



Ferrata Storti Foundation

Haematologica 2020
Volume 105(5):1294-1305

Meningioma 1 is indispensable for mixed lineage leukemia-rearranged acute myeloid leukemia

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ABSTRACT

Mixed lineage leukemia (*MLL/KMT2A*) rearrangements (MLL-r) are one of the most frequent chromosomal aberrations in acute myeloid leukemia. We evaluated the function of Meningioma 1 (MN1), a co-factor of HOXA9 and MEIS1, in human and murine MLL-rearranged leukemia by CRISPR-Cas9 mediated deletion of MN1. MN1 was required for *in vivo* leukemogenicity of MLL positive murine and human leukemia cells. Loss of MN1 inhibited cell cycle and proliferation, promoted apoptosis and induced differentiation of MLL-rearranged cells. Expression analysis and chromatin immunoprecipitation with sequencing from previously reported data sets demonstrated that MN1 primarily maintains active transcription of HOXA9 and HOXA10, which are critical downstream genes of MLL, and their target genes like BCL2, MCL1 and Survivin. Treatment of MLL-rearranged primary leukemia cells with anti-MN1 siRNA significantly reduced their clonogenic potential in contrast to normal CD34⁺ hematopoietic progenitor cells, suggesting a therapeutic window for MN1 targeting. In summary, our findings demonstrate that MN1 plays an essential role in MLL fusion leukemias and serve as a therapeutic target in MLL-rearranged acute myeloid leukemia.

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Received: November 6, 2018.

Accepted: August 8, 2019.

Pre-published: August 14, 2019.

doi:10.3324/haematol.2018.211201

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/105/5/1294

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Introduction

Acute myeloid leukemia (AML) is associated with a plethora of genetic alterations such as chromosomal rearrangements and mutations,¹ with some of them being generic for different types of leukemias and cancers.¹ MLL1 (Mixed lineage leukemia 1/KMT2A) rearrangements are one such example that are found in myeloid as well as lymphoid leukemias.² Approximately 135 different MLL rearrangements have been identified so far, but only nine specific gene fusions (including AF4, AF9, ENL, and AF6) account for more than 90% of all oncogenic recombinations.^{3,4} A unifying hallmark of all MLL-rearranged (MLL-r) leukemias is the deregulation of clustered HOXA/MEIS1 genes.² Transcriptional activation of MLL target genes (HOXA9/MEIS1) is associated with an increase in histone H3 lysine79 dimethylation (H3K79me2) across the respective gene locus, which is specifically mediated by histone methyltransferase DOT1.^{2,5} Recently, several studies in patients and murine models have highlighted the importance of co-operating genetic alterations in MLL-r leukemia progression. In 40-50% of MLL-r AML cases, RAS and FLT3 mutations have been shown to accelerate leukemogenesis, and Mn1, Bcl11a and Fosb have been identified as co-operating oncogenes in a murine leukemia virus insertional mutagenesis model.^{4,6}

MN1 (*Meningioma-1*) is frequently over-expressed in AML patients and is associated with a poor prognosis.⁷⁻¹³ However, in patients with *inv(16)*, highest *MN1* expression has been reported with favorable prognosis to current therapeutics.¹¹ *MN1* functions as a transcriptional regulator that co-operates with the nuclear receptors for retinoic acid (RAR) and vitamin D, by acting as co-activator or co-repressor, depending on the interacting partners.¹⁴⁻¹⁶ In addition, *MN1* is frequently over-expressed and occasionally fused to *TEL* as part of the rare *MN1-TEL* translocation.¹⁷ *Mn1* is known

to be co-operating partner of several oncogenic fusion genes (NUP98–HOXD13,¹⁸ CALM–AF10,¹⁹ MLL–AF9⁶ and MLL–ENL)²⁰ and mutated RUNX1,²¹ and as a common target of insertional mutagenesis in a hematopoietic stem cell (HSC) gene therapy trial,²² thereby promoting leukemogenesis.

Interestingly, MN1-induced AML is also dependent on Hoxa cluster genes and Meis1.²³ Multipotent progenitor cells (MPP) and common myeloid progenitors (CMP) have been identified as the cell of origin in MN1-induced AML, while granulocyte-macrophage progenitors (GMP) cannot be transformed.²³ We found that the differential expression of *Hoxa9*, *Hoxa10* and *Meis1* in MPP/CMP compared to GMP cells was responsible for the ability of MN1 to transform the more immature, but not the more mature, progenitor cells.²³ One important difference between MN1 and MLL-r leukemia is that MN1 cannot activate *Hox* gene expression by itself, while MLL-AF9 can.^{23,24} Therefore, MN1 is unable to transform GMP cells, while MLL-AF9 can transform myeloid progenitor cells down to the differentiation state of a GMP. Both MLL-AF9- and MN1-induced leukemias depend on the H3K79 methyltransferase DOT1L.^{14,25, 26} In addition, deletion of *Mll* and *Dot1l* in MN1-expressing cells abrogated the cell of origin-derived gene expression program, including the expression of Hoxa cluster genes, and impaired the leukemogenic activity of MN1 *in vivo*.¹⁴ However, it is not known whether MLL-AF9 transformed cells also depend on MN1.

High *MN1* expression confers resistance to all-trans retinoic acid (ATRA)-induced differentiation and chemotherapy-induced cytotoxicity.^{7,27} Recent studies have shown that pyrimethamine [a dihydrofolate reductase (DHFR) inhibitor] and DOT1L inhibitors possess anti-leukemic effects in MN1^{hi} AML cells.^{14,27} However, the mechanism of MN1-induced AML and drug resistance is still not completely understood due to its little structural/functional similarity to any other protein.¹⁴ Mn1 null mice have severe defects in bones of the cranial skeleton, yet the effects of its deletion in hematopoiesis/leukemia are not known.²⁸ Here, we show that CRISPR–Cas9-mediated deletion of MN1 in MLL-r leukemias, and consequently treatment of MLL-r leukemias with an anti-MN1 siRNA, led to strong anti-leukemic effects, including increased terminal myeloid differentiation and suppression of leukemic growth *in vitro* and *in vivo*.

Methods

Viral vectors, vector production, and CRISPR

Briefly, helper-free recombinant retrovirus harvested from supernatants of the transfected ecotropic Phoenix packaging cell line was used to transduce 5-fluoro-uracil treated mouse bone marrow cells to generate an immortalized MLL-AF9 cell line, as described before.^{15,29} CRISPR lentiviral vector L40C-CRISPR.EFS.dTomato (Addgene # 89392) was kindly provided by Dr. Dirk Heckl, Hannover Medical School, Hannover, Germany.³⁰ CRISPR-Cas9 target sites in the *Mn1*/*MN1* gene were selected using the CCTop selection tool.³¹ Single guide RNA (sgRNA) were cloned in the CRISPR-Cas9 vector. The list of sgRNA is summarized in *Online Supplementary Table S1*.

Cell lines and primary cells

Bone marrow from healthy donors was obtained from the transplantation unit of Hannover Medical School. Mononuclear

cells from peripheral blood or bone marrow were isolated by density gradient centrifugation. CD34⁺ cells were isolated using the CD34 microbead kit (Miltenyi Biotec, Bergisch Gladbach, Germany), as per manufacturer's protocol. AML patient samples were taken from the AML-myelodysplastic syndromes (AML-MDS) repository of Hannover Medical School. Written informed consent was obtained for the use of patient samples in accordance with the Declaration of Helsinki, and the study was approved by the institutional review board of Hannover Medical School (ethical vote 1187-2011).

Mouse transplantation and homing assay

NOD-SCID and NOD/SCID/IL2rgnull (NSG) mice were bred and maintained in pathogen-free conditions in the animal laboratory of Hannover Medical School, Hannover, Germany. All animal experiments were approved by the Lower Saxony state office for consumer protection, Oldenburg, Germany.

siRNA and lipid nanoparticles

All siRNAs (MN1 and control/AHA1) were purchased from Axolabs GmbH (Kulmbach, Germany). siRNAs were packaged in lipid nanoparticles (LNP), as described previously.³²

Gene expression and chromatin immunoprecipitation sequencing analysis

We performed gene expression profiling of *in vitro* cultured MLL-AF9/Mn1wt versus MLL-AF9/Mn1null cells in triplicate. RNA was extracted using the standard trizol method and was further used for gene expression profiling. Gene expression profiling using extracted RNA from MLL-AF9/MN1wt and MLL-AF9/MN1null cells was performed on Affymetrix GeneChip Mouse 430 2.0 arrays (43,000 probes). The whole dataset can be found at GEO (GSE130631) for public access.

