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The co-receptor Neuropilin-1 enhances proliferation in inv(16) acute myeloid leukemia via VEGF signaling

Mahesh Hegde¹, Mohd H. Ahmad¹, Roger Mulet Lazaro², Mayumi Sugita³, Rui Li¹, Kai Hu¹, Claudia Gebhard⁴, Monica L. Guzman³, John H. Bushweller⁵, Lihua J. Zhu¹, Michael Brehm⁶, Scot A. Wolfe¹, Ruud Delwel², Lucio H. Castilla^{1,✉}

¹Department of Molecular, Cell and Cancer Biology; University of Massachusetts Chan Medical School, 364 Plantation Street, Worcester, MA, USA.

²Department of Hematology, Erasmus University Medical Center and Onco Institute, Rotterdam, The Netherlands.

³Department of Medicine, Weill Cornell Medical College, 1300 York Ave, New York, NY, USA.

⁴Department of Internal Medicine III and Regensburg Centre for Interventional Immunology, University Hospital Regensburg, Regensburg, Germany.

⁵Department of Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, VA, USA.

⁶Program in Molecular Medicine, Diabetes Center of Excellence, University of Massachusetts Chan Medical School, 368 Plantation Street, Worcester, MA, USA.

Abstract

Oncogenic programs regulate the proliferation and maintenance of cancer stem cells, and can define pharmacologic dependencies. In acute myeloid leukemia (AML) with the chromosome inversion 16 (inv(16)), the fusion oncoprotein CBF β ::MYH11 regulates pathways associated with leukemia stem cell activity. Here we demonstrate that expression of Neuropilin-1 (NRP1) is regulated by the fusion oncoprotein, and promotes AML expansion. Mechanistically, we show that the *NRP1* locus has open chromatin in inv(16) AML, and that CBF β ::MYH11 modulates the local function of the transcription factors ERG, GATA2 and RUNX1 to sustain NRP1 levels. We found that ERG activates *NRP1* expression, and that CBF β ::MYH11 knockdown represses ERG expression, thereby allowing the repressive activity of GATA2/RUNX1 at three *NRP1* enhancers. Functionally, we demonstrate that NRP1 enhances the expansion of leukemic cells in vitro and in

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✉ Correspondence and requests for materials should be addressed to Lucio H. Castilla. Lucio.Castilla@umassmed.edu.

AUTHOR CONTRIBUTIONS

Conceptualization: MH and LHC. Methodology: MH, MHA, MS, MLG, MB, CG, SAW and LHC. Formal Analysis: LJZ, RL, KH, RML, CG, and RD. Investigation: MH, MHA, RML, MB, RL and LHC. Visualization: MH, MS, RL, KH, RML, RD and LHC. Supervision: RD, MLG and LHC. Writing—original draft: MH and LHC. Writing—review & editing: MH, MHA, RML, RL, LJZ, JHB, SAW, RD and LHC.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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mice, and that this activity is dependent on its VEGFR-associated FV/FVIII domain. Finally, we show that treatment with VEGF inhibitor axitinib reduces AML cell growth and delays median leukemia latency in vivo. Our findings reveal that the NRP1/VEGF axis mediates proliferation in inv(16) AML blasts, and suggest that targeting NRP1 function could be promising in combination AML therapy.

INTRODUCTION

Hematopoiesis is tightly regulated by the coordinated interaction of transcription factors and epigenetic complexes that define stem cell homeostasis and differentiation [1, 2]. Genetic mutations can rewire this integrated network to establish a new balance with unique transcription profiles that drive leukemia stem cell (LSC) self-renewal and cell viability [3]. In acute myeloid leukemia (AML), founding mutations in genes encoding transcription factors can subvert regulatory networks in the hematopoietic stem and progenitor cells (HSPCs) and drive leukemogenesis with specific transcription and epigenetic signatures [4, 5]. The chromosome 16 inversion inv(16)(p13q22), henceforth inv(16), is a founding mutation in AML that expresses the fusion transcript *CBFB::MYH11*, encoding the leukemia fusion protein CBF β ::MYH11 (also CBF β ::SMMHC). Inv(16) AML is associated with approximately 12% of pediatric and 5 to 9% of adult AML [6–8]. This fusion protein disrupts the function of the hematopoietic heterodimeric transcription factor RUNX1/CBF β , blocks myeloid differentiation and drives leukemia transformation [9].

Using a mouse model for inv(16) AML (*Cbfb*^{MYH11/+} knock in), we have demonstrated that CBF β ::MYH11 is necessary but not sufficient for transformation [10, 11]. Inv(16) AML results from the clonal expansion of HSPCs expressing CBF β ::MYH11 and “cooperating” mutations [12], frequently in components of signaling transduction pathways [13] that enhance LSC self-renewal [14]. Allelic expression of CBF β ::MYH11 in *Cbfb*^{MYH11/+} mice disrupts myelo-erythroid differentiation and creates pre-leukemic progenitor cells that progress to leukemia [15]. The viability of inv(16) AML blasts depend on CBF β ::MYH11 function following an oncogene addiction model, as targeted inhibition of CBF β ::MYH11/RUNX1 binding induces apoptosis in inv(16) AML [16].

CBF β ::MYH11 directs a specific integrated network of transcription factors and epigenetic signatures in AML [4, 5]. The hematopoietic heptad of transcription factors (RUNX1, ERG, GATA2, TAL1, FLI1, LYL1 and LMO2) play an important role in regulation of hematopoiesis [1], and form integrated networks in AML, including inv(16) AML [17, 18]. However, the mechanistic dynamics of how CBF β ::MYH11 associates with the heptad transcription factors in the regulation of specific pathways that control LSC function and viability remains poorly understood.

The transmembrane glycoprotein Neuropilin-1 (NRP1) is a co-receptor that binds to a variety of membrane receptors, enhancing their ligand-mediated signaling activity [19]. Its activity is mediated by two extracellular domains, the CUB domain that binds to semaphorins and the FV/VIII domain that binds to vascular-endothelial growth factor (VEGF) receptors. The NRP1 function has been implicated in a variety of biological processes, including immunity, vascular development and regeneration, and hematopoietic

reconstitution [20, 21]. In cancer, NRP1 expression has been associated with poor outcome in solid tumors [22–24], and it has been found to predict relapse in cytogenetically normal AML [25, 26]. However, the role of NRP1 in AML proliferation and maintenance is not known.

Here we demonstrate that CBFβ::MYH11 expression signature is associated with NRP1 and the cytokine membrane receptors CSF3R, FGFR1, RET, FLT3, and FLT4. Utilizing CRISPR/Cas9 editing and genomic approaches, we show that the fusion protein controls NRP1 expression at two local enhancer elements through the opposing function of ERG as an activator and RUNX1 and GATA2 as repressors. Furthermore, we show that the expression of a subset of CBFβ::MYH11 targets is mediated by NRP1 activity at the transcriptional level. Finally, we demonstrate the sensitivity of inv(16) AML cells for treatment with VEGF inhibitors. This study defines the complexity of CBFβ::MYH11 driven pathways that maintain the viability of the leukemic blasts, and identifies NRP1/VEGF as a potential pharmacologic target for therapeutic intervention in inv(16) AML.

MATERIAL AND METHODS

Cell assays

Cell lines. The inv(16) AML ME-1 cell line (DSMZ, cat. # ACC-537) was cultured in RPMI-1640 (Thermo Fisher Scientific, cat. # A10491–01) with 20% FBS. K562 (t(9;22) erythroleukemia, ATCC, cat. # CCL-243), THP-1 (t(9;11) AML, ATCC, cat. # TIB-202), Mono-MAC-6 (t(9;11) AML), MOLM-13 (t(9;11) AML), RPMI-8402 (t(11;14) T-ALL) and NB4 (t(15;17) APL, DSMZ, cat. # ACC-207) cells were cultured in RPMI-1640 (Thermo Fisher Scientific, cat. # 11875–093) with 10% FBS. SEM (t(4;11) ALL) cells were cultured in IMDM (Gibco, cat. # 12440053) with 10% FBS, and the HEK293T/17 cells (Human embryonic kidney; ATCC, cat. # CRL-11268) were cultured in DMEM (Thermo Fisher Scientific, cat. # 11995073) with 10% FBS. The cultures were maintained with 100 U/mL Penicillin, 100 mg/mL Streptomycin (Thermo Fisher Scientific, cat. # 15140122) and 1 μL/mL Plasmocin (Invivogen, cat. # ant-mpp).

