SUPPLEMENTARY APPENDIX

Protein arginine methyltransferase 6 controls erythroid gene expression and differentiation of human CD34+ progenitor cells

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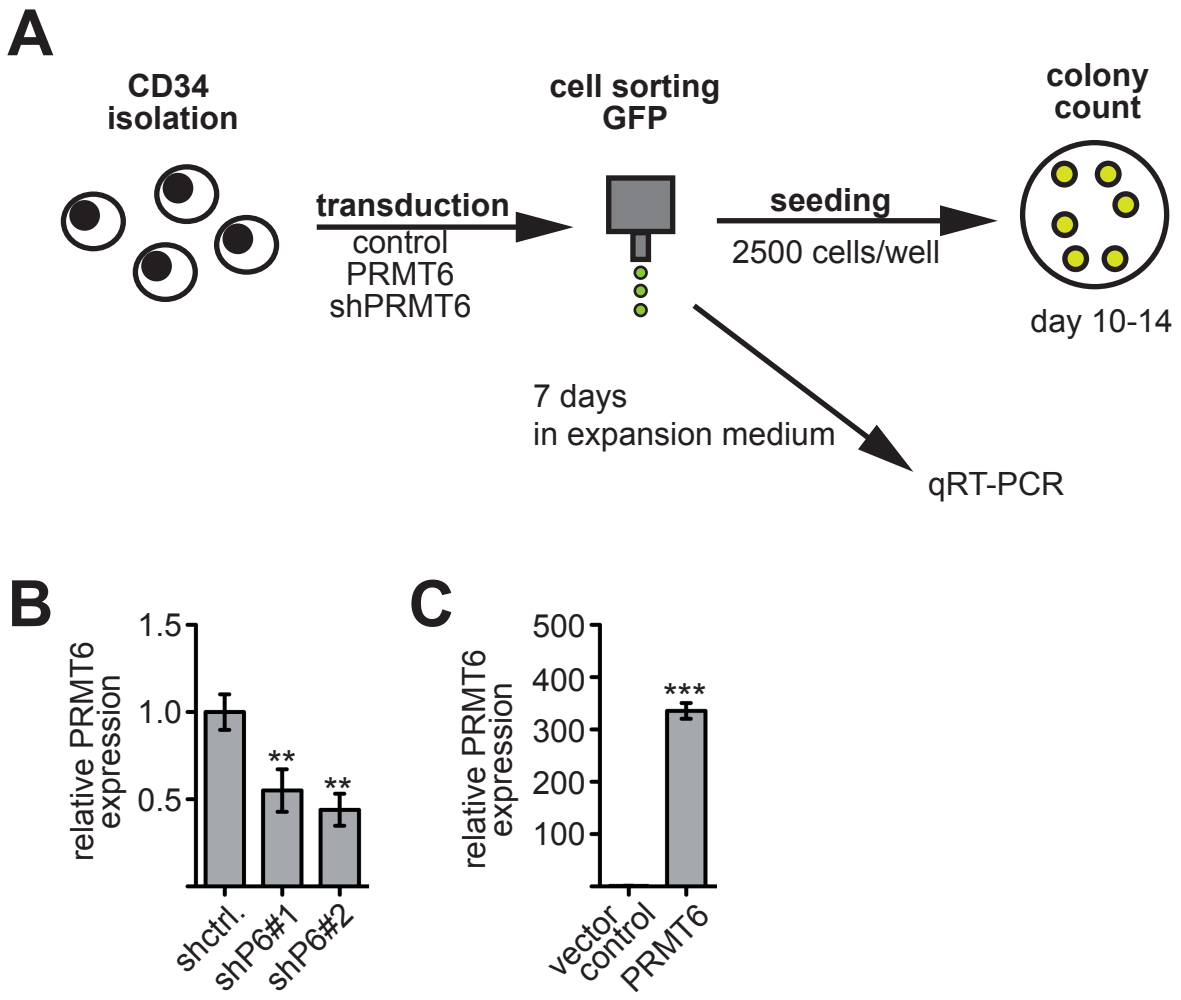
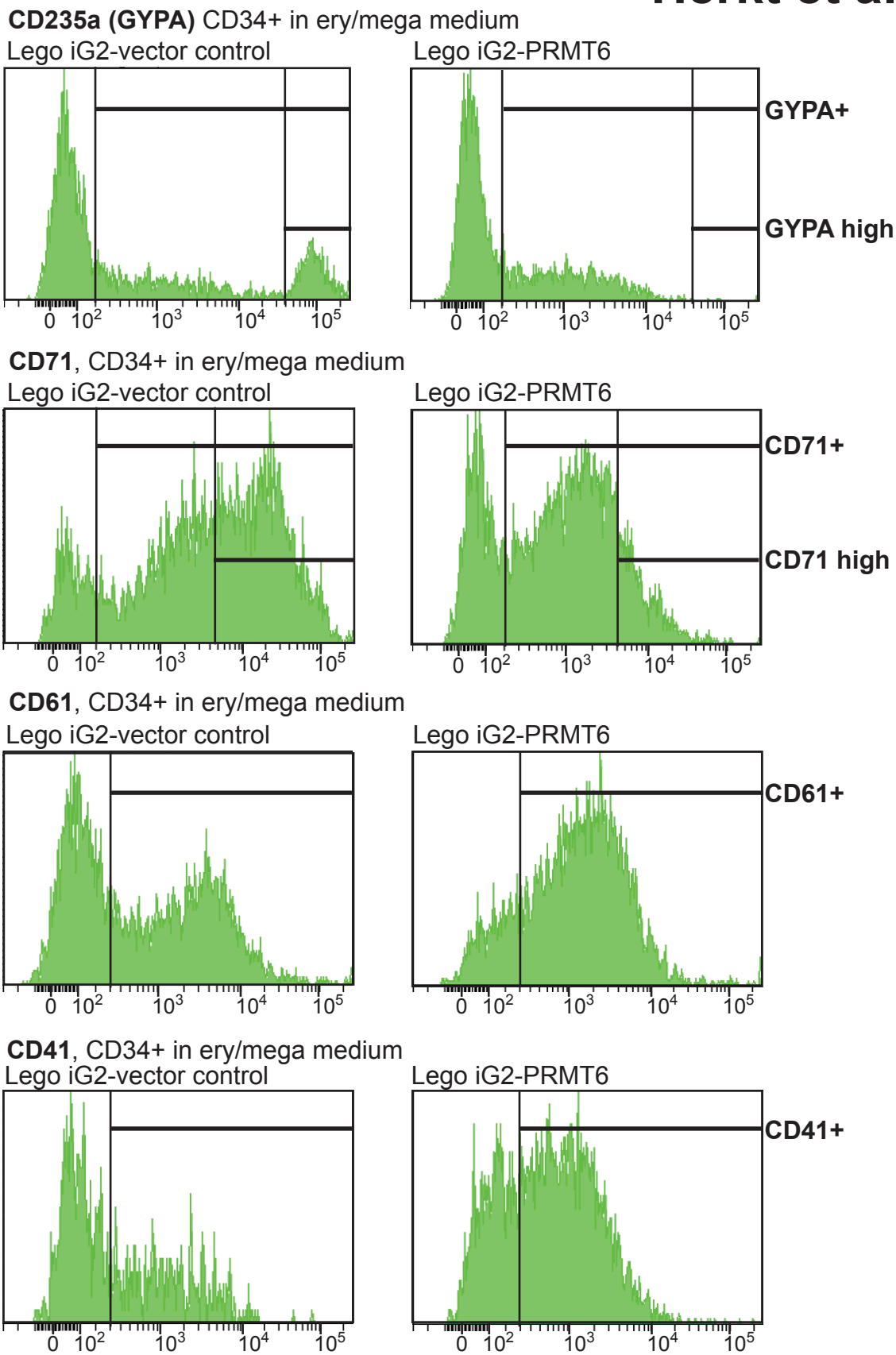