Chromatin immunoprecipitation sequencing (ChIP-Seq) DNA binding data were taken for H3K79me2 from GSE55038,³³ MLL-AF9 from GSE29130,²⁵ *Hoxa9* from GSE33518,³⁴ and MN1 and MEIS1 from our previous publication.²³

Statistical analysis

Pairwise comparisons were performed by Student t-test for continuous variables. Two-sided significance was set at $P < 0.05$. Comparison of survival curves were performed using the log rank test. Statistical analyses were performed with Microsoft Excel (Microsoft, Munich, Germany) and GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

More detailed information on the materials and methods used can be found in the *Online Supplementary Appendix*.

Results

MLL-AF9 cell proliferation depends on Meningioma 1

To study the effects of MN1 gene inactivation in leukemia cells, we generated CRISPR-Cas9 engineered MN1null murine and human leukemia cells and characterized these cells *in vitro* and *in vivo* (Figure 1A). MN1 was deleted in murine cells transformed by MLL-AF9, HOXA9, HOXA9MEIS1, E2A-HLF and 10 human leukemia cell lines: THP-1, MV-4-11, NB4, OCI-AML2, OCI-AML3, U937, K562, Kasumi-1, HL-60 and HEL. Ninety-six to 288 transduced cells per cell line were single cell sorted and the outgrowing clones were evaluated for MN1 deletion by qualitative RT-PCR (qRT-PCR), western blot and sequencing (*Online Supplementary Tables S2* and *S3* and *Online Supplementary Figure S1A-F*). While up to

30% of the single sorted cells grew out as clones, only 0–8.9% of the single sorted clones were MN1-deleted, and only one MN1-deleted clone was obtained from four different murine cell lines with HOX-dependent oncogene expression (*Online Supplementary Table S2*), suggesting that MN1 loss impairs clonogenicity of MLL-r and HOXA9-transformed cells. Subsequently, in parallel to MLL-AF9 mouse cells, we also studied THP-1 and MV-4-11 (positive for MLL-AF9 and MLL-AF4, respectively) and the non-MLL-r human cell line U937. Loss of MN1 more potently reduced proliferation in MLL-r cells as compared to non-MLL-r leukemia cells (Figure 1B–D and *Online Supplementary Figure S2A*). The inhibitory effect of MN1 loss in U937 cells may be due to high HOXA9 and MEIS1 expression (*Online Supplementary Figure S2B* and C). The

cell lines K562, Kasumi-1 and HL-60 had the lowest HOXA9 and MEIS1 expression levels, but had the highest rate of MN1-deleted clones (*Online Supplementary Table S2*), indicating a dependence on MN1 in MLL-r and HOXA9/MEIS1 expressing cells.

MN1 deletion drastically impaired the colony-forming potential up to 22-fold and reduced the size of the colonies in MLL-AF9 transformed murine cells (Figure 1E and 1F). Due to potential unpredictable off-target effects, we tested one additional sgRNA (sgRNA-4; positioned at a different location), which had a similar effect as the initial sgRNA (*Online Supplementary Figure S3A* and B). Thus, *in vitro* proliferation and colony-forming potential of MLL-rearranged cells require MN1 expression both in murine and human leukemia cells.

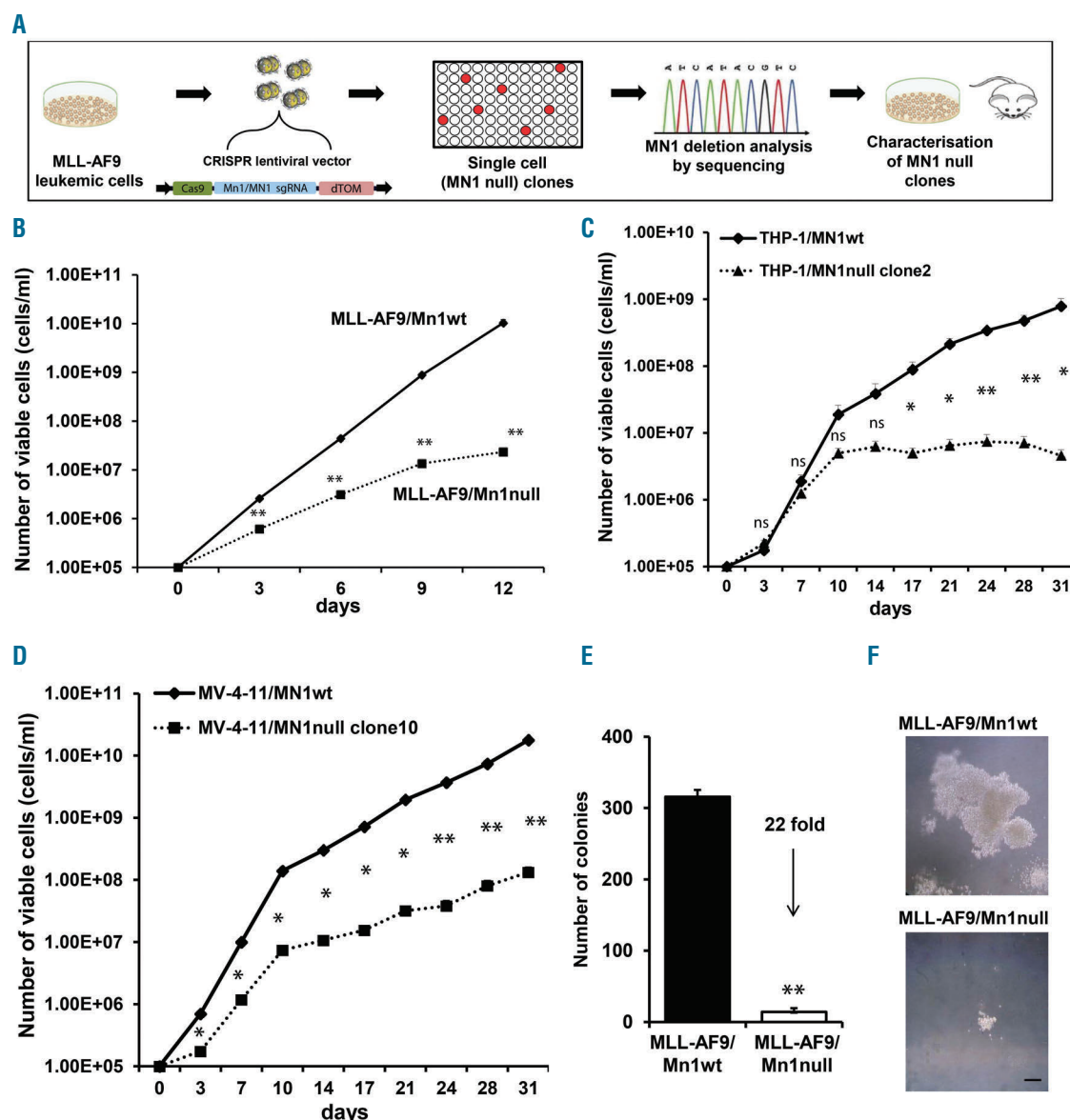


Figure 1. MLL-AF9 cell proliferation depends on Meningioma 1 (MN1). (A) Schematic outline of the strategy for deletion of MN1/Mn1 in murine and human cell lines. (B) Cumulative cell counts of MLL-AF9/Mn1wt and MLL-AF9/Mn1null cells [mean±standard error of mean (SEM), n=3]. (C) Cumulative cell counts of THP-1 MN1wt and MN1null cells (mean±SEM, n=3). (D) Cumulative cell counts of MV-4-11 MN1wt and MN1null cells (mean±SEM, n=3). (E) Colony-forming cell (CFC) counts of MLL-AF9/Mn1wt and MLL-AF9/Mn1null cells (mean±SEM, n=3). (F) Morphology of representative CFC colonies of MLL-AF9/Mn1wt and MLL-AF9/Mn1null cells. Black scale bar represents 0.25 mm. * $P < 0.05$; ** $P < 0.01$; ns: not significant.