Primary hematopoietic cell cultures. Human CD34⁺ HSPCs were isolated from cord blood samples utilizing the CD34 Microbead kit from Miltenyi Biotec, following protocols approved by The Ethics Committee of the University of Massachusetts Chan Medical School. Patient AML samples were received from AML collections (UMass, UPENN Tissue-Banks and Erasmus MC). Samples were cultured in StemSpan SFEM II (STEMCELL Technologies, cat. # 09605) serum-free expansion medium, 100 U/mL Penicillin and 100 mg/mL Streptomycin supplemented with 10 ng/mL human recombinant TPO (Peprotech, cat. # 300–18), 10 ng/mL human recombinant FLT3L (Peprotech, cat. # 300–19), 100 ng/mL human recombinant SCF (Peprotech, cat. # 300–07), 10 ng/mL human recombinant IL-3 (Peprotech, cat. # 200–03), and 20 ng/mL human recombinant IL-6 (Peprotech, cat. # 200–06).

Primary mouse leukemic cells were generated in the laboratory, utilizing *Cbfb*^{MYH11^{+/+}};*Nras*^{G12D^{+/+}} [14] or *MLL::AF9* transduction models [27], and cultured in StemSpan SFEM (STEMCELL Technologies, cat. # 09650) with 100 U/mL Penicillin

and 100 mg/mL Streptomycin supplemented with 10 ng/mL murine recombinant IL-3 (Peprotech, cat. # 213–13) and 50 ng/mL murine recombinant SCF (Peprotech, cat. # 250–03).

shRNA cloning and transduction. The lentiviral vectors for shRNA-knockdown were constructed by replacing the *CMV-MCS* region of the *pCDH-CMV-MCS-EF1-copGFP-P2A-Puro* lentiviral-vector with a fragment including the *U6 promoter* and hairpin insertion sites (*AgeI* and *EcoRI*), resulting in the *pCDH-U6-CS-EF1-copGFP-P2A-Puro* vector. The shRNA vectors were generated by first annealing each shRNA hairpin oligo, and cloned into *AgeI* and *EcoRI* sites. The shRNA lentiviral particles were prepared in HEK293T/17 cells along with packaging plasmids *psPAX2* and *pMD2.G* (gift from Didier Trono; Addgene, cat. # 12260 and #12259 respectively). The lentivirus supernatants were concentrated (Lenti-X™ concentrator, Takara Bio, cat. # 631231) and transduced twice in ME-1 cells, 24 h apart, by spin infection, and cultured for 5 days in Puromycin (5 µg/mL, Invivogen, cat. # ant-pr-1). Live cells were isolated using Ficoll-gradient centrifugation (Cytiva Life Sciences, cat. # 17144002). Alternatively, transduced ME-1 cells were cultured in complete media, and GFP-positive cells were estimated by flow cytometry (BD LSRII) every 4 days.

Statistical analysis

For mouse leukemia survival analysis, the leukemia latency and *p*-values were estimated using Log-Rank test using Survival package in R. Bar diagrams represented with standard deviation of triplicates and significance was calculated using an unpaired t test, **p* < 0.05 or ***p* < 0.005. The specific *P* values for each figure are given in Supplementary Table S5.

Additional methods section can be found in the Supplementary Information document.

RESULTS

CBFβ::MYH11 regulates expression of kinase signaling receptors

To study the role of CBFβ::MYH11 target genes in AML maintenance, we first characterized a cell-based system with CBFβ::MYH11 knockdown (shRNA-Puro-GFP) in ME-1 cells [inv(16) AML] using 3 *MYH11* shRNAs. This knockdown significantly reduced cell proliferation when cultured after puromycin selection or when traced for GFP for 40 days (Fig. 1A, Supplementary Fig. S1A, B and Supplementary Table S1). Cellular analysis at different timepoints revealed no significant changes in viability or differentiation in cell morphology at day 14 (Supplementary Fig. S1C–E). However, cell cycle analysis showed a significant increase in cells in G0/G1 phase at day 21 (Supplementary Fig. S1F), followed by an increase in apoptosis (Annexin V, 7AAD staining, and caspase-3 cleavage; Fig. 1B). These assays confirm that CBFβ::MYH11 drives proliferation and survival of inv(16) AML cells.

We utilized transcriptome profiling (RNA-sequencing) in day 7 *MYH11shRNA4* ME-1 cells to identify genes that are regulated by CBFβ::MYH11. Differential gene expression analysis (DESeq2) defined 438 differentially expressed genes (DEGs; FDR < 0.05; Log₂FC > 1; Fig. 1C). Gene Set Enrichment Analysis (GSEA) identified a significant negative correlation of the DEGs with the inv(16) AML gene set signature (NES: −4.05, FDR q-value < 0.001;

Fig. 1D). Gene Ontology (GO) Molecular Function analysis revealed a significant inhibition of genes encoding components of cytokine receptor signaling pathways, predominantly VEGF-activated receptor activity and receptor kinase activity, and including pathways such as PI3-AKT, MAPK, Ras, Jak-STAT, and TGF β (Fig. 1E, F). These signature pathways highlight the dependency of these pathways to CBF β ::MYH11 expression in inv(16) AML.

NRP1 expression is regulated by CBF β ::MYH11 in inv(16) AML

To identify dependencies critical for leukemia maintenance in inv(16) AML, we focused in the analysis of the top deregulated genes encoding membrane receptors (*NRP1*, *FLT4*, *CSF3R*, *FLT3*, *FGFR1*, and *RET*). First, we validated their expression and of two control genes (KIT and IGF1R) in *MYH11shRNA* ME-1 cells (Fig. 2A and Supplementary Table S1). Second, analysis of AML patient gene expression datasets revealed the *NRP1* expression was significantly higher in inv(16) AML when compared to other AML subtypes and to normal HSPCs (Fig. 2B and Supplementary Fig. S2A). Third, *NRP1* expression was higher in CBF β ::MYH11 expressing AML cells when compared to non-CBF β ::MYH11 expressing cells, in a panel of AML cell lines, in patient AML cells, and in mouse *Cbfb*^{MYH11^{+/+}} leukemic cells (Fig. 2C, D, Supplementary Fig. S2B, C, and Supplementary Table S2). Fourth, NRP1 protein levels in membrane (median fluorescent intensity, MFI) of *MYH11-shRNA* transduced ME-1 cells or gene edited (using fusion protein specific sgRNAs) inv(16) AML patient samples were significantly reduced (Fig. 2E, F and Supplementary Fig. S2D). These results demonstrate that NRP1 expression is regulated by CBF β ::MYH11 in AML.

Considering that CBF β ::MYH11 reduces RUNX1 function in AML [10], we tested whether the deregulation of RUNX1 modulates *NRP1* expression in inv(16) AML. *NRP1* expression was significantly increased in ME-1 cells transduced with *RUNX1-shRNA*, and in cells treated the small molecule AI-10-104 (CBF β inhibitor) but not with AI-10-88 (inactive analog; ref. [28]), showing that RUNX1 represses *NRP1* expression in inv(16) AML (Fig. 2G, H). Furthermore, treatment with AI-10-104 restored *NRP1* expression in cells transduced with *MYH11shRNAs* (Fig. 2I). Conversely, *NRP1* expression was not affected by RUNX1 knockdown or after treatment with CBF β inhibitor in MLL::AF9 expressing THP1 cells (Supplementary Fig. S2E–G). Finally, analysis of published RNA-seq datasets of preleukemic *Cbfb*^{MYH11^{+/+}} bone marrow cells showed that *Nrp1* levels were increased 4-fold in mouse bone marrow cells after allelic activation of *CBFB::MYH11* expression, and that loss of *Runx1* expression further increased *Nrp1* levels 2-fold in these cells (Supplementary Fig. S2H, I, refs. [9, 29]). These results demonstrate that RUNX1 mediates repression of *NRP1* levels in inv(16) AML.