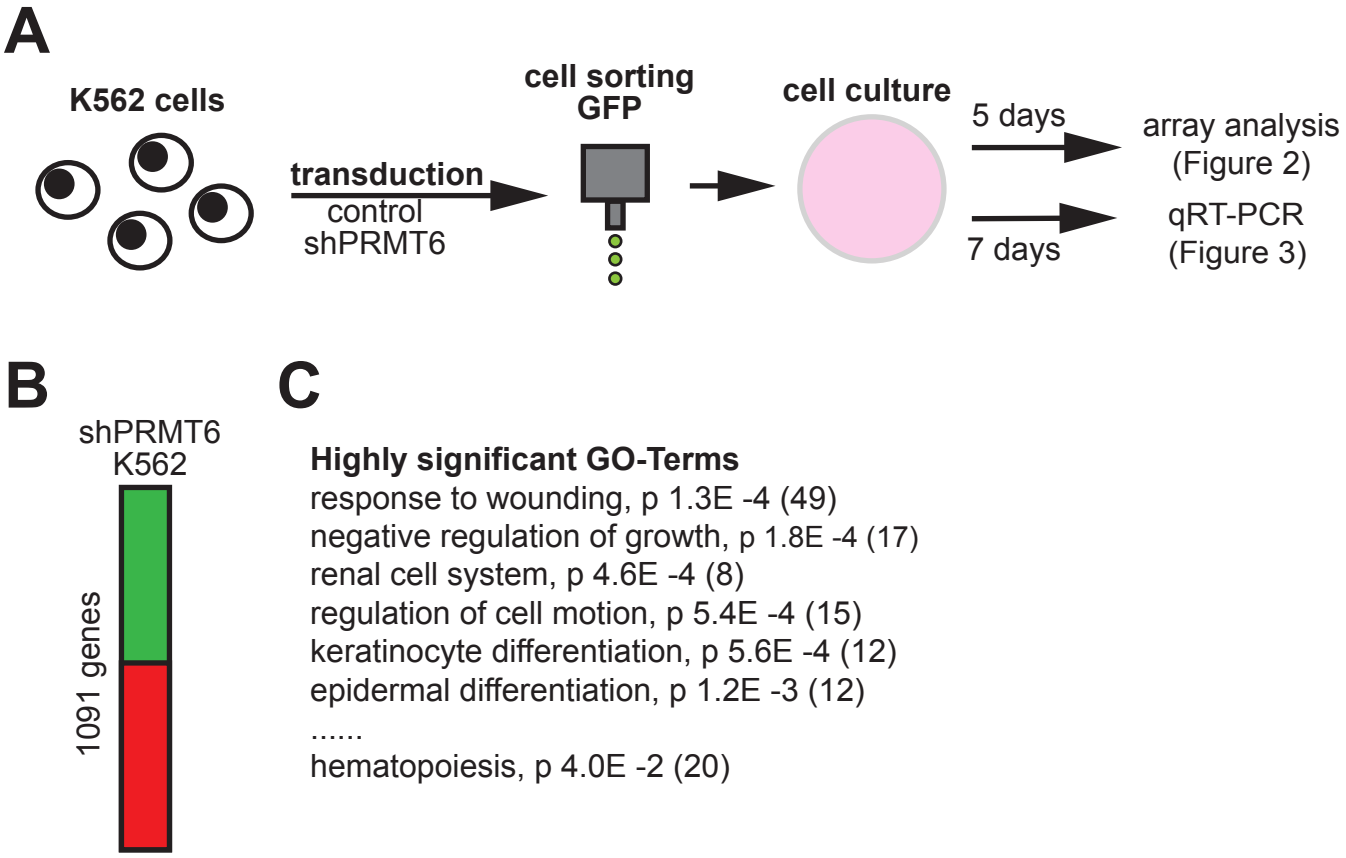
Supplementary Figure 1. (A) Schematic workflow of the colony forming units (CFU) assay. hCD34+ cells were transduced with PRMT6 knockdown (shPRMT6), PRMT6 expression, or control vectors. Transduced GFP-positive cells were sorted by FACS and subjected to a CFU assay. For determination of the knockdown or overexpression cells were cultivated for seven days in expansion medium and the *PRMT6* expression determined by qRT-PCR. **(B)** Analysis of the *PRMT6* expression after knockdown by qRT-PCR. **(C)** Analysis of PRMT6 overexpression by qRT-PCR. Expression values were normalised to *GAPDH* expression. The p-values were calculated using Student's t-test. **P < .01; ***P < .001.

Supplementary Figure 1

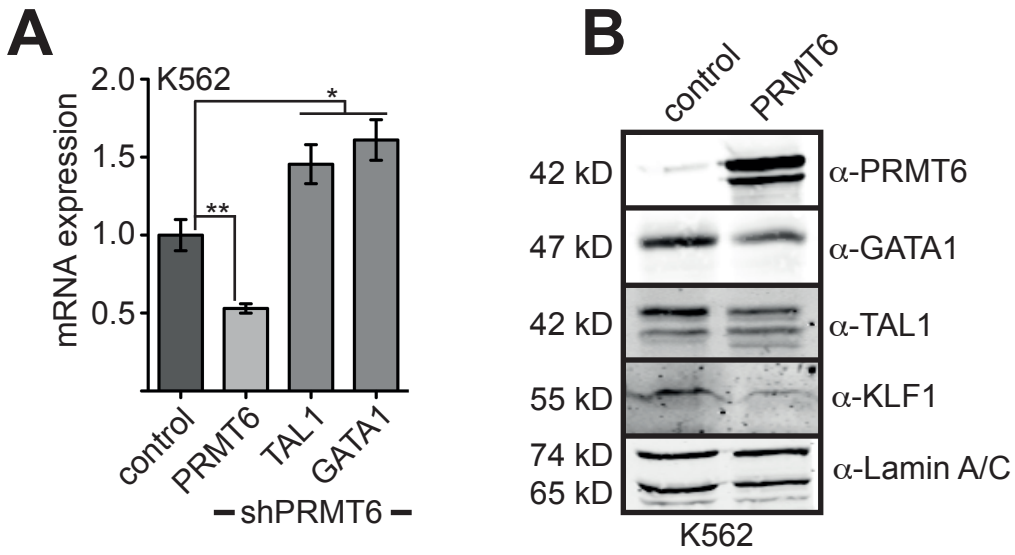


Supplementary Figure 2. hCD34+ cells were isolated and subjected to expansion medium. After two days cells were transduced with either a PRMT6-overexpression or control vector and finally transferred into ery/mega medium. After 10 days of differentiation, erythroid and mega-karyocytic differentiation marker like CD71, CD235a (GYPA), CD41 and CD61 were measured by flow cytometry. A histogram is shown, in which the different gating for the cells is depicted. One histogram for every differentiation marker is shown exemplarily. The FACS measurement was done in three determinations.