Meningioma 1 regulates cell cycle, apoptosis and differentiation in MLL-AF9 cells

Next, we explored the impact of *MN1* deletion on cell cycle, apoptosis and differentiation in MLL positive leukemic cell lines. Loss of MN1 inhibited cell cycle progression resulting in more cells in G0/G1 phase and fewer cells in S phase of the cell cycle compared to MN1wt cells (Figure 2A and *Online Supplementary Figure S4A*). In addition,

cell cycle progression was negatively associated with MN1 null cells by gene set enrichment analysis (GSEA) of gene expression profiling data (*Online Supplementary Tables S4* and *S5* and *Online Supplementary Figures S4B* and *S5A-D*). The proportion of Annexin V positive cells was significantly increased and the gene sets for apoptosis and the p53 pathway were highly enriched in MLL-AF9/Mn1null cells (Figure 2B, *Online Supplementary Table S4* and *Online*

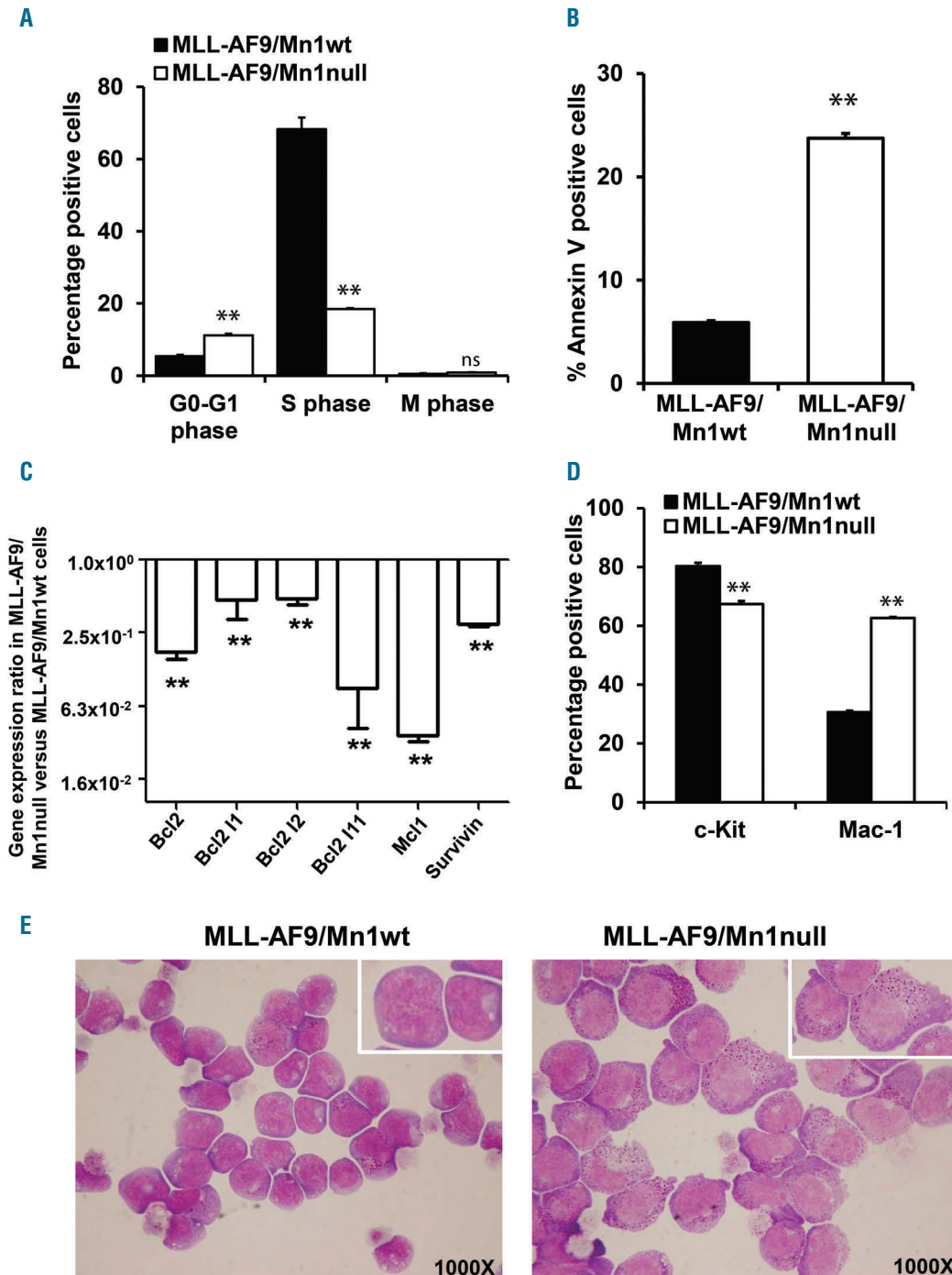


Figure 2. Meninoma 1 (MN1) regulates cell cycle, apoptosis and differentiation in MLL-AF9 cells. (A) Cell cycle analysis of MLL-AF9/Mn1wt and MLL-AF9/Mn1null cells [mean±standard error of mean (SEM), n=3]. (B) Proportion of apoptotic cells in MLL-AF9/Mn1wt and MLL-AF9/Mn1null cells (mean±SEM, n=3). (C) Relative gene expression of differentially expressed genes normalized to Abl1 in MLL-AF9/Mn1wt and MLL-AF9/Mn1null cells (mean±SEM, n=3). (D) Immunophenotype of *in vitro* cultured MLL-AF9/Mn1wt and MLL-AF9/Mn1null cells (mean±SEM, n=3). (E) Morphology of *in vitro* cultured MLL-AF9/Mn1wt and MLL-AF9/Mn1null cells (original magnification 1,000-fold). **P*<0.05; ***P*<0.01; ns: not significant.

Supplementary Figure S4E and F). In support of the GSEA analysis, a strong downregulation of the anti-apoptotic Bcl2 gene family, Mcl1 and Survivin was shown by qRT-PCR (Figure 2C). MLL-AF9/Mn1null cells showed low levels of the hematopoietic stem/progenitor cell marker c-Kit/CD117 but high expression of the mature myeloid cell marker Mac1/CD11b and Gr-1/Ly-6G in Mn1 null cells compared to MLL-AF9/Mn1wt cells (Figure 2D and *Online Supplementary Figure S6A*). This observation was also supported by morphological analysis, as Mn1 null cells had a lower nuclear-cytoplasmic ratio and were more granulated (Figure 2E), and gene sets were enriched for myeloid and lymphoid cell differentiation (*Online Supplementary Tables S4 and S5*, and *Online Supplementary Figures S4B and S6B and C*). MLL-AF9 oncogene expression was found to be unaltered or increased upon loss of Mn1 in MLL-AF9/Mn1null cells when compared with MLL-AF9/Mn1wt cells, excluding the possibility that downregulation of the oncogene is responsible for the anti-leukemic effects of MN1 loss (*Online Supplementary Figure S7A-C*). Hence, MN1 deletion suppresses cell cycle, promotes apoptosis, and induces differentiation in MLL positive leukemia cells.