CBF β ::MYH11/RUNX1 complex regulates *NRP1* expression at local enhancers in inv(16) AML

To define sequences that regulate *NRP1* locus expression in inv(16) AML, we first analyzed the RUNX1 occupancy peaks in a 400 kb genomic region (200 kb up- and down-stream of the transcription start site, TSS) utilizing RUNX1 ChIP-seq dataset in ME-1 cells treated with *MYH11shRNA* [30]. We found four RUNX1 peaks as candidate regulatory elements (RE) in this region, located at +74 kb (RE+74), +1 kb (RE+1), -30 kb (RE-30) and -174 kb

(RE -174) from the TSS (Supplementary Fig. S3A). These REs contain one to six RUNX1 consensus binding sites (Supplementary Table S3). Analysis of RUNX1 occupancy by ChIP-qPCR confirmed a significant increase at *RE+1*, *RE-30* and *RE-174* after CBFβ::MYH11 knockdown, while remaining unchanged at *RE+74* and the negative control (NC) sites (Fig. 3A).

We analyzed the chromatin accessibility at the *NRP1* locus in a set of inv(16) and non-inv(16) AML patient samples ($n = 8/\text{group}$) for which expression data were also available [31]. Expression analysis validated the strong correlation between *NRP1* and *CBFB::MYH11* transcript expression in the inv(16) AML group (Fig. 3B, Pearson's correlation analysis, $p\text{-value} = 1.37\text{e-}07$, and $R = 0.923$). Differential peak analysis of active chromatin revealed a significant increase in H3K27Ac-ChIPseq and ATAC-seq peaks in inv(16) AML when compared to non-inv(16) AML samples (Fig. 3C, D, Supplementary Figs. S3B and S3C; Supplementary Table S4). These results define the three RUNX1-dependent REs (*RE+1*, *RE-30* and *RE-174*) as active enhancers in the *NRP1* locus. Conversely, our results suggest that RE +74 is not RUNX1 dependent in this context, as it has low H3K27Ac marks and RUNX1 occupancy is unchanged.

We utilized CRISPR/Cas9-mediated editing to investigate whether the three REs are functionally active in regulating *NRP1* expression. Notably, single RE deletions (511–685 bp) encompassing their RUNX1 binding site(s) significantly reduced expression of *NRP1* transcript and protein (Fig. 3E, F, and Supplementary Tables S1 and S3), even in cases with incomplete editing efficiency (Fig. S3D). These results functionally demonstrate that these REs have enhancer activity regulating *NRP1* expression in inv(16) AML.

NRP1 expression is regulated by ERG and RUNX1/GATA2 complex in inv(16) AML

Considering that the dynamic interaction of the heptad of transcription factors control hematopoietic differentiation and define integrated circuits in AML [1, 32], we hypothesized that CBFβ::MYH11 may regulate the expression and/or function of these transcription factors. Expression analysis of the seven transcription factors showed that CBFβ::MYH11 knockdown significantly reduced *ERG* (−2.39 fold) and increased *GATA2* (+3.34 fold) expression (Fig. 4A and Supplementary Fig. S4A). Furthermore, *ERG* knockdown significantly inhibited *NRP1* expression (Fig. 4B) while *GATA2* knockdown increased *NRP1* expression in ME-1 cells (Fig. 4C). We validated *GATA2* repressive function in *NRP1* expression by analyzing two published systems. First, *Gata2* haploinsufficiency increased *Nrp1* levels 3 fold in *Gata2*^{+/-} *Cbfb*^{MYH11/+} mouse leukemic cells (Supplementary Fig. S4B; ref. [33]). Second, *Gata2* expression decreased 4.7-fold and *Nrp1* levels increased over 4-fold in CMP/GMP hematopoietic cells sorted from *Gata2* −77 kb enhancer-deleted E14.5 fetal liver (Supplementary Fig. S4C; ref. [34]). Together, these results support the model that *ERG* acts as an activator of *NRP1* expression in inv(16) AML cells, while *GATA2*/RUNX1 function as a repressor complex.

The REs have multiple consensus binding sites for these three transcription factors, varying from 1 to 6 sites (Supplementary Table S3). To evaluate if these transcription factors occupy the *NRP1* enhancers, we analyzed their occupancy using ChIP-qPCR. We found that *ERG* binds to the four REs, and that this binding is reduced after *MYH11* knockdown (Fig.

4D), presumably because of its reduced expression. GATA2 occupies the RE+1, RE-30 and RE-174, but not the RE+74 enhancer (Fig. 4E). Interestingly, its occupancy is significantly increased after *MYH11*-knockdown at the three REs. Considering that the three REs have two to three GATA binding sites, it is possible that CBF β ::MYH11 knockdown may induce an increase in GATA2 occupancy at some of these sites.

Since *RE+1* maps close to the promoter region, we tested whether *RE+1* functions as a discrete regulatory unit. This enhancer is located in intron 1, spanning 655 bp (+755 bp to +1410 bp) of the *TSS* (Supplementary Fig. S4D), based on the published heptad transcription factor ChIP-seq peaks in the *NRP1* promoter region [18], we defined a 1035 bp proximal *P1* region and a 792 bp distal *P2* region. The ChIP-qPCR analysis using the three regions independently revealed that ERG was bound at RE+1 and the promoter in inv(16) AML cells, and its occupancy was significantly reduced at RE+1 and *P2*, after *MYH11* *shRNA* treatment (Fig. 4F). Conversely, RUNX1 and GATA2 binding were significantly increased only at RE+1 after CBF β ::MYH11 knockdown (Fig. 4G, H). These results reveal that *RE+1* functions as a discrete enhancer in which RUNX1/GATA2 dock after CBF β ::MYH11 knockdown.

Analysis of ChIP-sequencing data for the other heptad transcription factors (TAL1, FLI1, LMO2, and LYL1) in CD34⁺ human hematopoietic progenitor cells and in ME-1 cells revealed that they do not bind to the *NRP1* locus (Supplementary Fig. S4E). Based on these results, we propose a model by which ERG activity is essential for NRP1 expression in inv(16) AML, bridging the activity between these enhancers and the promoter. In this context, CBF β ::MYH11 inhibits RUNX1/GATA2 repressive function to maintain NRP1 expression. CBF β ::MYH11-knockdown reduces ERG expression and increases RUNX1 availability thereby promoting the occupancy of the repressors RUNX1 and GATA2 at *RE+1*, RE-30 and *RE-174* enhancers and inhibiting NRP1 expression (Fig. 4I).

NRP1 enhances leukemia proliferation in inv(16) AML

To test whether NRP1 function is required for inv(16) AML cell viability, we investigated the growth of ME-1 cells transduced with two *NRP1-shRNA*/GFP lentiviruses, which reduced NRP1 transcript and protein levels (Supplementary Figs. S5A and S5B). *NRP1-shRNA* transduced ME-1 cells showed significant reduction in proliferation and increased apoptotic cell death, as evidenced by increased caspase-3 cleavage (Fig. 5A, B). The dependency of inv(16) AML cells in NRP1 expression was validated in ME-1 cells with CRISPR/Cas9-mediated NRP1 deletion (sgRNA9 + 11 and sgRNA10 + 12; Fig. 5C, Supplementary Figs. S5C and S5D).

Next, we tested the dependency of primary mouse leukemias to NRP1 in vivo. The expression of Nrp1 in *Cbfb*^{MYH11/+} mouse leukemic cells [14] was significantly higher than in *MLL::AF9*-expressing cells (Supplementary Figs. S5E and S5G). Upon CRISPR/Cas9-mediated deletion of *Nrp1* gene exons 3 and 4, the Nrp1 MFI was significantly reduced 72% and 81% in *Cbfb*^{MYH11/+} leukemic cells and modestly changed in *MLL::AF9* leukemic cells (Supplementary Figs. S5E and S5G). In vitro analysis of the colony forming unit (CFU) capacity in *Nrp1*-edited leukemic cells showed a significant reduction (60%) in CFUs in *Cbfb*^{MYH11/+} cells and in the colony size (Fig. 5D and Supplementary Fig.

