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Therapeutic targeting of preleukemia cells in a mouse model of *NPM1* mutant acute myeloid leukemia

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Abstract

The initiating mutations that contribute to cancer development are sometimes present in pre-malignant cells. Whether therapies targeting these mutations can eradicate pre-malignant cells is unclear. Acute myeloid leukemia (AML) is an attractive system for investigating the effect of preventative treatment as this disease is often preceded by a pre-malignant state (clonal hematopoiesis or myelodysplastic syndrome). In *Npm1c/Dnmt3a* mutant knock-in mice, a model of AML development, leukemia is preceded by a period of extended myeloid progenitor cell proliferation/self-renewal. We found that this self-renewal can be reversed by oral administration

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Competing interests: SAA has been a consultant and/or shareholder for Vitae/Allergan Pharmaceuticals, Epizyme Inc, Imago Biosciences, Cyteir Therapeutics, C4 Therapeutics, Syros Pharmaceuticals, OxStem Oncology, Accent Therapeutics and Mana Therapeutics. SAA has received research support from Janssen, Novartis, and AstraZeneca. RLL is on the supervisory board of Qiagen and is a scientific advisor to Loxo, Imago, C4 Therapeutics and Isoplexis, which each include an equity interest. He receives research support from and consulted for Celgene and Roche, he has received research support from Prelude Therapeutics, and consulted for Incyte, Novartis, Astellas, Morphosys and Janssen. He has received honoraria from Lilly and Amgen for invited lectures and from Gilead for grant reviews. GSV is a consultant for Oxstem and a consultant for and minor stockholder in Kyma. H.G. owns stock in Theravance Biopharma.

Data and materials availability: VTP50469 can be obtained via MTA from GMM/Syndax. All data of this study are deposited in the NCBI Gene Expression Omnibus (GEO) under accession number GSE129638.

of a small molecule (VTP-50469) that targets the MLL1-Menin chromatin complex. These preclinical results support the hypothesis that individuals at high risk of developing AML might benefit from targeted epigenetic therapy in a preventative setting.

Main Text:

Nucleophosmin (*NPM1*) mutant actute myeloid leukemia (AML) is one of the most common types of AML (1–3). Despite its high prevalence, the mechanism of leukemogenesis is still poorly understood, and targeted therapy options are lacking (4). *NPM1* gene mutations (*NPM1c*) induce cytoplasmic localization of NPM1 and often co-occur with other mutations in genes such as *DNA methyltransferase 3A* (*DNMT3A*^{R882H}). *NPM1c* leukemias express a distinctive stem cell like gene expression pattern which includes homeobox cluster A and B (*HOXA/B)* genes and their DNA-binding co-factor *MEIS1* (5–8). In humans *DNMT3A* mutations are detected in the most primitive hematopoietic stem cell compartment, often long before the development of leukemia, a condition often referred to as clonal hematopoiesis of indeterminate potential (CHIP) (9). *NPM1* mutations are found in committed progenitors and differentiated myeloid cells in AML, but are absent from the stem cell and lymphoid compartments (9, 10). This suggests that *NPM1c* may induce self-renewal in myeloid progenitors as a critical step in the development of AML, and that this aberrant progenitor self-renewal may represent a critical step in the progression from CHIP to AML.