MLL-AF9-induced leukemogenesis requires Meningioma 1

To further investigate the role of Mn1 deletion in MLL-AF9 leukemogenesis *in vivo*, we transplanted MLL-AF9/Mn1wt and MLL-AF9/Mn1null cells in syngeneic mice and monitored onset of leukemia and survival. Mice transplanted with MLL-AF9/Mn1null cells had <1% engraftment in all ten mice, whereas mice transplanted with MLL-AF9/Mn1wt cells showed >90% engraftment at week 4 (Figure 3A). At eight weeks, engraftment in MLL-AF9/Mn1null was below 2% (except for one mouse with 88% MLL-AF9 expressing cells, which consequently died), while 8 of 10 MLL-AF9/Mn1wt mice died from leukemia and the two surviving mice showed >90% engraftment (Figure 3A). Correspondingly, white blood cell (WBC) counts were significantly lower in MLL-AF9/Mn1null than in MLL-AF9/Mn1wt mice (Figure 3B). Loss of Mn1 significantly prolonged survival as compared to Mn1wt mice (Figure 3C). At death, mice transplanted with MLL-AF9/Mn1null cells had significantly lower spleen weight (Figure 3D). Since loss of Mn1 in MLL-AF9 cells did not show a potent engraftment ability, and to exclude the possibility of homing defects, we transplanted equal numbers of MLL-AF9 (Mn1wt/null) cells intravenously in sub-lethally irradiated mice. Mice were sacrificed after 8 and 24 hours and MLL-AF9/Mn1null cells showed better homing in bone marrow than mice transplanted with MLL-AF9/Mn1wt cells, suggesting that differences in homing could not account for the differences in survival (Figure 3E). In addition, to rule out the presence of any immunological effects against MLL-AF9/Mn1null cells, we transplanted MLL-AF9 (Mn1wt or null) cells in NSG mice (lacking cellular and humoral immunity) and monitored leukemia onset and survival. Similar to syngeneic mouse transplantation studies, MLL-AF9/Mn1null mice did not develop leukemia, while their MLL-AF9/Mn1wt counterpart quickly died from leukemia (*Online Supplementary Figures S8A and B and S9A-H* for blood counts). Thus, MLL-AF9 positive murine leukemia requires *MN1* expression to induce leukemia *in vivo*.

MLL-AF9 and MLL-AF4 rearranged human leukemias also depend on Meningioma 1

Besides syngeneic MLL-AF9 transplantation studies, we also assessed the role of MN1 deletion in MLL-AF4 (MV-4-11) and MLL-AF9 (THP-1) leukemias *in vivo*. Equal numbers of THP-1 and MV-4-11 (MN1wt or MN1null) cells were transplanted intravenously in NSG mice, and the onset of leukemia and survival were assessed. MN1null cells showed lower engraftment of transplanted cells in peripheral blood at four weeks and improved blood counts compared to their wild-type counterparts (Figure 4A and B and *Online Supplementary Figures S10 and S11*). MN1 deletion significantly prolonged survival of mice transplanted with THP-1/MN1null and MV-4-11/MN1null clones (Figure 4C and D). At death, engraftment in bone marrow and spleen weight were also found lower in mice transplanted with MN1 deletion clones of THP-1 and MV-4-11 (Figure 4E and F and *Online Supplementary Figure S12A and B*). In addition, we also evaluated the tumor-forming ability of THP-1/MN1null and MV-4-11/MN1null clones by subcutaneous transplantation in NOD-SCID mice. Tumor volumes were monitored every five days starting 15 days after transplantation. Deletion of MN1 led to significantly reduced tumor volumes for 2 of 3 MN1null clones as compared to wild-type MN1 human leukemic cell lines (*Online Supplementary Figure S13A and B*). Our *in vivo* transplantation studies suggest that loss of MN1 critically affects leukemia proliferation in human MLL-rearranged AML.

Meningioma 1 overexpression restores leukemogenicity in MLL-AF9/Mn1null cells

In an attempt to rescue the deleterious effects caused by Mn1 deletion, we over-expressed control (MY) vector or MN1 in MLL-AF9/Mn1 null cells and characterized its properties *in vitro* and *in vivo*. MLL-AF9/Mn1null cells transduced with MN1 overcame the reduced proliferative capacity of MLL-AF9/Mn1null cells similarly to MLL-AF9/Mn1wt cells (Figure 5A). Similarly, MLL-AF9/Mn1null cells with MN1 expression restored the reduced colony-forming potential of MLL-AF9/Mn1null cells akin to MLL-AF9/Mn1wt cells (Figure 5B and C). We also studied the leukemogenic potential of MLL-AF9/Mn1null cells rescued by MN1 expression in comparison with MLL-AF9 (Mn1wt or null) cells transduced with vector control. As previously mentioned in our manuscript, MLL-AF9/Mn1null cells do not possess the ability to engraft in mice. However, MLL-AF9/Mn1null cells with ectopic MN1 expression engrafted in mice and induced a leukemic phenotype with short survival similar to MLL-AF9/Mn1wt mice (Figure 5D and E and *Online Supplementary Figure S14*). Hence, deleterious effects caused by loss of MN1 expression can be rescued by restoring the expression of MN1.

Meningioma 1 maintains expression of the distal HOXA cluster and MEIS1

MLL-AF9-mediated leukemogenesis is primarily mediated by upregulation of the Hox/Meis1 gene cluster and their target genes.^{33,35} DOT1L methylates histone H3 on lysine 79 (H3K79me2), which has been associated with MLL-AF9 binding and expression of Hoxa cluster genes and Meis1 in normal hematopoietic progenitors and MLL-r leukemias.²⁵ Gene expression analysis by qRT-PCR in MLL-AF9/Mn1null and Mn1wt cells showed that *Hoxa3*

through *Hoxa7* were not significantly dysregulated, while *Hoxa9*, *Hoxa10* and *Meis1* were strongly down-regulated in MLL-AF9/Mn1null cells (Figure 6A). We also performed global gene expression analysis in MLL-AF9/Mn1wt versus MLL-AF9/Mn1null cells with significant differentially expressed genes (Online Supplementary Table S6 and Online Supplementary Figure S15A).

To evaluate a direct transcriptional effect of MLL-AF9 and MN1 on the distal Hoxa cluster genes, we analyzed the binding of MN1, MLL-AF9, Hoxa9, MEIS1 and dimethyl marks of H3K79 (representing DOT1L binding) at the Hoxa cluster locus from available chromatin immunoprecipitation-sequencing (ChIP-Seq) data sets.

MN1 chromatin marks were enriched at distal Hoxa cluster genes (*Hoxa7* to *Hoxa10*) similar to MLL-AF9, MEIS1 and H3K79me2 marks (Figure 6B), suggesting that MN1 is a direct regulator of *Hoxa* cluster gene expression. However, genome-wide co-localization of MLL-AF9 and MN1 chromatin did not show any overlap of a significant number of chromatin marks, in contrast to the high overlap of MN1 with *Hoxa9* and MEIS1 (Figure 6C-E), suggesting that MN1 is essential for MLL-AF9 primarily due to its role as a co-factor of *Hoxa9* and *Meis1*.

To substantiate this observation, we evaluated the expression of Bcl2 in MLL-AF9/Mn1 wild-type and null cells, as Bcl2 is a known target of *Hoxa9* but not of MLL-