S5F). In contrast, the CFU capacity of *Nrp1*-edited *MLL::AF9* leukemic cells was reduced 20% (Supplementary Fig. S5F), with similar colony size as of the *Rosa* control group (Supplementary Fig. S5H).

In vivo, the median leukemia latency in mice transplanted with *Cbfb^{MYH11}*⁺ leukemic cells pre-edited with *NRP1*-sgRNAs was significantly delayed (*Rosa*-sgRNAs: 29 days, *NRP1sg1* + 4: 41 days, and *NRP1sg2* + 3: 42 days; $p = 0.002$; Fig. 5E). Conversely, the median leukemia latency in mice transplanted with *Nrp1*-edited *MLL::AF9* cells was not changed when compared to *Rosa* control group (*Rosa*: 39 days, *sg1* + 4: 41 days, and *sg2* + 3: 41 days; Fig. 5G). These results demonstrate that NRP1 expression and function increase the leukemogenic potential in inv(16) AML while being dispensable in *MLL::AF9* associated AML.

Inv(16) AML is sensitive to VEGF receptor activity

As a co-receptor, NRP1 enhances the activity of membrane signal transduction receptors in cancer, including TGF β RI and RII, VEGFR2, growth factor receptors HGF and PDGFR, and Plexins [19]. However, despite the multiple implications of NRP1 function in normal and tumor biology, its function in AML is not known. To this end, we analyzed the transcription profile of ME-1 cells treated with *NRP1*-shRNA by RNA-seq. We identified 503 up- and 189-down-regulated genes (FDR < 0.05, Log₂FC > 0.585; Supplementary Fig. S6A).

Gene Set Enrichment Analysis showed that *NRP1*-knockdown was significantly correlated with the “apoptosis” gene set (Supplementary Fig. S6B), supporting the pro-apoptotic functional results described above. GO analysis for molecular function identified significant association with “transmembrane signaling receptor activity” and “signaling receptor activity” functions (Fig. 6A), which were also identified by *MYH11* knockdown.

The NRP1 coreceptor enhances receptor signal transduction through one of two extracellular domains: the CUB domain enhances semaphoring/plexin1 signaling and the FV/VIII domain enhances VEGF receptor signaling. To define the domain that mediates NRP1 activity in inv(16) AML, we tested the proliferation of a panel of human leukemia cell lines after treatment with blocking antibodies with specificity for the CUB domain (anti-NRP1^A) or the FV/FVIII domain (anti-NRP1^B) [35]. ME-1 cells were the only cells sensitive to anti-NRP1^B, while the CUB domain did not seem to be active (Fig. 6B and Supplementary Fig. S6C). These results demonstrate that the VEGF associated FV/VIII domain mediates NRP1 activity in the viability of inv(16) AML.

To elucidate whether this axis can reveal a pharmacologic sensitivity, we analyzed the drug dependencies of the BeatAML2 dataset, for which drug response results are available [36]. The set of 671 AML samples were divided into four groups based on their NRP1 levels, and analyzed for their sensitivity to drug treatment (Fig. 6C). Of the 41 inv(16) AML samples tested, 38 clustered in the High group, while the remaining 3 were in the Medium group. The NRP1-high group showed significantly strong dependency to VEGF inhibitors axitinib, linifanib, motesanib and cediranib (p value < 0.05, Fig. 6D and Supplementary Table S5), highlighting the role of NRP1/VEGF function in inv(16) AML proliferation. In addition,

this analysis revealed that inv(16) AML samples are significantly dependent on inhibitors for components of MEK, AKT and mTOR signal transduction pathways, pathways that are downstream of the NRP1/VEGF axis (Supplementary Fig. S6D).

We tested the sensitivity of inv(16) AML primary samples to treatment with axitinib, a VEGF inhibitor that is currently in clinical trials for treatment of other cancers. The median inhibitory concentration (IC₅₀) of axitinib treatment in inv(16) AML patient samples in dose response assays was 5 times lower than that of CD34⁺ HSPCs or normal karyotype AML samples ($n = 3/\text{group}$; Fig. 6E, F and Supplementary Fig. S6E). In addition, mouse *Cbfb*^{MYH11/+} leukemic cells showed high sensitivity to axitinib treatment in vitro (IC₅₀: 38 nM; Fig. 6G). Finally, we tested the efficacy of axitinib treatment in vivo using a transplantation model (Supplementary Fig. S6F). Mice transplanted with 2×10^3 *Cbfb*^{MYH11/+} leukemic cells were treated with axitinib for 16 days. Analysis of leukemic (c-kit⁺) cells in peripheral blood 4 weeks after treatment showed that axitinib treatment induced a significant reduction in the leukemia burden (Fig. 6H). Furthermore, the median leukemia latency was significantly delayed from 37.5 to 57.5 days ($p = 0.016$; Fig. 6I). These in vitro and in vivo results in human AML cells and in mice demonstrate that inhibition of the VEGF receptor has anti-proliferative role in inv(16) AML.

DISCUSSION

The identification of signaling pathways activated by founding oncoproteins provide a promising platform for targeted therapies in cancer. The CBFβ::MYH11 fusion protein predisposes to leukemogenesis and to the maintenance of LSC activity [37]. This study reveals that NRP1/VEGF signaling promotes proliferation and survival in inv(16) AML.

This study shows that CBFβ::MYH11 enhances the expression of the co-receptor NRP1, and that this regulation correlates with the deregulation of the sub-circuitry of hematopoietic transcription factors ERG, RUNX1 and GATA2 at *NRP1* local enhancers. The expression of ERG correlates with LSC programs and is associated with poor prognosis in normal karyotype AML [17, 38]. Accordingly, we show that ERG plays an important oncogenic function in inv(16) AML by mediating the activation of NRP1 expression.

The mechanism by which CBFβ::MYH11 regulates this locus probably also implicates changes in chromatin dynamics. CBFβ/RUNX1 regulates gene expression in T cells and megakaryocytes in association with the polycomb repressive complex 1 (PRC1) [39]. We have shown that pharmacologic inhibition of CBFβ::MYH11 triggers RUNX1/PRC1 mediated exclusion of chromatin activating complex SWI/SNF at the MYC super-enhancer BDME, and subsequent repression of MYC expression [1]. The PRC1 involvement in inv(16) AML was later validated at other genomic loci [40]. It is therefore likely that RUNX1/GATA2 repressive function may also involve PRC1 chromatin complex at the NRP1 locus. This regulatory dynamics is consistent with the tumor suppressor function of these transcription factors in familial and somatic AML [41]. In addition, it correlates with the finding that *GATA2* haploinsufficiency accelerates leukemogenesis in inv3q21q26 AML and in *Cbfb*^{MYH11/+} mice [33, 42].

NRP1 does not have a cytosolic domain that triggers signal transduction. As a co-receptor, it binds to membrane receptors and enhance their activity. Functionally, we demonstrate that in inv(16) AML, NRP1 co-receptor activity regulates the expression of membrane receptors via its FV/VIII domain. This domain binds to and enhances the activity of the VEGF receptors, a signal transduction pathway associated with proliferation and survival of AML blasts [43, 44]. Pharmacologically, we demonstrate that inv(16) AML has a dependency to the VEGF inhibitor axitinib, which is in clinical trials for the treatment of solid tumors. In addition, our study also shows that inv(16) AML are also highly sensitive to NRP1 activated pathways, including PI3K/AKT and MAPK/ERK.

In conclusion, we demonstrate that NRP1/VEGF signaling is regulated by CBF β ::MYH11, and that enhances proliferative pathways in inv(16) AML. These results support the use of NRP1 inhibitors in combination therapy protocols for the reduction of LSCs in inv(16) AML. Finally, since NRP1 expression correlates with poor prognosis in AML [45], the therapeutic value of NRP1 inhibitors may extend to other AML subtypes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY

The data generated for this study are available in the main text or in the Supplementary Material files of the manuscript. The RNA-sequencing data generated are available on NCBI Gene Expression Omnibus (GEO) under accession code GSE215613.