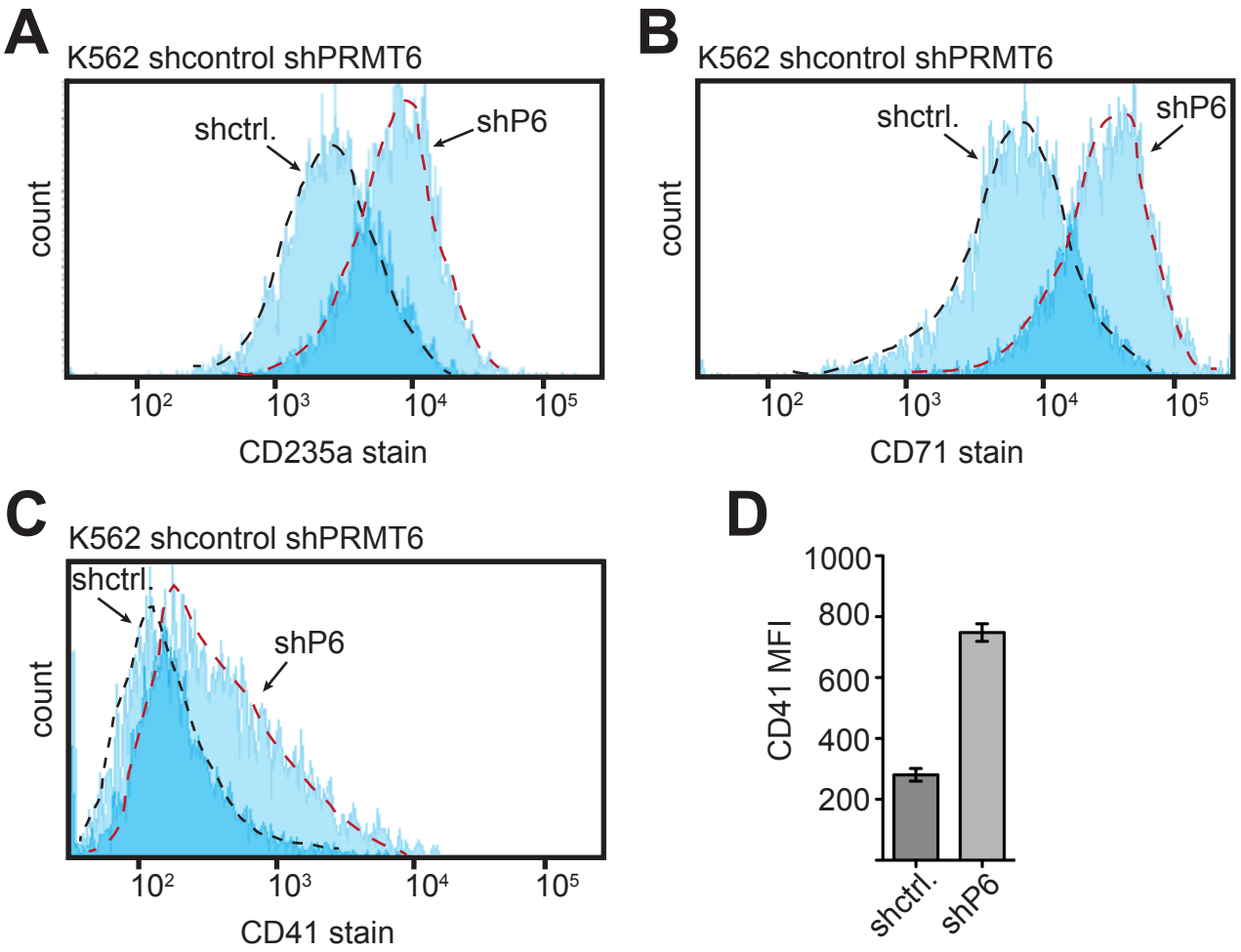
Supplementary Figure 2



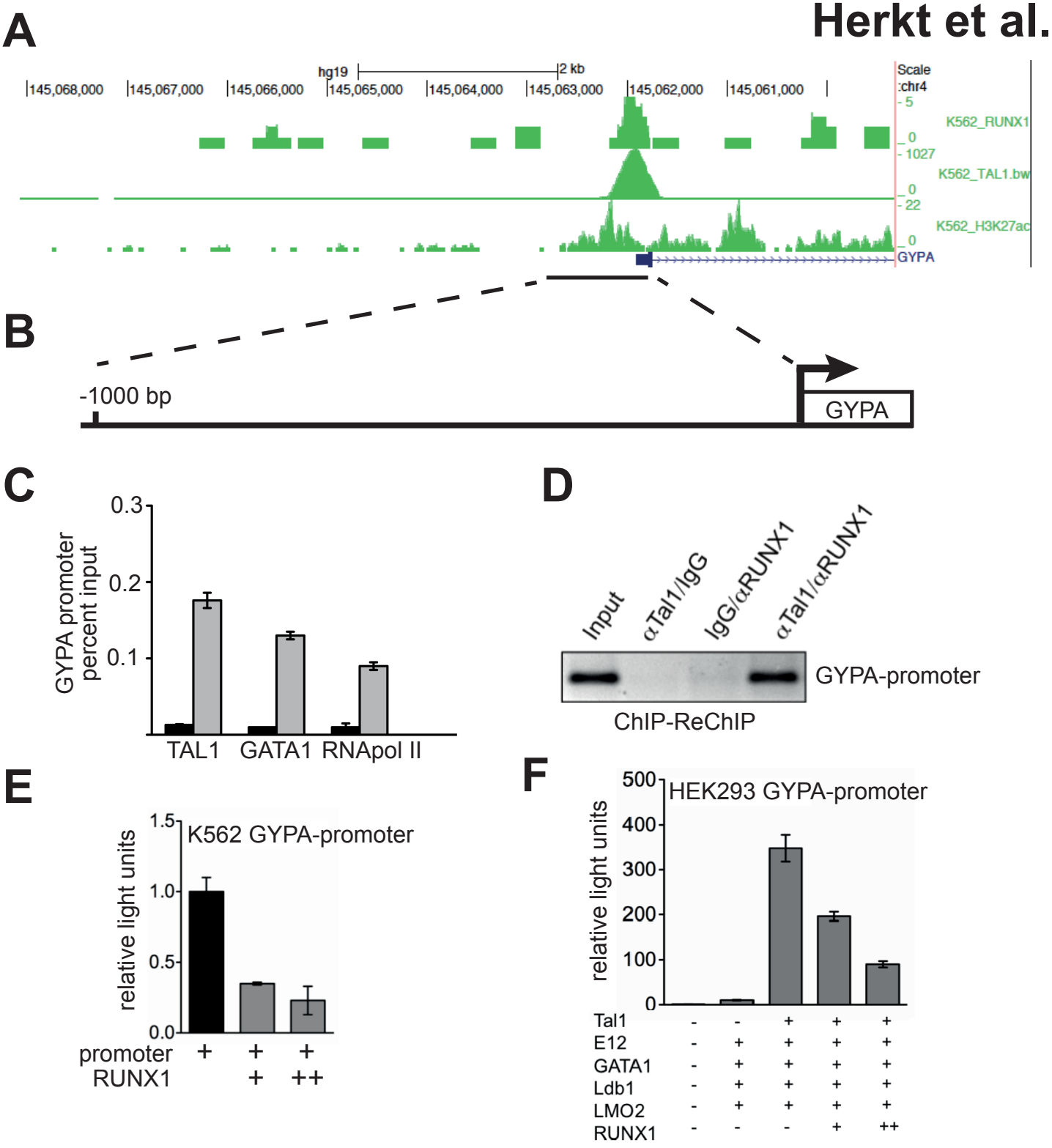
Supplementary Figure 3. Gene expression analysis upon PRMT6 knockdown. (A) K562 cells were transduced with a control vector expressing a non targeting shRNA and shPRMT6 constructs, respectively. Upon transduction the cells were sorted to a purity of >95% according to the GFP signal. After sorting cells were cultured in RPMI culture medium and harvested at the given time points for gene expression analysis. **(B)** Gene expression array analysis was performed with shPRMT6 K562 cells. Knockdown of PRMT6 altered expression of 1091 genes. **(C)** Gene ontology analysis of the 1091 genes changed upon PRMT6 knockdown. Analysis was performed using DAVID with standard settings. Highly significantly enriched GO-terms are shown. The p-values are given and the digits in brackets give the number of changed genes.