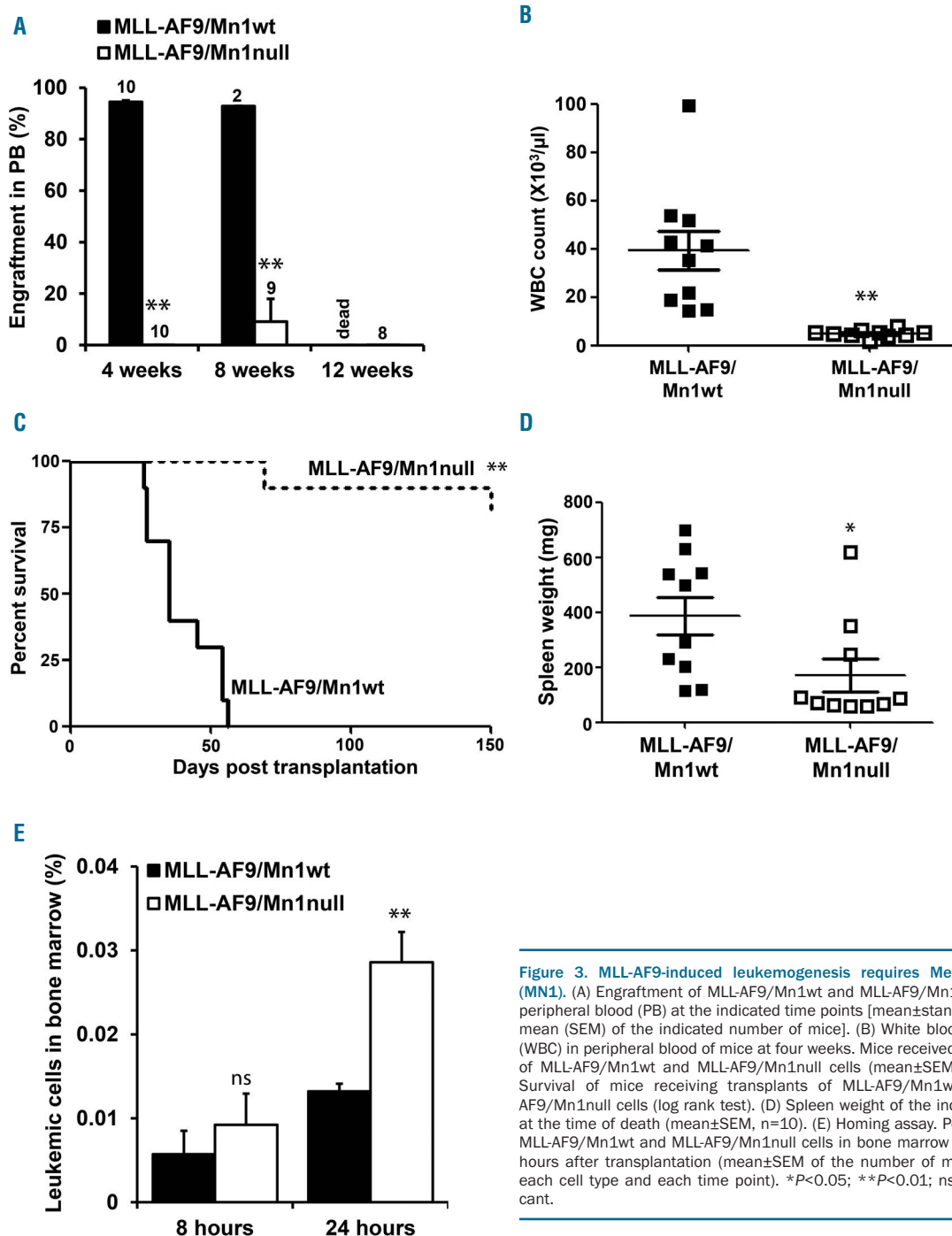


Figure 3. MLL-AF9-induced leukemogenesis requires Meningioma 1 (MN1). (A) Engraftment of MLL-AF9/Mn1wt and MLL-AF9/Mn1null cells in peripheral blood (PB) at the indicated time points [mean±standard error of mean (SEM) of the indicated number of mice]. (B) White blood cell count (WBC) in peripheral blood of mice at four weeks. Mice received transplants of MLL-AF9/Mn1wt and MLL-AF9/Mn1null cells (mean±SEM, n=10). (C) Survival of mice receiving transplants of MLL-AF9/Mn1wt and MLL-AF9/Mn1null cells (log rank test). (D) Spleen weight of the indicated mice at the time of death (mean±SEM, n=10). (E) Homing assay. Percentage of MLL-AF9/Mn1wt and MLL-AF9/Mn1null cells in bone marrow at 8 and 24 hours after transplantation (mean±SEM of the number of mice, n=5 for each cell type and each time point). *P<0.05; **P<0.01; ns: not significant.

AF9 (Figure 6F).³⁶ In agreement with this, Bcl2 and related genes were strongly down-regulated upon deletion of Mn1 in MLL-AF9 cells (Figure 2C), confirming the critical role of MN1 in *Hoxa9/Meis1* regulated gene expression. We also identified putative *Hoxa9/Hoxa10* targets differentially expressed and regulated in our gene expression data

and GSEA analysis in MLL-AF9 (Mn1wt vs. Mn1null) cells (*Online Supplementary Table S7* and *Online Supplementary Figure S15B* and C). Significantly, more *Hoxa9/Hoxa10* target genes were down-regulated in MLL-AF9/Mn1null ($P=0.027$, from hypergeometric distribution). Thus, our data suggest that MN1 is required in MLL-r leukemia to