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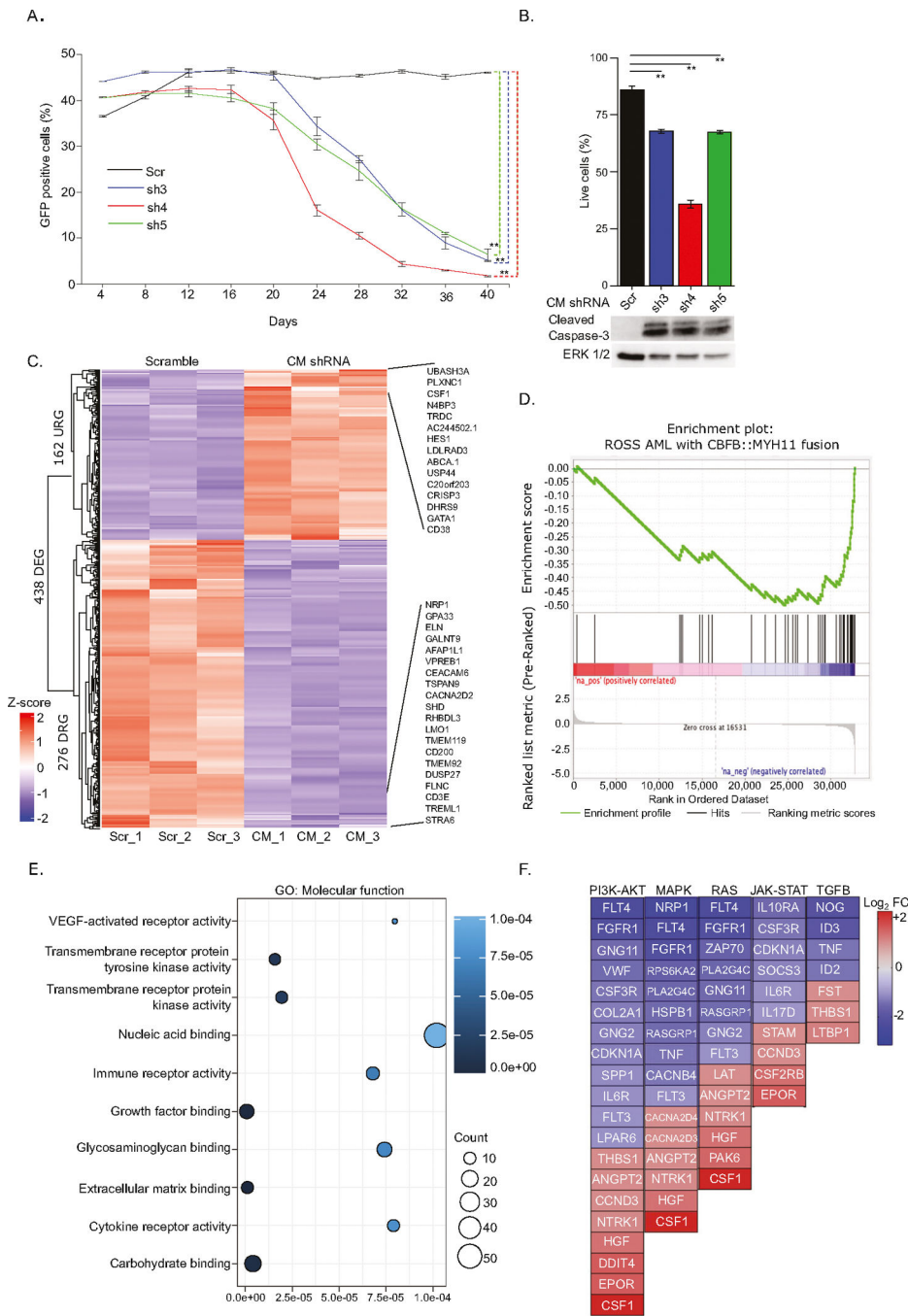


Fig. 1. CBFb::MYH11 knockdown deregulates expression of signal transduction genes and induces apoptosis in *inv(16)* AML cells.
A Time-course curve of GFP⁺ ME-1 cells transduced with 3 *MYH11* shRNAs (sh3-sh5) and control (Scr), with LD₅₀*MYH11* = 29.5, 22.7 and 30.2 days, respectively (*n* = 3). **B** Estimation of cell viability (Annexin V⁻, 7AAD⁻) and immunoblotting assay for cleaved caspase3 antibody, at day 28 post *shRNA* transduction. Significance was calculated using an unpaired t-test, ***p* < 0.005 (*n* = 3). **C** Heatmap representing intensity of differentially expressed genes on day 7 in Scramble (Scr) and *MYH11*sh4 (CM) shRNAs, experiments are

shown in biological triplicates ($L_2FC \geq 1$, $FDR \leq 0.05$). Top upregulated or downregulated genes are shown. **D** GSEA showing the correlation with *CBFB::MYH11* AML. **E** GO analysis of DEGs. **F** Heat map representation of signal transduction pathways gene expression affected by *MYH11*shRNA treatment.

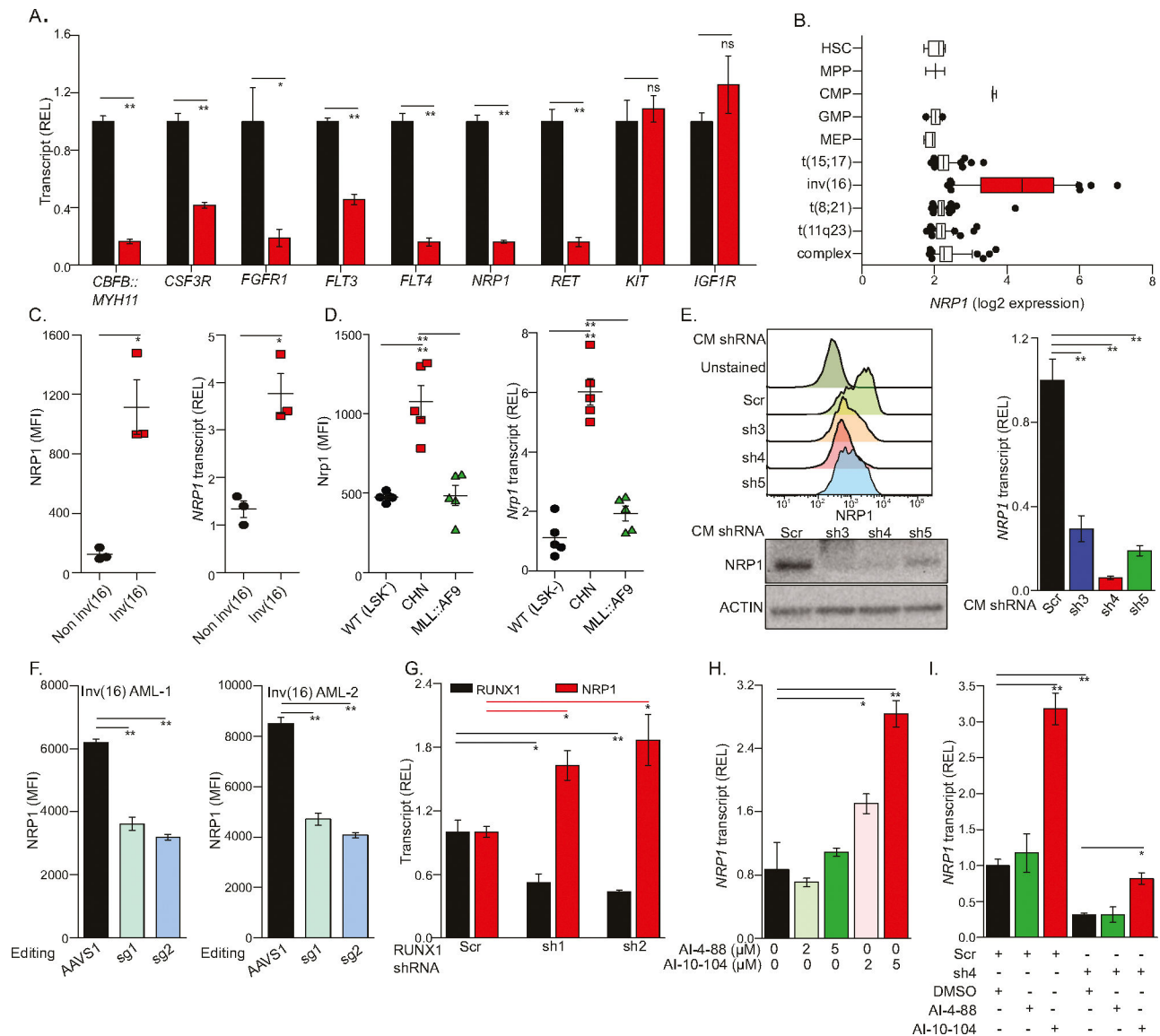


Fig. 2. NRP1 expression is regulated by CBFβ::MYH11/RUNX1 in inv(16) AML.