Supplementary Figure 4. (A) Analysis of TAL1 and GATA1 expression after knockdown of PRMT6 in K562 cells. The mRNA levels were evaluated by qRT-PCR. Error bars show the standard deviation of four independent evaluations. The p-values were calculated using Student's t-test. *P < .05; **P < .001. **(B)** Evaluation of TAL1 and GATA1 expression on protein level upon PRMT6 overexpression in K562 cells by Western blot. A representative blot is shown (n=2).

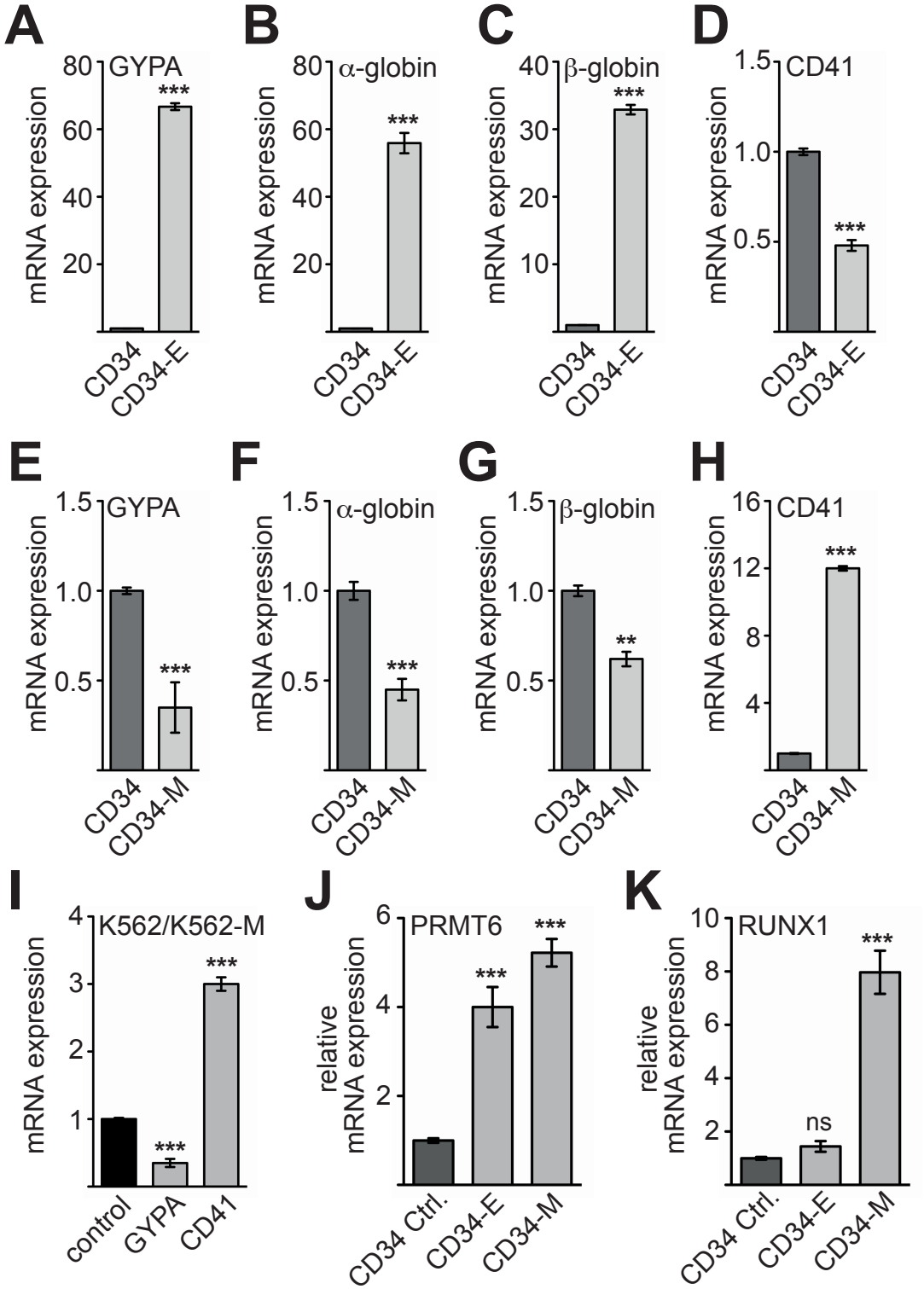


Supplementary Figure 5. FACS analysis of CD235a (GYPA), CD71 and CD41 surface expression upon knockdown of PRMT6 in K562 cells. K562 cells were transduced and the transduced cell population was analysed by FACS using a fluorescence labeled antibody against **(A)** GYPA (CD235a), **(B)** CD71 and **(C-D)** CD41. The distinct populations are marked by a dotted line, as a guidance. **(E)** The K562 cell pellet displays a redish colour upon PRMT6 knockdown. Cells transduced with unspecific shRNA construct were used as a control. **(F)** K562 cells display a redish colour upon treatment with the indicated concentrations of PRMT6 inhibitor for three days.