To identify the leukemia-initiating cellular population in *NPM1c* AML, we used previously developed mouse models with an inducible Cre recombinase (*MxCre)* and heterozygous conditional knock-in of the humanized *Npm1* mutation (*Npm1*$^{flox\text{-}cA/+}$; hereafter called *Npm1c* mutant mice), alone or in combination with *Dnmt3a*^{R878H} mutation (*Dnmt3a*$^{R878H/+}$; hereafter called *Dnmt3a* mutant mice) (5, 11). We confirmed *Hox* gene upregulation in different hematopoietic stem and progenitor populations of *Npm1c* single and *Npm1c/Dnmt3a* double mutant mice 16 weeks after induction of the knock-in allele by polyinosinic:polycytidylic acid (pIpC) injection (Fig. 1A). At this time, mutant mice showed no signs of leukemia, had normal blood counts, and only the double mutant showed a slight increase in granulocyte and macrophage progenitor (GMP) frequencies (Fig. S1A and B). Sorted wildtype (WT) and *Dnmt3a* single mutant cells showed a stepwise decrease of *Hoxa9* mRNA expression from long term hematopoietic stem cells (LT-HSCs) to GMPs which coincides with their loss of self-renewal properties (Fig. 1A). *Npm1c* or *Npm1c/Dnmt3a* mutant cells maintained inappropriately high levels of *Hoxa9* across the different progenitor cell types (Fig. 1A). RNAseq analysis 4 weeks after activation of the *Npm1c* allele, revealed that half of the top 20 upregulated genes in *Npm1c* GMPs were *Hoxa/b* genes. The HSC-enriched Lin^{-}, Sca1^{+}, Kit^{+} population (LSK) showed much lower fold changes due to their high baseline expression of *Hoxa/b* genes (Fig. 1B and table S1). The gene expression programs induced in *Npm1c* mutant GMPs were also enriched for LT-HSC and human *Npm1c* mutated AML signatures which include *Hoxa/b* genes and *Meis1* (Fig. S1, C to I). Based on these gene expression data, we conclude that *Npm1c* supports the inappropriate expression of genes associated with normal stem cell self-renewal such as *Hoxa/b* cluster genes, throughout myeloid differentiation.

We next investigated whether *Npm1c* can induce stem-cell associated gene expression *de novo* in committed progenitor cells, which lack self-renewal and have low levels of *Hox/Meis1* expression. For this, we sorted Cre-negative *Npm1c/Dnmt3a* single or double mutant GMPs and LSK cells and then used retroviral Cre overexpression to induce the mutant knock-in alleles *in vitro* (Fig. 1C). *Npm1c* expression induced *Hoxa9* expression in GMPs *in vitro*, suggesting that the *Npm1c*-driven stem cell-associated program can be turned on at different stages of myeloid differentiation (Fig. 1C). Induction of $Dnmt3a^{R878H}$ knock-in alone did not induce or enhance the *Hoxa9* induction activated by *Npm1c* in progenitors, indicating that mutant *Npm1c* and not *Dnmt3a* is responsible for the observed upregulation of stem-cell associated genes.

Our gene expression data suggest that *Npm1c* induces stem cell properties in non-stem cells. To examine whether these transcriptional changes coincide with functional self-renewal properties in *Npm1c* progenitors, we first performed colony forming (CFU) assays. *Npm1c* mutant GMPs displayed increased *in vitro* self-renewal capacity as shown by their ability to replate up to 4 rounds in CFU assays (Fig. S2, A and B). *In vivo* transplantation experiments performed using *in vivo* pIpC-induced and *in vitro Cre*- transduced mutant GMPs demonstrated that *Npm1c* enhances engraftment and self-renewal of GMPs (Fig.1D, and fig. S2C). While some of the initially engrafted GMPs were depleted over time, about half of the mice retained self-renewing GMPs for >12 weeks (Fig. 1E). These long-term engrafting GMPs (LT-GMPs) showed $CD11b^{+}Gr1^{+}$ peripheral blood engraftment, and recipient mice showed no signs of leukemia for over 6 months (Fig. S2D). These experiments demonstrate that self-renewal properties induced by *Npm1c* in myeloid progenitors are sufficient to give rise to a preleukemic population that stably engrafts long-term.