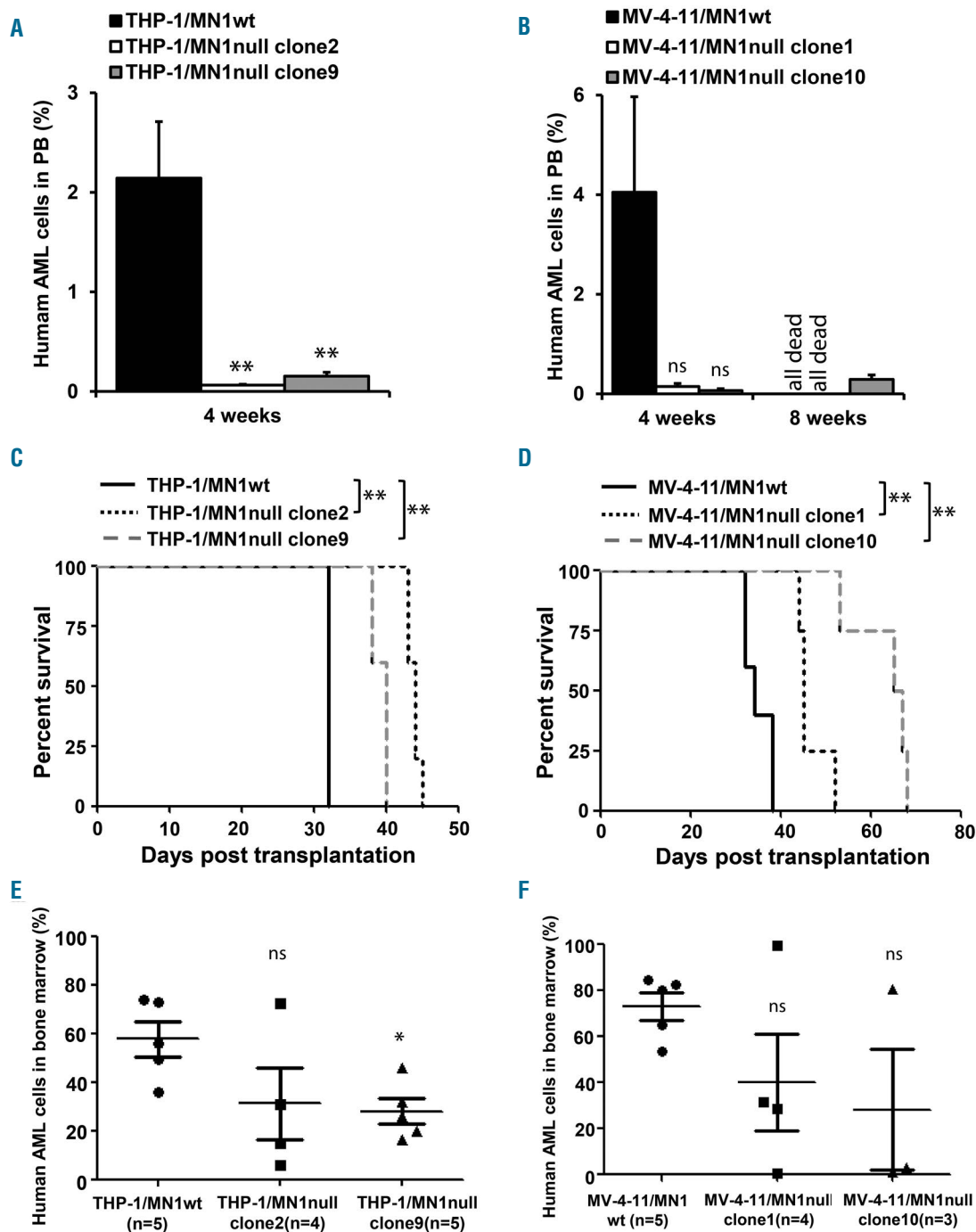


Figure 4. MLL-AF9 and MLL-AF4 rearranged human leukemias also depend on Meninoma 1 (MN1). (A) Engraftment of THP-1/MN1wt and THP-1/MN1null cells in peripheral blood (PB) at four weeks [mean±standard error of mean (SEM), n=5 for THP-1/MN1wt, THP-1/MN1null clone2 and 9]. (B) Engraftment of MV-4-11/MN1wt and MV-4-11/MN1null cells in peripheral blood (PB) at 4 and 8 weeks (mean±SEM; n=5 for MV-4-11/MN1wt, n=4 for MV-4-11/MN1null clone1 and 10). (C) Survival of mice receiving transplants of THP-1/MN1wt and THP-1/MN1null cells (log rank test); (n=5 for THP-1/MN1wt, THP-1/MN1null clone2 and 9). (D) Survival of mice receiving transplants of MV-4-11/MN1wt and MV-4-11/MN1null cells (log rank test); (n=5 for MV-4-11/MN1wt, n=4 for MV-4-11/MN1null clone1 and 10). (E) Percentage of THP-1 (MN1wt or MN1null clone) cells in bone marrow at death (mean±SEM; n=5 for THP-1/MN1wt and THP-1/MN1null clone9, n=4 for THP-1/MN1null clone2). (F) Percentage of MV-4-11 (MN1wt or MN1null clone) cells in bone marrow at death (mean±SEM; n=5 for MV-4-11/MN1wt, n=4 for MV-4-11/MN1null clone1 and n=3 for MV-4-11/MN1null clone10). AML: acute myeloid leukemia; ** $P<0.01$; ns: not significant.

maintain expression of the distal *Hoxa* cluster and *Meis1* and as a co-factor of the *Hoxa9/Meis1* transcriptional complex and their target genes, which also includes MLL.

Meningioma 1 as therapeutic target in MLL-AF9 acute myeloid leukemia

To evaluate MN1 as a therapeutic target in MLL positive AML, we tested the effect of MN1 siRNA in primary

human AML cells. As a proof of principle, we first tested *MN1* knockdown using LNP packaged with anti-*MN1* siRNA in the ME-1 cell line, which expresses MN1 at high levels. *MN1* expression was reduced up to 4-fold in anti-*MN1* siRNA treated cells as compared to control siRNA treated cells (Online Supplementary Figure S16A). Furthermore, we evaluated the effect of MN1 knockdown in CD34⁺ hematopoietic progenitor cells from four

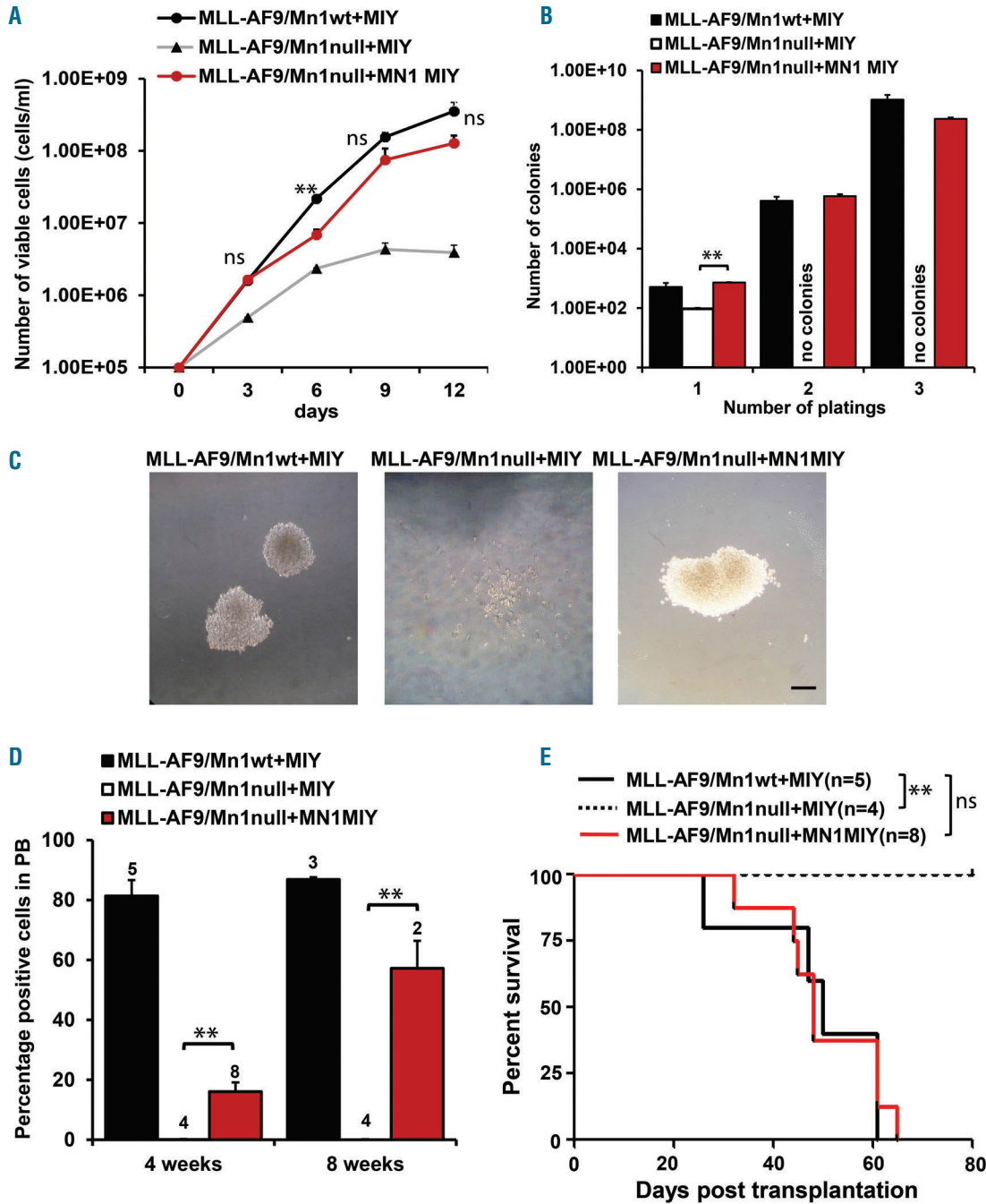


Figure 5. Meningioma 1 (MN1) overexpression restores leukemogenicity in MLL-AF9/Mn1null cells. (A) Cumulative cell counts of MLL-AF9/Mn1wt and MLL-AF9/Mn1null cells transduced with control or MN1MIY plasmid [mean±standard error of mean (SEM), n=3]. (B) Colony-forming cell (CFC) counts of MLL-AF9/Mn1wt and MLL-AF9/Mn1null cells transduced with control or MN1MIY plasmid (mean±SEM, n=3). (C) Morphology of representative CFC colonies of MLL-AF9/Mn1wt and MLL-AF9/Mn1null cells transduced with control or MN1MIY plasmid. Black scale bar represents 0.25 mm. (D) Engraftment of MLL-AF9/Mn1wt and MLL-AF9/Mn1null cells transduced with control or MN1MIY plasmid in peripheral blood (PB) at 4 and 8 weeks after transplantation (mean±SEM of the indicated number of mice). (E) Survival of mice receiving transplants of MLL-AF9/Mn1wt and MLL-AF9/Mn1null cells transduced with control or MN1MIY plasmid (log-rank test). ***P*<0.01; ns: not significant.

healthy stem cell donors and five MLL-AF9 positive AML patients in colony forming cell (CFC) assays. Colony numbers and size were significantly reduced in MLL-AF9 positive AML cells treated with anti-MN1 siRNA compared to CD34⁺ cells from healthy donors when normalized to control siRNA treated cells (Figure 7A and B). Cell numbers from CFC assays were also found significantly reduced in MLL-AF9 positive AML cells treated with anti-MN1 siRNA (Figure 7C). In order to exclude toxic effects

of the LNP/siRNA formulation, we tested an additional anti-MN1 siRNA (MN1 siRNA-3) and found similar specific inhibitory effects with anti-MN1 siRNA-3 against MLL-AF9 positive primary human AML cells (Online Supplementary Figure S16B and C). Thus, *MN1* expression is critical for proliferation of MLL-transformed leukemic cells while it has no effect on CD34⁺ cells from healthy donors, suggesting MN1 as a therapeutic target in MLL-transformed AML.