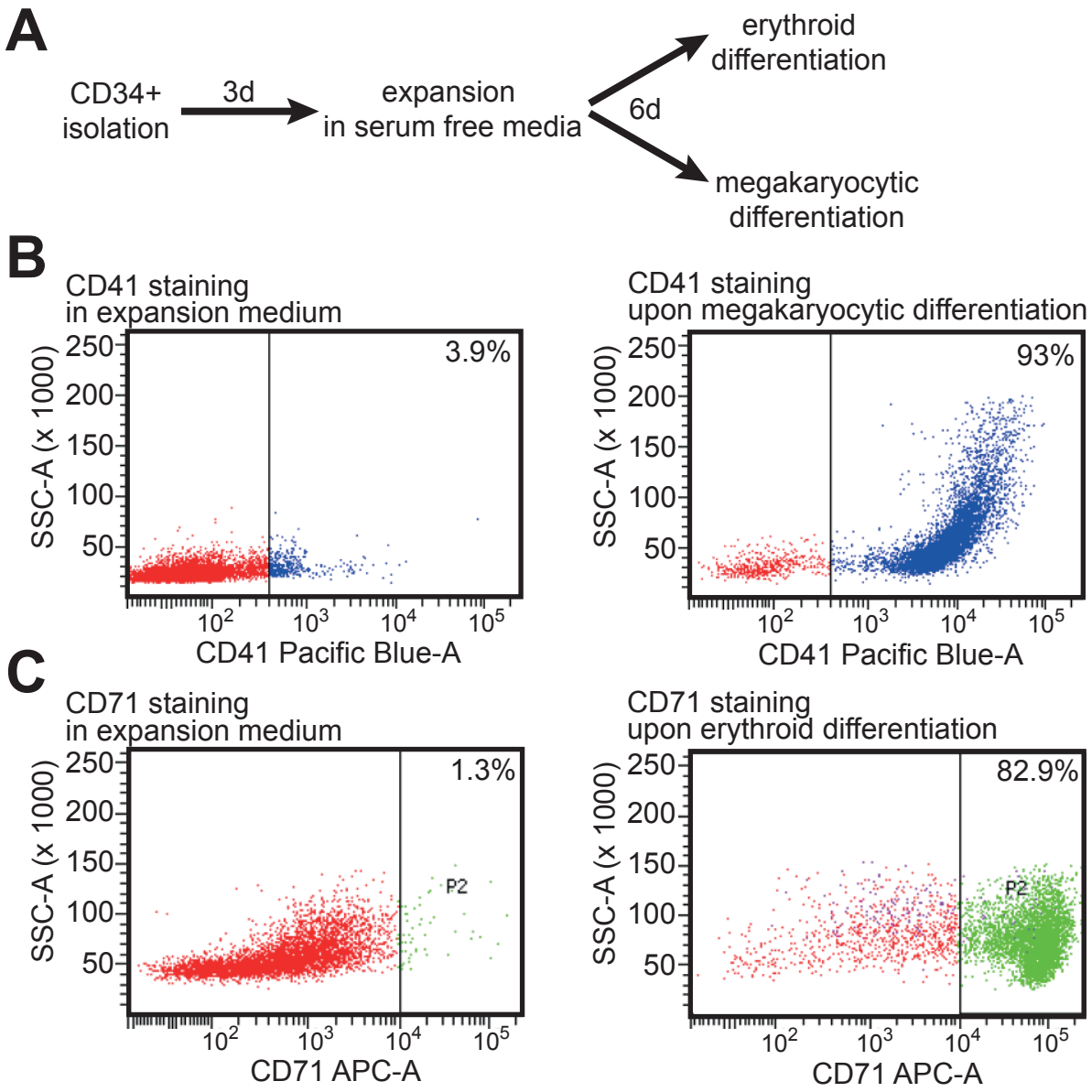
Supplementary Figure 6. (A) Analysis of a ChIP-Seq dataset of TAL1, RUNX1 and H3K27me3 gathered from K562 cells (Chacon *et al.*, 2017). GALAXY was used for analysis. **(B)** Scheme of the *GYPA* promoter region. **(C)** ChIP assay showing TAL1, GATA1 and RNA pol II binding close to the transcriptional start site. **(D)** ChIP-ReChIP-PCR analysis of RUNX1 and TAL1 co-occupancy of the *GYPA* promoter in K562 cells. **(E)** *GYPA* promoter assay reveals inhibitory effect of RUNX1 on the activity of the promoter luciferase construct in K562 cells. Luciferase values were normalized to internal control (β-Gal expression) and are shown in fold change related to the activity of the promoter construct alone. **(F)** A complex containing TAL1, E47, LMO2, Ldb1, and GATA1 activates the *GYPA* promoter in HEK293T cells. This activation is repressed by cotransfection of RUNX1. Luciferase values were normalized to internal control (β-Gal expression) and are shown in fold change related to the activity of the promoter construct alone.