To determine whether these preleukemic *Npm1c* mutant clones would progress to leukemia, we performed secondary transplants of LT-GMPs (Fig. 2A). Secondary recipients of *Npm1c* mutant or *Npm1c/Dnmt3a* double mutant LT-GMPs developed AML 3–5 months post-secondary transplant similar to mice that received mutant LSK cells (Fig. 2B). LSK and GMP-derived secondary mice presented with high WBC counts, enlarged spleens, and extramedullary hematopoiesis suggesting that *Npm1c* is sufficient to give GMPs enough self-renewal capacity to ultimately generate AML. (Fig. 2, C and D, and fig. S3, A and B). The long latency indicates that *Npm1c* mutant LT-GMPs may acquire further mutations over time, which has been shown to occur in this and other *Npm1c* knock-in mouse models (7, 12). Furthermore, mouse *Npm1c/Dnmt3a* mutant leukemia cells presented with highly upregulated *Hoxa/b* expression resembling expression patterns observed in human *NPM1c* AML and other *HOX*-associated AMLs such as *MLL*-AF9 AML (Fig. 2E, and S3, C and D). These results confirm that preleukemic *Npm1c* mutant LT-GMP eventually give rise to leukemia.

We have previously shown that inhibition of the interaction between the histone methyltransferase *MLL1* and adaptor protein *Menin* reverses leukemogenic gene expression in *NPM1c* AML cell line OCI-AML3 (13). Menin-MLL interaction inhibitors were originally developed to target the oncogenic MLL-fusion complexes, by directly disrupting the oncogene complex from assembling on chromatin. Our findings however, suggests that the wildtype MLL1-Menin interaction is essential to maintain *NPM1c*-driven leukemia. To

test this, we used an orally bioavailable inhibitor of the Menin-MLL1 interaction (VTP-50469). This compound has been used to treat established disease in models of *MLL*-rearranged AML and B-ALL [see (14) for details on the chemical synthesis of VTP-50469]. We assessed whether *Npm1c* mutant mouse cells respond to Menin inhibition in serial CFU replating assays of double and single mutant cell lines (Fig. 3A, and fig. S4A). Menin inhibition led to a rapid loss of replating capacity and upregulation of myeloid differentiation marker CD11b with no significant increase in apoptosis (Fig. S4, B and C). Gene expression analysis of *Npm1c/Dnmt3a* mouse cells after Menin inhibition revealed a rapid repression of stem cell genes including *Meis1* and *Pbx3* (Fig. S5A, left panel). *Meis1/Pbx3* are important co-factors of *Hoxa/b* transcription factors and play essential roles in *Hoxa9* driven leukemogenesis and maintenance of leukemic stem cell gene expression programs (15–17). Even though many *Npm1c*-induced genes, including *Hoxa/b*, remained highly expressed, the loss of essential co-factors such as *Meis1* could account for the loss of self-renewal observed upon Menin inhibition.

To confirm that reduced *Meis1* expression is crucial for the drastic differentiation of *Npm1c/Dnmt3a* mutant cells observed after VTP-50469 treatment, we first attempted to rescue the VTP-50469 induced loss of leukemic stem cell gene expression by retroviral overexpression of *Meis1*. Maintaining *Meis1* expression rescued the replating capacity of *Npm1c/Dnmt3a* mutant cells in the presence of Menin inhibitor and increased the IC50 values significantly (Fig 3B, and fig. S5B). While control cells lost essential components of their self-renewal program in response to VTP-50469, *Meis1*-expressing cells showed increased expression of a group of stem-cell associated genes including *Mecom* and *Pbx3* and retained them in the presence of Menin inhibitor (Fig S5, B to G). Conversely, Cas9 mediated knock-out of *Meis1* led to a rapid loss of out-of-frame edited cells in culture as well as a reduction in CFU replating capacity confirming *Meis1* as a dependency in *NPM1c* mutated AML (Fig 3C and fig S5H). These data confirm the essential role of *Meis1* in maintaining leukemic stem cell programs.

