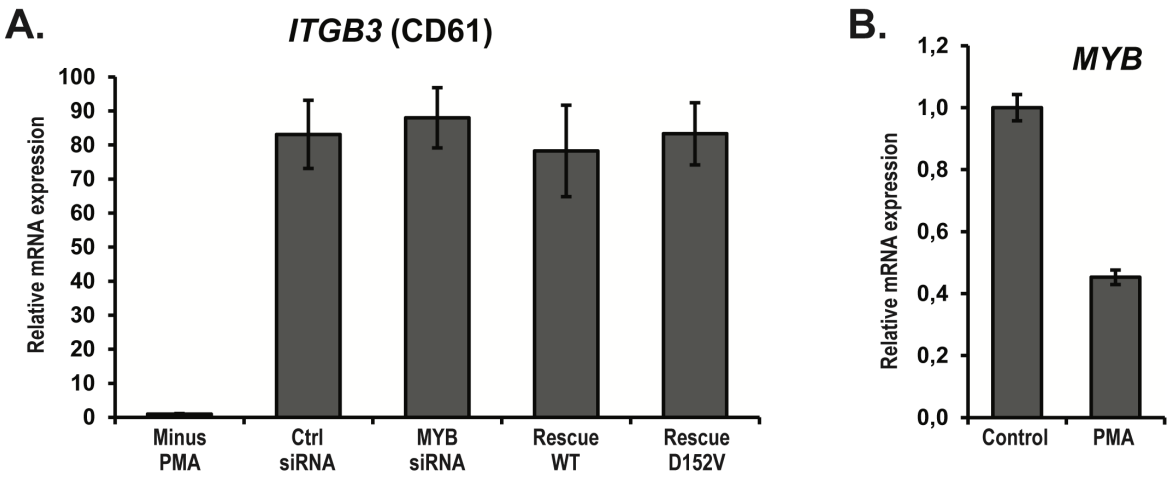
Molecular and cellular functions	P-value range
Cellular growth and proliferation	9,21E-03 - 1,60E-06
Cellular function and maintenance	9,21E-03 - 2,06E-05
Molecular transport	9,21E-03 - 2,06E-05
Small molecule biochemistry	9,21E-03 - 2,06E-05
Cellular movement	9,21E-03 - 2,31E-05

B.



Supplementary Figure S4. IPA analysis of the two groups of c-Myb target genes.
A) Enriched biological processes and **B)** top five canonical pathways (based on P-value).The analyses were carried out using the Ingenuity Pathway Analysis (IPA) tool (Qiagen).

Supplementary Figure S5



Supplementary Figure S5. PMA-induced megakaryocytic differentiation of K562 cells. **A)** K562 cells were induced for megakaryocytic differentiation with 1 nM PMA for 48 hours. The level of megakaryocytic differentiation was analysed by qRT-PCR using primers specific to *ITGB3* (CD61). **B)** Expression of *MYB* analysed by qRT-PCR.

Supplementary Materials and Methods

RNA isolation, reverse transcription and qRT-PCR

Total RNA was isolated 24 hours after transfection using the RNeasy RNA isolation kit (Qiagen) and 1 µg RNA was used for reverse transcription with the AffinityScript cDNA synthesis kit (Agilent technologies). qRT-PCR was performed using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent technologies) in a LightCycler 96 instrument (Roche). The values of RNA expression for the different genes were normalized to the relative amount of the reference genes *POLR2A* (K562) or *hprt* (HD-11). All qRT-PCR experiments were done in triplicates.

Primer sequences:

Gene	Forward (5'-3')	Reverse (5'-3')
POLR2A	TGCCACAGACAGACAACAAG	GACATAGGAGCCATCAAAGGAG
MYB	AAATACGGTCCCCTGAAGATGCTA	GTCTGCGTGAACAGTTGGGTATTC
ITGB3 (CD61)	CGAGTACTAGAGGACAGGCC	CATGGAGTAAGACAGGTCCA
hprt	GGATACGCCCTCGACTACAA	GGGCTGGGGTGTTCTACAAT
mim-1	GCAACAGTGTATGCTCCCTTTT	ATCTGAGCGATCGCAGTTCT

Protein extraction and Western blotting

Cellular protein extracts from K562 cells were generated by harvesting the cells 24 hours after siRNA transfection and incubating for 1 hour in lysis buffer (20 mM HEPES [pH 7.6], 300 mM NaCl, 0.2 mM EDTA, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.5 mM DTT) supplemented with Complete protease inhibitor cocktail (Roche) at 4 °C. SDS loading dye was added to the lysates and they were subjected to Western blotting. Transfected HD-11 cells and CV-1 cells were lysed in parallel to the functional assays for validation of protein expression by Western blotting. SDS loading dye was added to pelleted cells and they were sonicated (8 sec on, 8 sec off, 20 cycles, high intensity) before Western blotting. Western blotting was performed using PVDF membranes and blocking with 3% milk in TBS. After blocking, the membranes were incubated overnight at 4 °C with primary antibody in TBS-T containing 3% milk. The membranes were then incubated with the corresponding secondary antibody in TBS-T containing 3% milk for 1 hour at room temperature. Antibodies applied: anti-c-Myb (ab45150, Abcam), anti-GAPDH (AM4300, Invitrogen), anti-HA (H6908, Sigma-Aldrich), anti-GFP (ab6556, Abcam), anti-FLAG (F3165, Sigma-Aldrich), anti-H3 (ab1791, Abcam).