Supplementary Figure 6

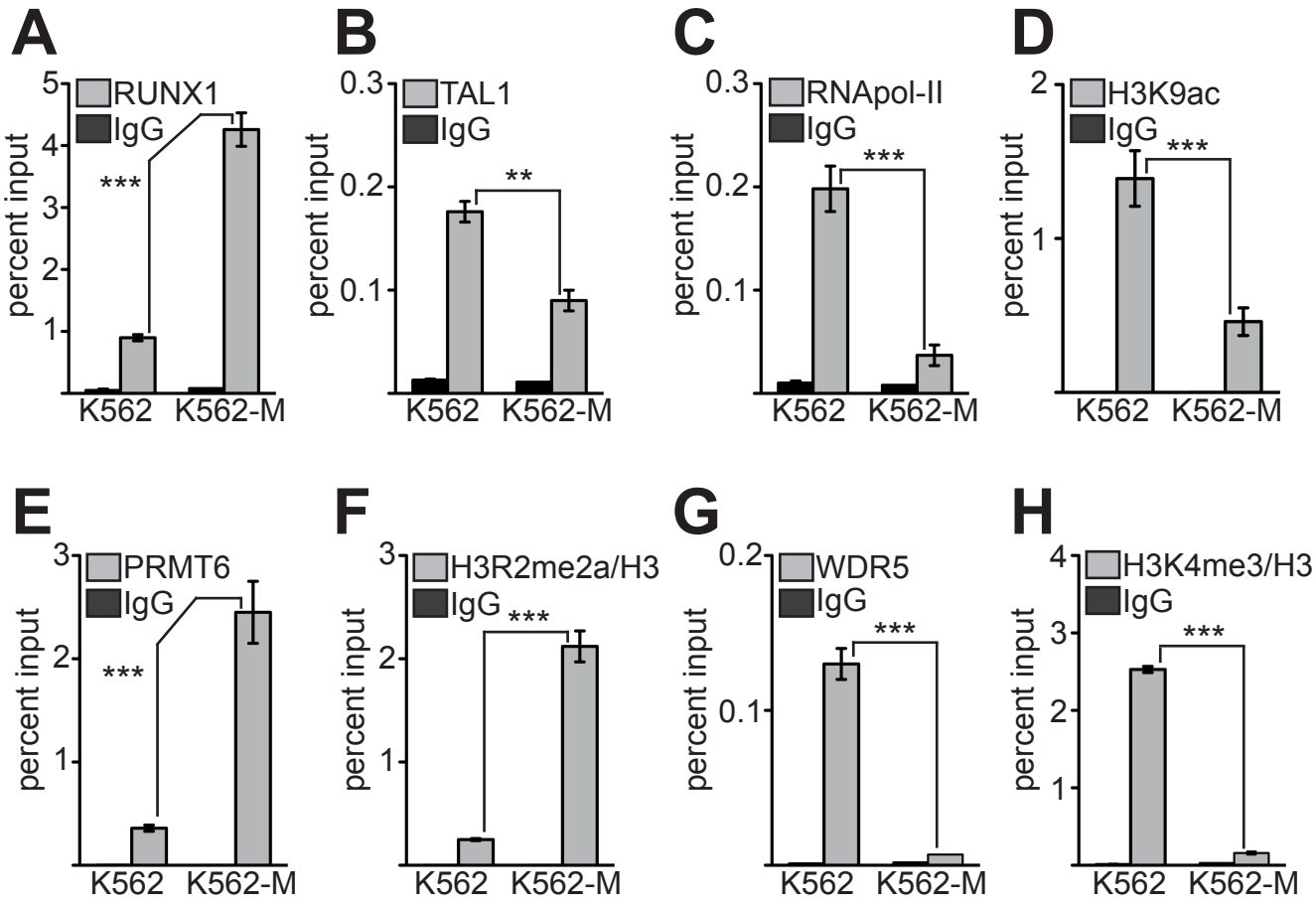


Supplementary Figure 7. Gene expression changes upon differentiation. (A-C) Expression of *GYPA*, α-*globin* and β-*globin* upon erythroid differentiation of hCD34+ cells (CD34-E). **(D)** Expression of *CD41* upon erythroid differentiation of CD34+ cells. **(E-G)** Expression of *GYPA*, α-*globin* and β-*globin* upon megakaryocytic differentiation of hCD34+ cells (CD34-M). **(H)** Expression of CD41 upon megakaryocytic differentiation of hCD34+ cells (CD34-M). **(I)** Expression of *GYPA* and *CD41* upon megakaryocytic differentiation of K562 cells (K562-M). **(J)** Expression of *PRMT6* upon erythroid and megakaryocytic differentiation. **(K)** Expression of *RUNX1* uopn erythroid and megakaryocytic differentiation. Error bars represent the standard deviation from at least four determinations. P-values are given according to Student's t-test. **P < .01; ***P < .001.

Supplementary Figure 7

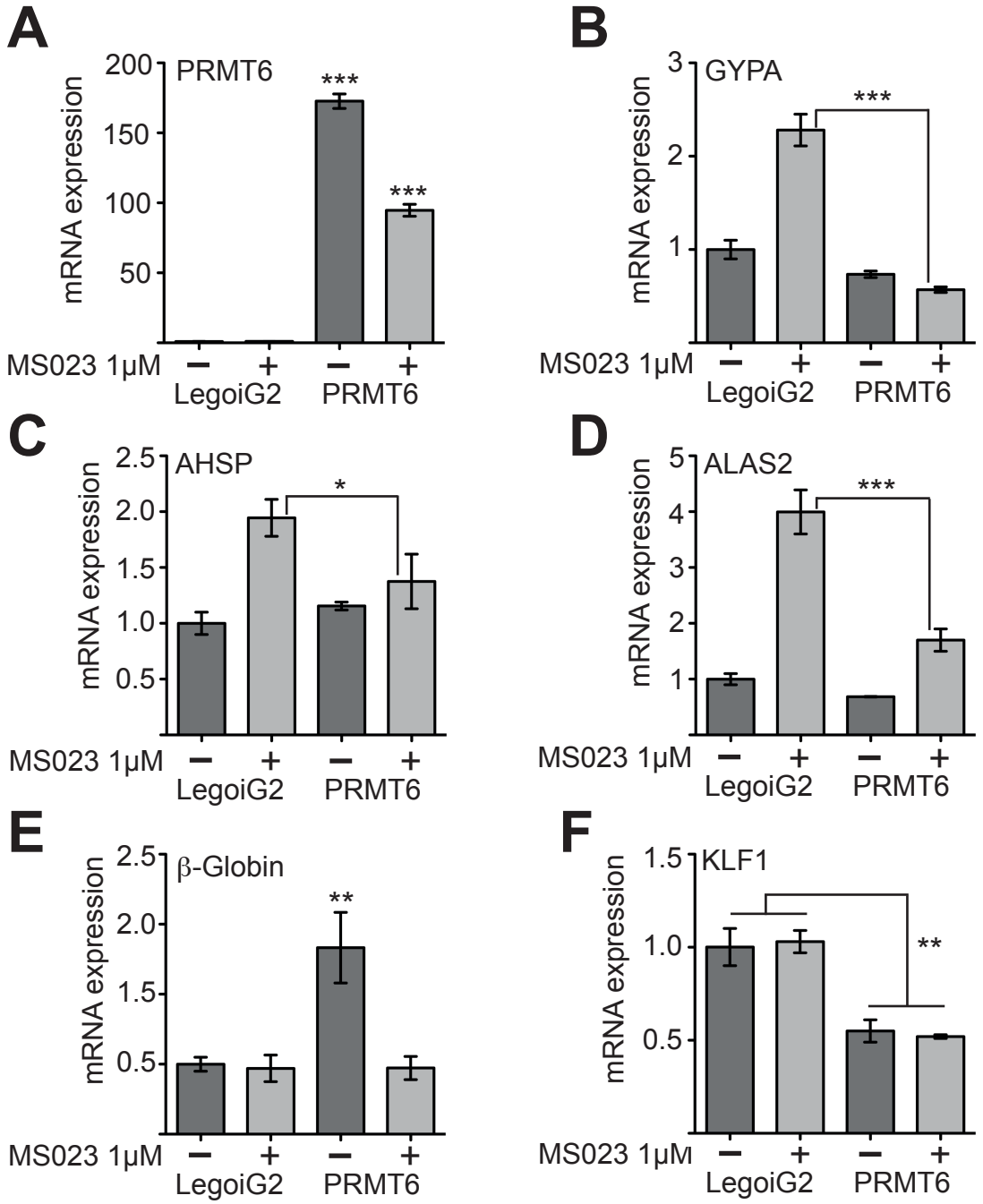


Supplementary Figure 8. Scheme of hCD34+ treatment and differentiation. (A) Human CD34+ cells were isolated from bone marrow or from peripheral blood mobilized with G-CSF using magnetic beads. After expansion for three days the medium was exchanged with differentiation medium. Erythroid and megakaryocytic differentiation was induced with a medium containing EPO or TPO for six days. **(B-C)** The differentiation efficiency was verified via flow cytometry with a CD41-PacificBlue or CD71-APC antibody. **(B)** Due to differentiation towards megakaryocytes CD41 positive cells are increasing compared to cells in expansion cells. **(C)** Upon erythroid differentiation the CD71-marker is increased compared to undifferentiated cells.