A Expression analysis of signaling receptors in ME-1 cells transduced with *Scr*- (black) or *MYH11*-sh4RNA (red). **B** *NRP1* expression in a panel of normal blood cells (GSE42519) and patient AML samples (GSE13159) (www.bloodspot.eu). **C, D** NRP1 surface expression (left; mean fluorescence intensity, MFI) and transcript levels (right) in patient AML samples (**C**), and in wild-type bone marrow LKS- (WT), and *Cbfb*^{MYH11^{+/+}}; *Nras*^{+/G12D} (CHN) and *MLL::AF9* mouse leukemic cells (**D**). Each experiment was done in triplicate, and standard deviation of three to five samples is shown. **E** NRP1 protein levels by FACS and immunoblotting (left), and *NRP1* transcript levels (right) in ME-1 cells transduced with 3 *MYH11* shRNAs (sh3–sh5) and *Scr* control. **F** NRP1 levels (MFI) calculated by FACS analysis in inv(16) AML patient samples edited with *CBFB::MYH11* guide RNAs (sg1- and sg2-*MYH11* or sg-*AAVS1*). **G** *NRP1* and *RUNX1* transcript level in ME-1 cells transduced with *RUNX1* shRNA (sh1 and sh2) and *Scr*-shRNA. **H, I** *NRP1* transcript level in ME-1

cells treated with AI-10–104 (0, 2 and 5 μ M) or AI-4–88 (inactive) for 24 h (**H**), or after transduction with *Scr*- or *MYH11sh4*-RNA (**I**). Bar plots are represented with error bars of standard deviation in triplicate; significance was calculated using an unpaired t-test, * $p < 0.05$ or ** $p < 0.005$.

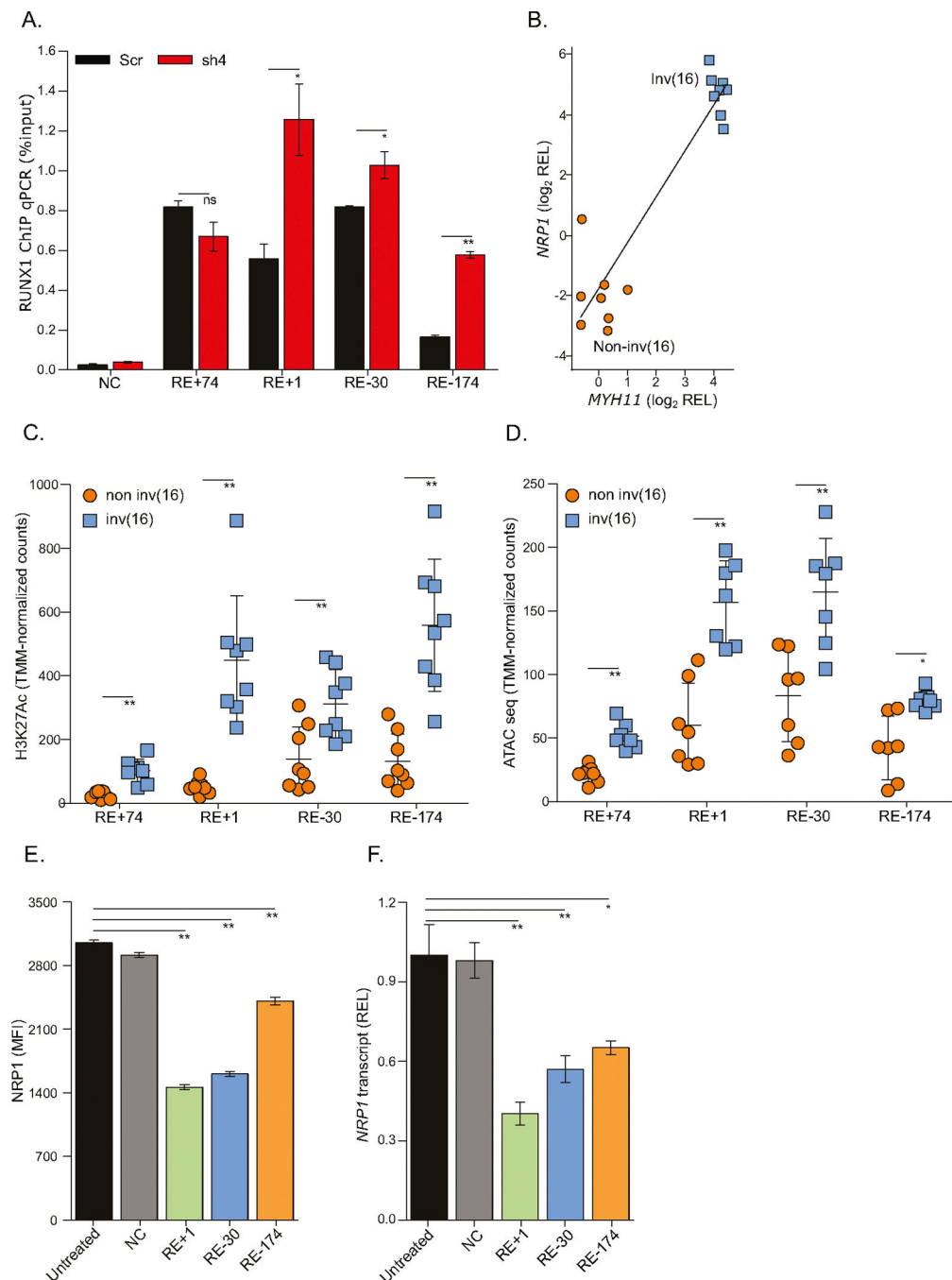


Fig. 3. Identification of NRP1 regulatory elements in *inv(16)* AML cells.

A Analysis of RUNX1 occupancy (ChIP-qPCR) at four regulatory elements (REs) of the *NRP1* locus in ME-1 cells transduced with *MYH11sh4*- or *Scr-shRNA*. **B** Correlation between *MYH11* and *NRP1* expression in the patient samples used in this H3K27Ac ChIP and ATAC sequencing study. **C** Quantification of H3K27Ac at REs in primary *inv(16)* and non-*inv(16)* AML samples ($n = 8$). **D** Quantification of ATAC sequencing peaks at REs in primary *inv(16)* and non-*inv(16)* AML samples ($n = 7$). NRP1 protein (**E**) and transcript (**F**) levels in single element deleted ME-1 cells, by SpCas9-editing (NC, RE+1, RE-30 and

RE-174). Bar graphs are represented with standard deviation of triplicates and significance was calculated using unpaired t-test, $*p < 0.05$ or $***p < 0.005$.