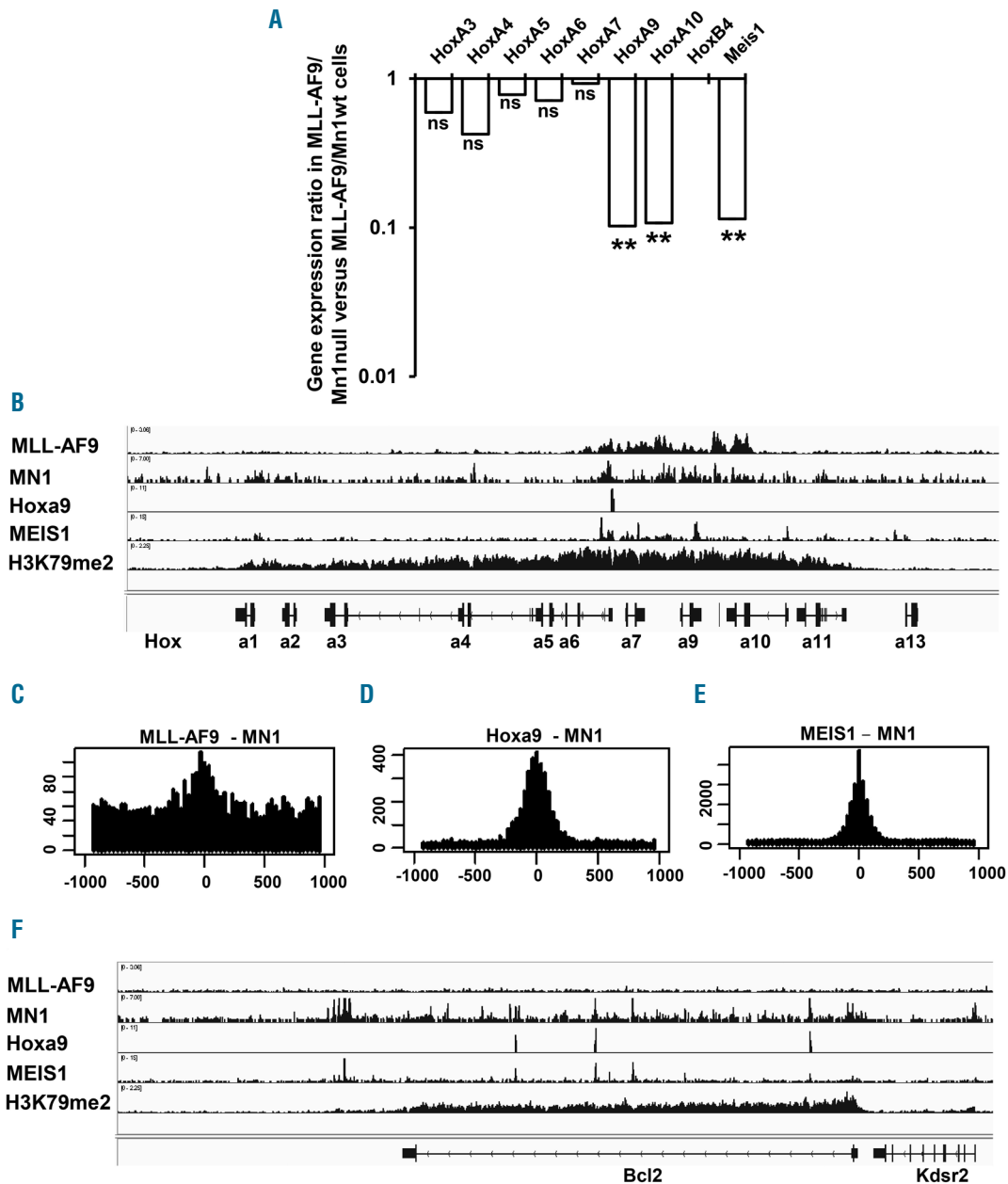


Figure 6. Mn1 maintains distal Hoxa cluster and Meis1 expression. (A) Relative gene expression of differentially expressed genes normalized to Ab1 in MLL-AF9/Mn1wt and MLL-AF9/Mn1null murine cells [mean \pm standard error of mean (SEM), n=3]. (B) Chromatin immunoprecipitation-sequencing (ChIP-Seq) for MLL-AF9, MN1, Hoxa9, MEIS1 and H3K79me2 in different leukemic cell lines (details in Methods). Screen shot of the Hoxa cluster with binding peaks of the above mentioned proteins. (C) Histogram showing the distance between peak maxima of MLL-AF9 peaks to their closest MN1 peak, indicating that MN1-binding sites are less frequently enriched at MLL-AF9-binding sites. (D) Histogram showing the distance between peak maxima of Hoxa9 peaks to their closest MN1 peak, indicating that MN1-binding sites are highly enriched at Hoxa9-binding sites. (E) Histogram showing the distance between peak maxima of MEIS1 peaks to their closest MN1 peak, indicating that MN1-binding sites are highly enriched at MEIS1-binding sites. (F) ChIP-Seq for MLL-AF9, MN1, Hoxa9, MEIS1 and H3K79me2 in different leukemic cell lines (details in Methods). A screen shot of the Bcl2 gene with binding peaks of the aforementioned proteins. * $P < 0.05$; ** $P < 0.01$; ns: not significant.

Discussion

Acute myeloid leukemia pathogenesis requires a multitude of genetic and epigenetic alterations for its initiation and progression.¹ One such genetic alteration is high

expression of MN1, which is widely reported as an independent poor prognostic marker in AML patients, and its induced overexpression in mouse cells induces aggressive AML.^{7-9,12,13,23} In the knockout studies of MN1, mice died young from cleft palate and severe defects in development