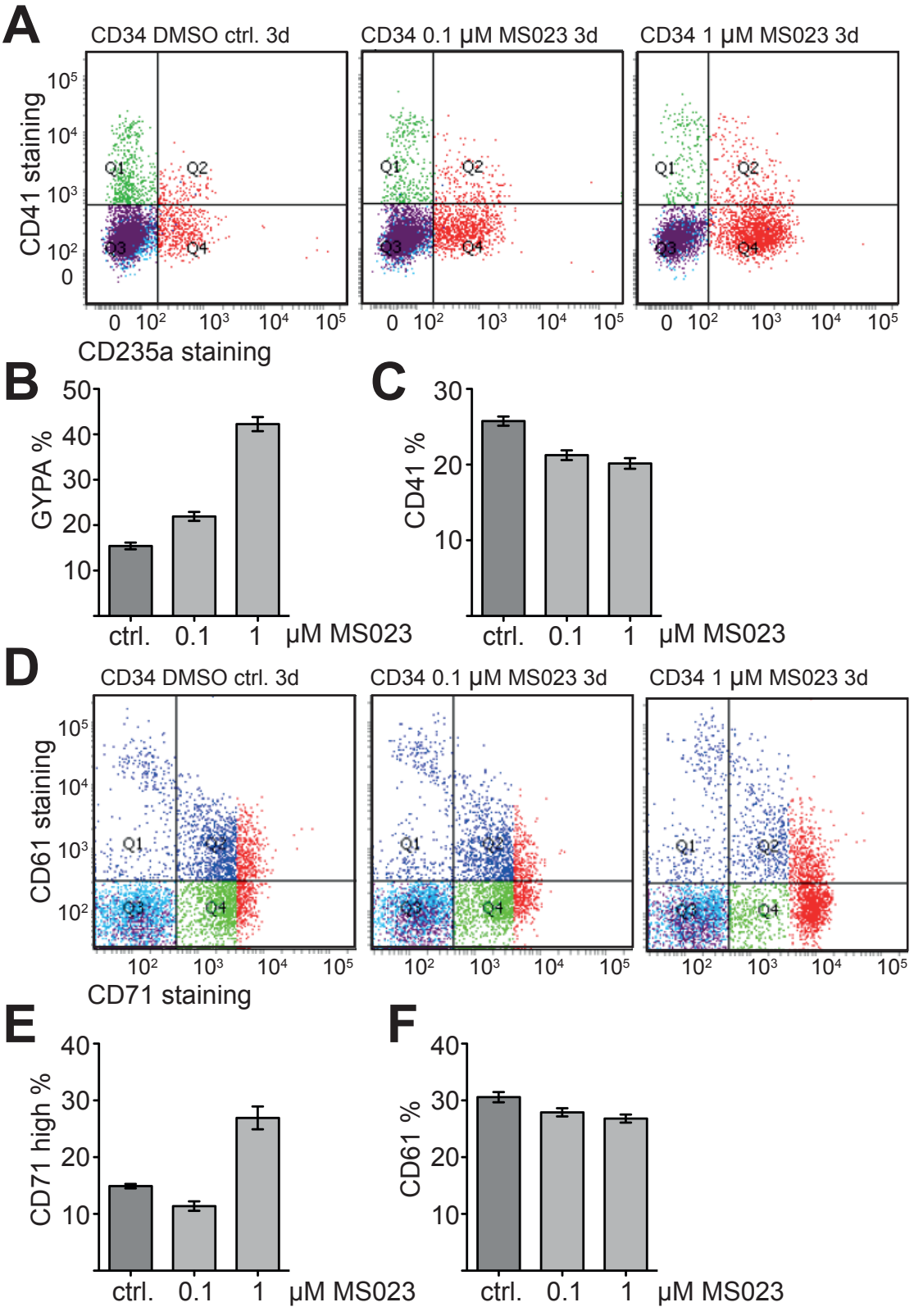


Supplementary Figure 9. Changes at the *GYPA* promoter upon megakaryocytic differentiation of K562 cells. (**A**) RUNX1 binding to the *GYPA* promotor is increased upon megakaryocytic differentiation of K562 cells. (**B**) TAL1 binding to the *GYPA* promotor is decreased upon megakaryocytic differentiation of K562 cells. (**C**) Upon megakaryocytic differentiation RNApol-II binding is decreased. (**D**) The activating H3K9ac is reduced upon megakaryocytic differentiation of K562 cells. (**E**) PRMT6 binding increases upon megakaryocytic differentiation of K562 cells. (**F**) H3R2me2 is increased upon megakaryocytic differentiation. (**G**) WDR5 binding is decreased upon megakaryocytic differentiation. (**H**) H3K4me3 is decreased upon megakaryocytic differentiation of K562 cells. Cells were differentiated with 30 nM TPA towards megakaryocytic differentiation for three days. Error bars give the standard deviation from at least for determinations. P-values are given according to Students t-test.
**P < .01; ***P < .001