Plasmids

The mammalian expression vectors pEF1neo-3xTY-hcM and pEF1neo-3xTY-hcM (D152V) as well as "empty vector" (pEF1neo-3xTY) were used to generate K562 cells stably expressing TY-tagged c-Myb and c-Myb D152V. pEF1neo is a mammalian expression vector derived from pCIneo, but with the CMV promoter region replaced by the promoter region of human EF-1-alpha, the latter PCR amplified and inserted between the BglII and EcoICRI sites of pCIneo. From this, plasmids for mammalian expression of 3xTY1-tagged human c-Myb were derived by standard methods.

The reporter plasmids used in the luciferase assays are previously described, pGL4b-3xMRE(GG)-myc (1), pmim3mim (2, 3), pGL3-LMO2, pGL3-STAT5A, and pGL3-MYC (4). The mammalian expression vectors pCIneo-hcM-HA and pCIneo- hcM (D152V)-HA,

pCIneoB-3FLAG-CHD3 as well as “empty vector” pCIneo-HA were used for expression of HA-tagged c-Myb or c-Myb D152V in the luciferase assays in CV-1 cells as well as in HD-11 cells. pCIneo-hcM-HA and pCIneoB-3FLAG-CHD3 has been previously described in (5, 6). The D152V mutation was generated using the QuikChange Site-directed Mutagenesis kit (Stratagene) on a subfragment of human c-Myb.

pCIneo-3FLAG-hcM was used for expression of Flag-tagged c-Myb in COS-cells for GST pulldown and has been described (7). The C/EBPβ construct was kindly provided by Karl-Heinz Klempnauer (University of Münster, Germany) and is described (8).

The expression vectors for the GST fusion proteins GST-H2A (aa 1-36), GST-H2B (aa 1-36), GST-H3 (aa 1-47) and GST-H4 (aa 1-34) were kindly provided by Achim Leutz (Max-Delbrueck-Center for Molecular Medicine, Berlin, Germany) and are described in (9). Plasmid for recombinant expression of histone H3.1 were kindly gifted from Gunnar Schotta (LMU, Munich, Germany). The pGEX constructs encoding GST in fusion with c-Myb fragments (NR123 (aa 1-192), R2R3 (aa 89-192), R2 (aa 89-137), R3 (aa 138-192), R2R3 D152V and R3 D152V) were generated by subcloning of PCR fragments into pGEX-6P2.

Supplementary References

1. Molværsmyr,A.-K., Saether,T., Gilfillan,S., Lorenzo,P.I., Kvaløy,H., Matre,V. and Gabrielsen,O.S. (2010) A SUMO-regulated activation function controls synergy of c-Myb through a repressor-activator switch leading to differential p300 recruitment. *Nucleic Acids Res.*, **38**, 4970–4984.

2. Wilczek,C., Chayka,O., Plachetka,A. and Klempnauer,K.-H. (2009) Myb-induced chromatin remodeling at a dual enhancer/promoter element involves non-coding rna transcription and is disrupted by oncogenic mutations of v-myb. *J. Biol. Chem.*, **284**, 35314–35324.

3. Chayka,O., Kintscher,J., Braas,D. and Klempnauer,K.-H. (2005) v-Myb mediates cooperation of a cell-specific enhancer with the mim-1 promoter. *Molecular and Cellular Biology*, **25**, 499–511.

4. Lorenzo,P.I., Brendeford,E.M., Gilfillan,S., Gavrilov,A.A., Leedsak,M., Razin,S.V., Eskeland,R., Saether,T. and Gabrielsen,O.S. (2011) Identification of c-Myb Target Genes in K562 Cells Reveals a Role for c-Myb as a Master Regulator. *Genes & Cancer*, **2**, 805–817.

5. Dahle,Ø., Andersen,T.Ø., Nordgård,O., Matre,V., del Sal,G. and Gabrielsen,O.S. (2003) Transactivation properties of c-Myb are critically dependent on two SUMO-1 acceptor sites that are conjugated in a PIASy enhanced manner. *Eur J Biochem*, **270**, 1338–1348.

6. Saether,T., Berge,T., Ledsaak,M., Matre,V., Alm-Kristiansen,A.H., Dahle,Ø., Aubry,F. and Gabrielsen,O.S. (2007) The chromatin remodeling factor Mi-2alpha acts as a novel co-activator for human c-Myb. *J. Biol. Chem.*, **282**, 13994–14005.

7. Saether,T., Pattabiraman,D.R., Alm-Kristiansen,A.H., Vogt-Kielland,L.T., Gonda,T.J. and Gabrielsen,O.S. (2011) A functional SUMO-interacting motif in the transactivation domain of c-Myb regulates its myeloid transforming ability. *Oncogene*, **30**, 212–222.

8. Dukare,S. and Klempnauer,K.-H. (2016) A conserved patch of hydrophobic amino acids modulates Myb activity by mediating protein-protein interactions. *Biochim Biophys Acta*, 10.1016/j.bbagrm.2016.04.004.

9. Mo,X., Kowenz-Leutz,E., Laumonnier,Y., Xu,H. and Leutz,A. (2005) Histone H3 tail positioning and acetylation by the c-Myb but not the v-Myb DNA-binding SANT domain. *Genes Dev*, **19**, 2447–2457.