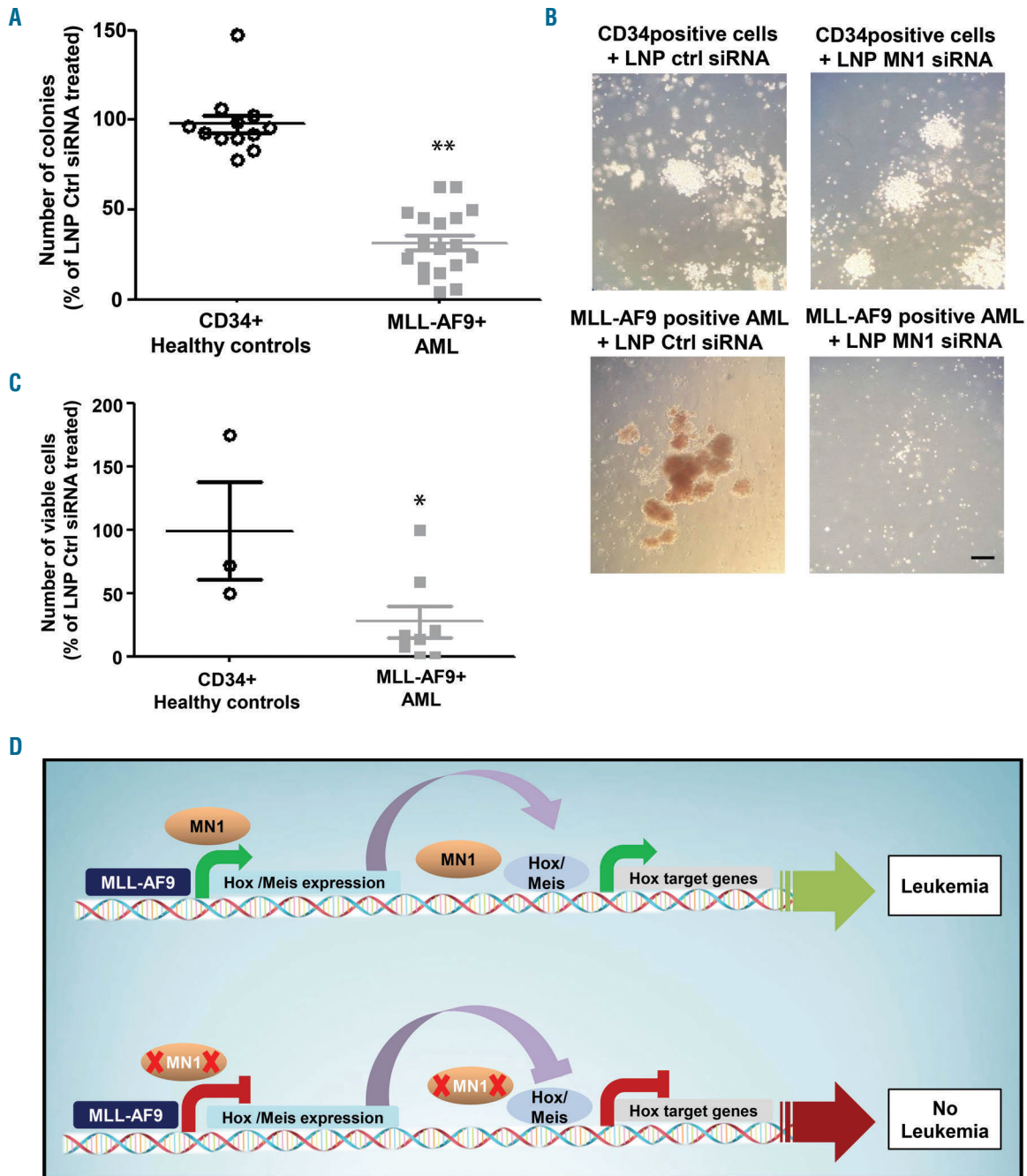


Figure 7. Meningioma 1 (MN1) as therapeutic target in MLL-AF9 acute myeloid leukemia (AML). (A) Colony-forming cell (CFC) counts of CD34⁺ healthy controls (Ctrl) and MLL-AF9 positive AML patient cells treated with anti-MN1 siRNA/LNP formulation normalized to control siRNA/lipid nanoparticle (LNP) formulation [mean±standard error of mean (SEM), cells from n=4 CD34⁺ healthy donors with 3 repeats, and cells from n=4 MLL-AF9 positive AML patients with 4 repeats and 2 repeats from 1 patient]. (B) Morphology of representative CFC colonies of CD34⁺ healthy controls and MLL-AF9 positive AML patient cells treated with control or anti-MN1 siRNA/LNP formulation. Black scale bar represents 0.25 mm. (C) Number of viable cells from colonies of CD34⁺ healthy controls and MLL-AF9 positive AML patient cells treated with anti-MN1 siRNA/LNP formulation normalized to control siRNA/LNP treated cells (mean±SEM, cells from n=1 CD34⁺ healthy donor in triplicate, and cells from n=4 MLL-AF9 positive AML patients in duplicate from each patient). *P<0.05; **P<0.01; ns: not significant. (D) Model of MN1-dependency in MLL-rearranged AML. MLL-AF9 and MN1 induces expression of HOXA cluster genes and MEIS1. MN1 is a critical co-factor of the HOXA9/MEIS1 transcriptional complex. Loss of MN1 affects HOXA expression and renders the HOXA9/MEIS1 complex inactive, and loss of this critical downstream complex of MLL-AF9 blocks its transforming ability.

of various bones in the skull. In addition, MN1 KO mice apparently did not develop hematopoietic defects during the first few weeks of life until they died, suggesting that MN1 inhibition or deletion has a therapeutic window when targeted in leukemic cells.²⁸ It has been previously reported that transient *MN1* knockdown *via* siRNA in human leukemia cell lines reduces their proliferation;²⁰ however, perpetual loss of *MN1* expression has not been comprehensively studied in murine or human leukemias. Our study shows that CRISPR-Cas-mediated loss of MN1 substantially impairs leukemogenesis in MLL-r leukemias. MN1 loss prevents *in vitro* proliferation and colony-forming potential of MLL-AF9 and MLL-AF4-dependent cells. Correspondingly, we demonstrate that MN1 inactivation inhibits cell cycle, promotes apoptosis and induces differentiation in MLL-r leukemic cells. In addition, loss of MN1 significantly impairs MLL-AF9-mediated murine leukemogenesis and reduces leukemic and tumor growth of human MLL-rearranged leukemias *in vivo*. Our results are supported by a previous study, which found that *Mn1* cooperated with MLL-AF9 in leukemogenesis in an *in vivo* transplantation assay.⁶ The homing potential of MLL-AF9/*Mn1* null cells is not affected by *Mn1* deletion. We also show that re-expression of MN1 in MLL-AF9/*Mn1* null cells restores the leukemic potential of MLL-AF9.

Both MN1 and MLL-AF9-induced leukemias rely on expression of the *Hoxa9/Meis1* complex.^{2,25,33,35,37,38} On a genome-wide level chromatin binding of MN1 largely overlapped with HOXA9 and MEIS1 binding, suggesting that the previously described function of MN1 as a co-factor of HOXA9/MEIS1 is its major contribution to the leukemogenic activity of MLL-AF9, as illustrated in our study (Figure 7D). This is supported by our gene expression data showing that HOXA9 target genes are primarily down-regulated upon MN1 deletion. Previous studies have found that both MLL-AF9 and MN1-induced leukemias are dependent on *Dot1l*.^{14,25,26} MLL fusions misdirect DOT1L to the promoters of Hoxa cluster genes and *Meis1* leading to H3K79 methylation and constitutive activation of these genes.^{2,25,33} It is important to note that MN1 transformed HSC have high expression of HOXA9 irrespective of DOT1L expression.¹⁴ In support of this observation, we show that loss of MN1 can overcome the effect of MLL-AF9 and DOT1L-directed dysregulation of HOXA cluster genes and MEIS1.

The leukemogenicity of MLL-positive leukemias is dependent on genes such as the Mll wild-type allele, AF9, Dot1l, JMJD1C, PU.1, CBX8, Hoxa7, Hoxa9 and S6K1, which mediate the leukemogenic activity of MLL-AF9

predominantly by Dot1l gene activation.^{26,33,37-41} Our data provide additional insights into MLL-rearranged leukemia through the addition of MN1 as a crucial co-factor regulating the HOXA cluster, MEIS1 and their target genes. As U937 cells were moderately affected by MN1 deletion, we speculate that cells with high HOXA9/MEIS1 expression may also depend on MN1 like MLL-r cells. Therefore, we predict that MN1 is not only required for MLL-transformed leukemias, but for approximately 50% of AML cases, which show dysregulated expression of the clustered homeobox genes.

High MN1 expression has been reported as a poor prognostic marker in AML patients, and its overexpression induces ATRA resistance and AML in mouse cells.^{7-9,12,13,23} Also, it is most highly expressed in CMP, and scantily expressed in HSC and more differentiated cells.²³ Interestingly, induced MN1 expression cannot transform stem cells, as previously described,²³ and we hereby report that proliferation of CD34⁺ cells remain unaffected by depletion of MN1 expression. Hence, the expression characteristics of MN1 seem to be favorable for therapeutic targeting. A potential therapeutic index is indicated by lack of an inhibitory effect in normal stem/progenitor cells and a specific inhibitory effect in AML cells. Therefore, targeting MN1 by LNP/siRNA formulations holds a strong potential therapeutic avenue, as suggested by our previous proof-of-principle study in a PDX model of acute lymphoblastic leukemia *in vivo*.³²

In summary, we identify MN1 as an essential protein in MLL fusion gene leukemia *in vivo* and highlight its importance as a co-factor of the HOXA9/MEIS1 complex. An LNP/siRNA formulation against MN1 effectively inhibits the colony-forming potential of MLL-r primary AML cells, suggesting that MN1 is a valid and druggable target in AML.

Acknowledgments

We thank Silke Glowotz, Annett Reinsch, Basem Othman, Kerstin Goerlich, Martin Wichmann, Nadine Kattre and Renate Schottmann for technical help. We also thank Dr. Matthias Ballmaier and the staff of the Cell Sorting Core Facility of Hannover Medical School for their excellent service (supported in part by the Braukmann-Wittenberg-Herz-Stiftung).

Funding

This study was supported by the Rudolf-Bartling Stiftung, an ERC grant under the European Union's Horizon 2020 research and innovation program (n. 638035), Deutsche Krebshilfe (70112697) and DFG grants HE 5240/5-1, HE 5240/5-2, HE 5240/6-1 and HE 5240/6-2.

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