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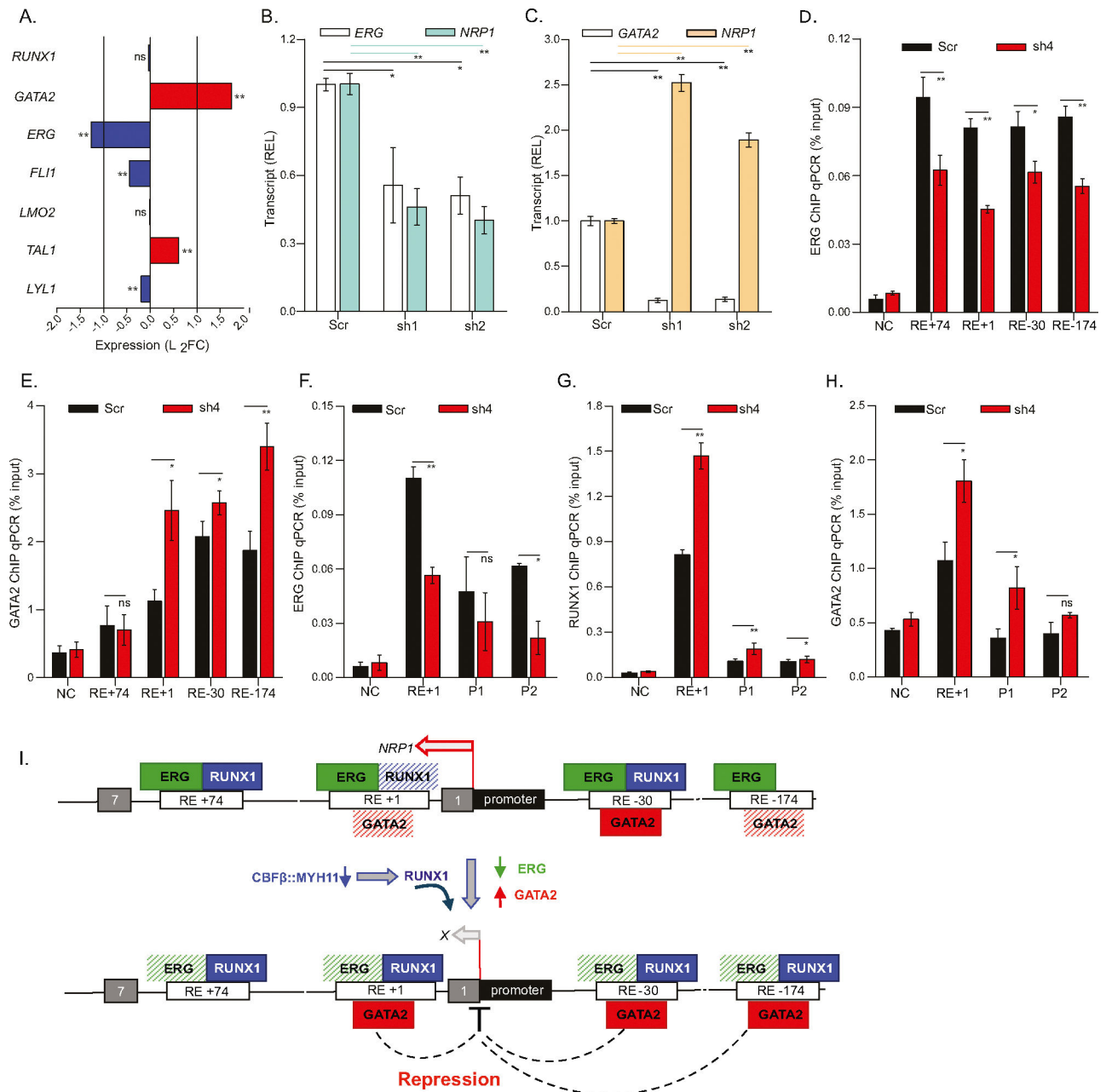


Fig. 4. Regulation of the *NRP1* locus in *inv(16)* AML.

A Fold change (log2) expression of heptad transcription factors (RNA-seq) after *MYH11*-shRNA treatment in ME-1 cells. **B** *NRP1* and *ERG* transcript levels in ME-1 cells transduced with *ERG*- (sh1 and sh2) and *Scr*-shRNA. **C** *NRP1* and *GATA2* transcript levels in ME-1 cells transduced with *GATA2*- (sh1 and sh2) and *Scr*-shRNA. **D**, **E** *ERG* and *GATA2* occupancy (ChIP-qPCR) at *NRP1* sites in cells transduced with *MYH11*sh4- and *Scr*-shRNAs. **F**, **G** and **H** *ERG*, *RUNX1* and *GATA2* occupancy (ChIP-qPCR) at NC, RE+1 and promoter regions in *MYH11*sh4- and *Scr*-shRNA treated ME-1 cells. **I** Proposed model for the regulation of *NRP1* expression in *inv(16)* AML, before and after *CBFβ::MYH11* knockdown. The *NRP1* gene is depicted in black, with REs (open boxes), exons (gray

boxes), and promoter region (black box). Transcription factors RUNX1 (blue), ERG (green) and GATA2 (red) are shown. Reduced occupancy (diagonal stripes). Arc (black dashed) lines depict the repressive function of REs on NRP1 promoter. Bar plots are represented with standard deviation of triplicates and significance was calculated using an unpaired t test, $*p < 0.05$ or $**p < 0.005$.

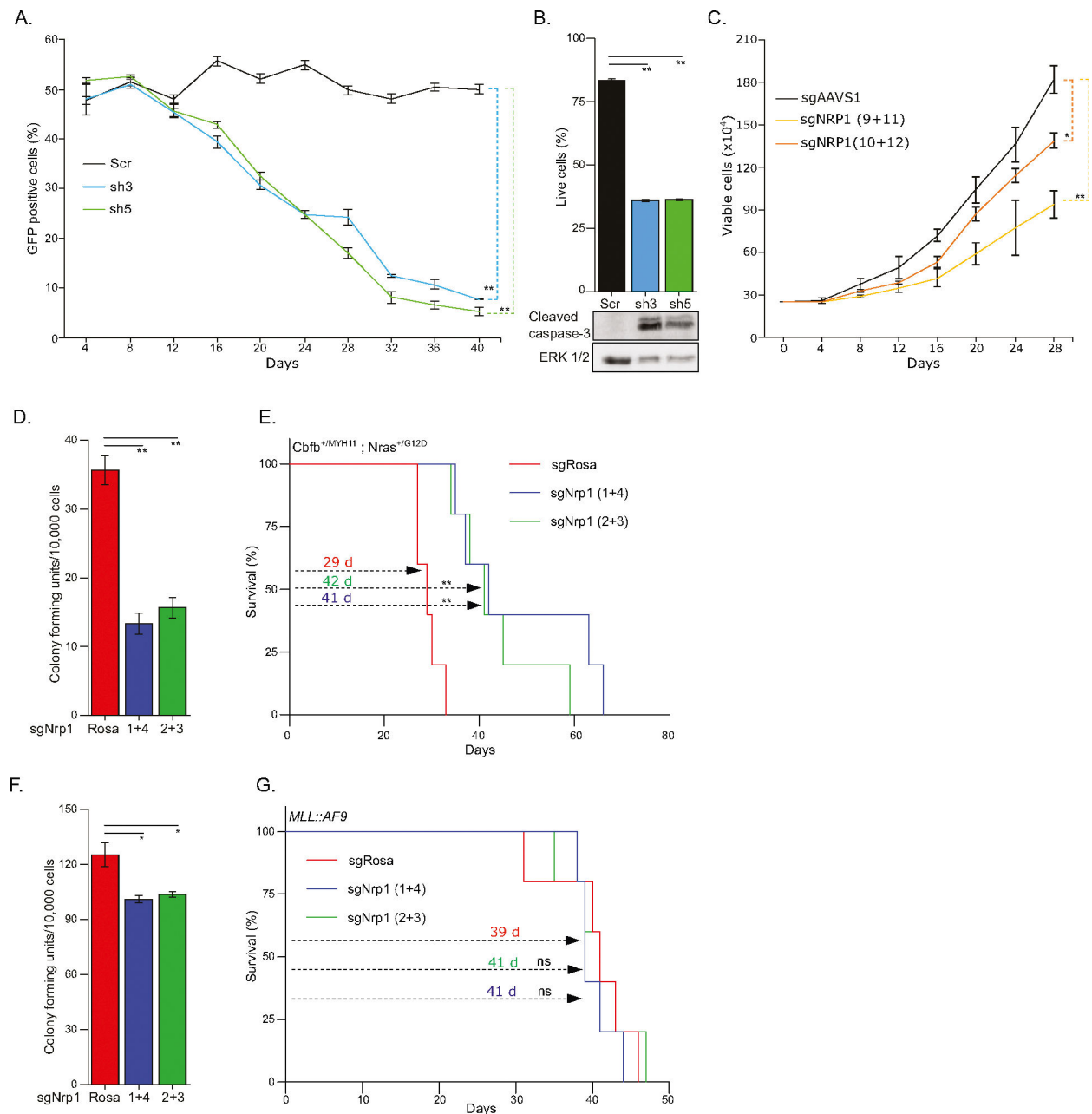


Fig. 5. NRP1 activity is essential for the expansion of inv(16) AML cells.