Supplementary Figure 9

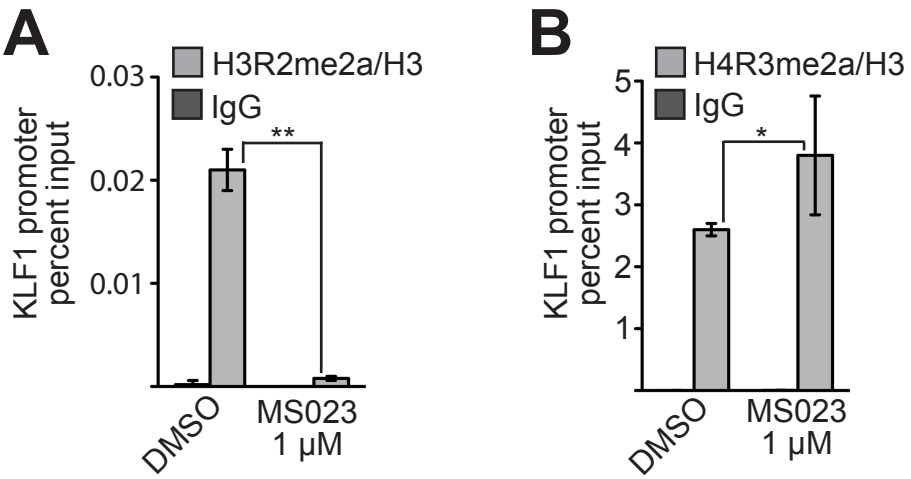


Supplementary Figure 10. PRMT6 overexpression reduces the influence of PRMT6-inhibition. K562 cells were transduced with empty LegoiG2 control vector or PRMT6 expression vector. These cells were treated with 1 μM PRMT6 inhibitor. The mRNA expression of *PRMT6*, *GYPA*, *AHSP*, *ALAS2*, *beta-Globin* and *KLF1* was determined. **(A)** Expression of PRMT6 was determined. **(B)** *GYPA* expression is induced by PRMT6 inhibitor. This induction is not seen in PRMT6 overexpressing cells. **(C-D)** *AHSP* and *ALAS2* expression is induced by PRMT6 inhibition.This increase is not seen in PRMT6 overexpession in K562 cells. **(E-F)** The rescue of the PRMT6 inhibitor effect is not seen in case of *beta-Globin* and *KLF1*. We speculate that in these cases the transduction of the K562 cells in combination with the treatment (DMSO in the control or MS023) had an influence on expression of these genes. qRT-PCR values were normalised against *GAPDH* expression. The expression level of the empty vector transduced K562 cells, which were treated with the DMSO control was set as one.The error bars represent the standard deviation from at least four determinations. The stars give the significants between control and PRMT6 overexpression, if not otherwise depicted. P-values were calculated using Student‘s t-test. P-values are given according to Student‘s t-test. *P < .05; **P < .01; ***P < .001



Supplementary Figure 11. FACS analysis of CD235a (GYPA), CD71, CD61 and CD41 surface expression of CD34+ cells upon MS023 inhibitor treatment. Cells were treated for 3 days with 0.1 µM or 1 µM MS023 inhibitor. Cells treated with DMSO served as a control. **(A-C)** Cells were stained with CD235a (GYPA) and CD41. **(A)** Dot blot representation of the FACS measurement. **(B)** Percentage of the GYPA-positive cells is shown. **(C)** Percentage of the CD41-positive cells is shown. **(D-F)** Cells were stained with CD71 and CD61. **(D)** Dot blot of the CD61/CD71 cells is shown. **(E)** Percentage of the CD71-high positive cells is shown. **(F)** Percentage of the CD61-positive cells is shown.

Supplementary Figure 11



Supplementary Figure 12. ChIP-Assay on the KLF1 promotor in hCD34 cells upon inhibitor treatment.
(A) ChIP assay upon PRMT6 inhibitor treatment of CD34+ cells for three days. H3Rme2a was decreased upon inhibitor treatment. **(B)** H4R3me2a was increased upon inhibitor treatment. Error bars give the standard deviation from four independent determinations. P-values were calculated using Student‘s t-test. *P < .05; **P < .001.

Antibodies
ChIP: anti-PRMT6 (sc55702, Santa Cruz und ABE124, Millipore), anti-RUNX1 (ab23980, Abcam), anti-TAL1 (ab75739, Abcam), anti-GATA1 (sc1233, Santa Cruz), anti-PADI4 (ab96758, Abcam), anti-RNAPol-II (ab5408, Abcam), anti-H3K4me3 (ab1012, Abcam), anti-H3R2me2 (ab80075, Abcam und 07-585, Millipore), anti H3K27me3 (ab6002), Abcam), anti-H3K9ac (ab10812, Abcam), anti-H3 (ab1791, Abcam)
As control IgG antibodies were used: IgG goat (sc2028, Santa Cruz), IgG rabbit (sc2027, Santa Cruz), IgG mouse (sc2025, Santa Cruz)

FACS: hCD41-APC (303710, Biolegend), hCD41-PacificBlue (303714, Biolegend), hCD61-APC (130-098-578, Miltenyi Biotec), hCD235a-APC (130-100-270, Miltenyi Biotec), hCD235a-APC (349107, Biolegend), hCD235a-PE (561051, BD Bioscience), hCD71-PE (334106, Biolegend), hCD71-APC (130-091-721, Miltenyi Biotec), CD34-FITC (343503, Biolegend)

Western Blot
For protein detection by western blot whole cell lysates were prepared with RIPA-buffer (50 mM Tris HCl pH 8, 0.1 % SDS, 150 mM NaCl, 2 mM EDTA pH 8, 1 % NP-40, 0.5 % sodium deoxy-cholate, 1 x Protease-Inhibitor). For histone western blot, lysates were resolved on 15% SDS-polyacrylamide gel and for other proteins on a 10% SDS-polyacrylamide gel. Proteins were blotted onto nitrocellulose membrane (HP40.1, Carl Roth), blocked for 1h in 5% dry-milk (T145.1, Carl Roth) in TBS/T. The incubation with the primary antibody in 5% dry-milk in TBS/T lasted over night at 4°C, whereas for histone proteins for 2 h at RT. The incubation with labelled secondary antibody was in a 1:15000 dilution in blocking solution. The signals were measured with the Odyssey system from LI-COR.
Western blot antibodies: anti-PRMT6 (sc365018, Santa Cruz), anti-Lamin B1 (ab16048), anti-Lamin A/C (612163, BD Bioscience), anti H3R2me2a (07-585, Millipore), anti-H3 (ab1791, Abcam), anti-Actin (ab1801, Abcam), anti-KLF1 (ab2483, Abcam).

Vector systems
For overexpression: For overexpression of PRMT6 the cDNA was cloned into LeGOiG2 lentiviral vector (Weber *et al.* 2008).

For knockdown: Sequences of the cloned shRNAs of PRMT6 were cloned into the backbone of the SEW vector (Demaison *et al.*, 2002), in which the SFFV promoter and EGFP were exch-anged with a sh-expression cassette and a GFP-Zeocin fusion expression cassette from psiR-NA-h7SKGFPzeo. A none-specific shRNA was used as a control.
shRNA-sequences for PRMT6:
 # 1:
5`-ACCTCGGCATTCTGAGCATCTTCTGTTCAAGAGACAGAAGATGCTCAGAATGCCTT-3
 # 2:
5'-ACCTCGGAGGAGAAGACCAAAGACTTTCAAGAGAAGTCTTTGGTCTTCTCCTCCTT-3'.

Demaison C. Parsley K. Brouns G., et al. High-level transduction and gene expression in hematopoietic repopulating cells using a human immunodeficiency virus type 1-based lentiviral vector containing an internal spleen focus forming virus promoter. Hum. Gene Ther. 2002;13:803–813

Weber K, Bartsch U, Stocking C, Fehse B. A multicolor panel of novel lentiviral "gene ontology" (LeGO) vectors for functional gene analysis. Mol Ther. 2008 Apr . 16(4):698-706.

Supplementary Material