A Growth curve of ME-1 cells transduced with GFP⁺ *NRP1*-sh3 and sh5 and *Scr*-shRNAs. **B** Estimation of ME-1 cell viability (Annexin V⁻, 7AAD⁻) by flow cytometry (top) and immunoblotting assay for cleaved caspase3 antibody (bottom), 28 days after *NRP1*-shRNA transduction. **C** Growth curve of ME-1 cells transfected with *AAVS1* or *NRP1* sgRNA/SpCas9 electroporation ($n = 3$). **D** CFU assay of *Cbfb*^{MYH11/+}; *Nras*^{+/G12D} leukemic cells after gene editing deletion of *Rosa* or *Nrp1* (sgRNA1 + 4 or sgRNA2 + 3). **E** Kaplan-Meier survival curve of mice transplanted with *Cbfb*^{MYH11/+}; *Nras*^{+/G12D} leukemic cells after *Rosa* or *Nrp1* editing. **F** CFU assay of *MLL::AF9* leukemic cells after *Rosa* or *Nrp1* editing. **G** Kaplan-Meier survival curve of mice transplanted with *MLL::AF9* leukemic

cells after *Nrp1* editing ($n = 5$). The significance was calculated using Log-Rank test in **E** and **F**, $*p < 0.05$ or $**p < 0.005$. Bar diagrams represented with standard deviation of triplicates and significance were calculated using an unpaired t test, $*p < 0.05$ or $**p < 0.005$ in **A–D** and **F**.

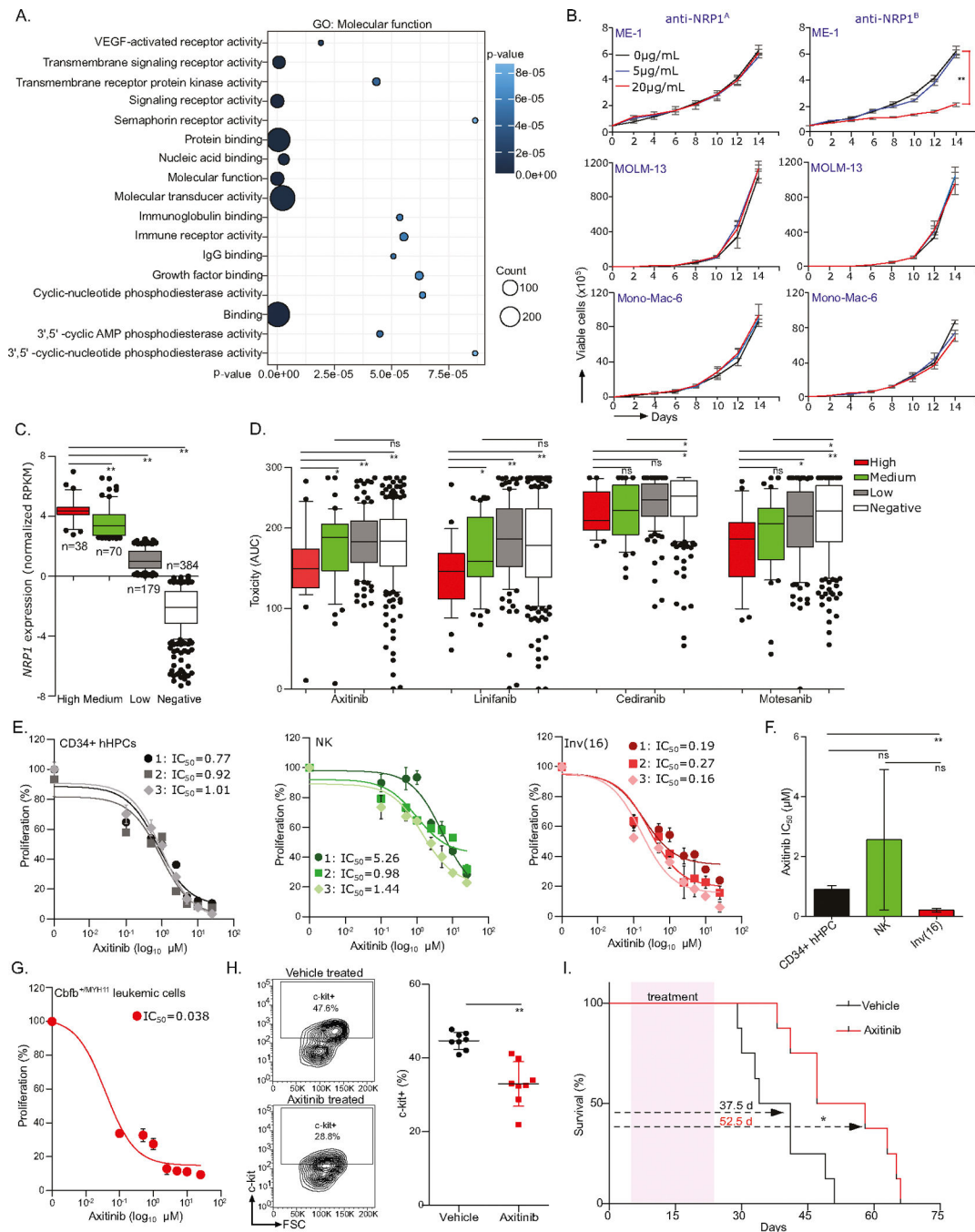


Fig. 6. The inv(16) AML cells are sensitive for VEGF inhibitors.

A Gene ontology analysis of DEGs (day 7) in *NRP1*-sh3 and *Scr*-shRNA. **B** Dose-response viability analysis of cell lines treated with blocking antibodies for the extracellular CUB (anti-NRP1^A) or FV/VIII (anti-NRP1^B) domains (0, 5 and 20 $\mu\text{g/mL}$) ($n = 3$). **C** Expression levels of NRP1 in High (inv-16) Medium (non-inv-16), Low and Negative groups from primary patient samples retrieved from BeatAML2 dataset. **D** Toxicity(AUC) levels of VEGFR inhibitors, Axitinib, Linifanib Cediranib and Motesanib in the BeatAML2 dataset. **E, F** Dose response curve of MTS assay depicting dose dependent effect of Axitinib on

human CD34⁺ cord blood samples, normal karyotype AML samples and inv(16) primary patient samples proliferation (48 h, $n = 3$) (**E**) and quantification of IC₅₀ values (**F**) are shown. **G** Dose response curve (MTS assay) depicting sensitivity of *Cbfb*^{MYH11^{+/+}}; *Nras*^{+/G12D} leukemic cells to axitinib treatment, and quantification of IC₅₀ value. **H** Representative FACS analysis contour plots (left) and quantification (right) of *Cbfb*^{MYH11^{+/+}}; *Nras*^{+/G12D} leukemic (c-kit⁺) cells in peripheral blood collected from vehicle (CMC)- or axitinib-treated mice ($n = 8$ /group) at week 4 after transplantation. **I** Kaplan–Meier survival curve of mice transplanted with *Cbfb*^{MYH11^{+/+}}; *Nras*^{+/G12D} leukemic cells followed by vehicle (CMC) or axitinib treatment (30 mg/kg, PO, BID) for 16 days ($n = 8$). Values are represented with standard deviation and significance was calculated using an unpaired t test, $*p < 0.05$ or $**p < 0.005$ in panels **C–H**. The significance was calculated using Log-Rank test in **I**, $*p < 0.05$ or $**p < 0.005$.