Next, we confirmed that human *NPM1c* mutant leukemia cells line, OCI-AML3 also respond to VTP-50469. OCI-AML3 were highly sensitive to Menin-MLL inhibition, as demonstrated by their low IC50 value (3nM on day 6) and rapid downregulation of *MEIS1* and *PBX3* upon VTP-50469 treatment (Fig. S6, A to C). In contrast to previously published Menin inhibitor molecules such as MI-2–2 and MI-503 that were shown to reduce expression of *HOXA/B* cluster genes as well as *MEIS1*, *HOX* genes were not repressed in response to VTP-50469 in OCI-AML3 cells (S6, B and C). In mouse cells, a modest repressive effect on some *Hox* genes was observed while others were upregulated (S7, D and E and S10, A to D)(18, 19). Furthermore, we observed a reduction of Menin and MLL1 chromatin occupancy at the *MEIS1* and *PBX3* transcriptional start sites (TSS), while MLL1 binding at *HOXA/B* TSSs was retained in regions where Menin was depleted (Fig. 3D, and fig. S6E, and table S2). Globally, Menin chromatin occupancy was decreased, while MLL1 and H3K4me3 were lost only at specific sites which were highly enriched for genes downregulated in response to Menin inhibition (Fig. S6, F to H, S7, A to C). To verify that MLL1 loss is responsible for the observed loss of stem cell associated gene expression, we generated CRISPR Cas9 mediated OCI-AML3 knock-out (KO) cell lines of *MLL1, MLL2* and *Menin* (Fig S8, A to C, Table S4). *Menin* KO mimicked the expression changes

observed upon VTP-50469 treatment, with reduced *MEIS1*/*PBX3* expression and upregulation of *HOXB5* and *HOXA5* (Fig. 3, E and F, and fig. S8, D to E). Loss of *MLL1,* however, also resulted in a reduction in *HOX* expression, while *MLL2* disruption showed no or only minor effects on *HOX/MEIS1* (Fig. 3F and fig. S8E). In agreement with this, *Menin* and *MLL1* KO cells were rapidly depleted in competition assays while *MLL2* KO cells were not (Fig S8F). Our findings confirm *MLL1* as the main driver of oncogenic *HOX/MEIS1* gene expression in *NPM1c* mutated AML and show that only a subset of MLL1 target genes are also Menin dependent.

DNMT3A mutations are frequently found in patients with CHIP and are associated with increased risk for hematologic malignancies (9, 20–23). By contrast, *NPM1c* mutations have not been reported in CHIP suggesting that their acquisition is rapidly followed by leukemic progression. This was demonstrated in at least one patient with *IDH2* mutant CHIP that developed AML shortly after *NPM1c* was detected (8, 24). Our mouse model of preleukemic *Npm1c/Dnmt3a* LT-GMPs allowed us to test whether we can interrupt leukemia progression via eradication of *Npm1c* mutant preleukemic clones. We evaluated the *in vivo* efficacy of VTP-50469 using secondary transplants of *Npm1c* single and *Npm1c/Dnmt3a* double mutant LT-GMPs (Fig. S9A). Engraftment was confirmed 3 weeks post-transplant and mice were treated with 0.1% VTP-50469 spiked chow for 9 weeks (Fig. S9, B and C). In control animals we observed an expansion of the LT-GMP engraftment and eventually mice succumbed to AML (Fig. 4, A and B, and fig. S9, D and E). After three weeks of Menin inhibitor-treated preleukemic mice showed a rapid decrease in engraftment (<1%) (Fig 4B, and fig. S9, D and E). Strikingly, no relapse of LT-GMPs was observed more than 6 months after the treatment was discontinued and VTP-50469 treated groups showed prolonged survival of over 9 months versus and average of 5 month in the untreated groups. Furthermore, when VTP-50469 treated mice were sacrificed 300 days post-transplant, no *Npm1c* mutant cells were detected in bone marrow spleen or liver (Fig. S9, F to H). Wildtype stem cell self-renewal was not affected by VTP-50469 treatment as demonstrated by stable engraftment of WT HSCs (Fig. S9I). Repression of *Meis1/Pbx3* and other stem cell factors was validated by RNAseq analysis of sorted *Npm1c/Dnmt3a* LT-GMPs after 5 days *in vivo* treatment (Fig S10, A to D). VTP-50469 was well tolerated even when administered for long periods (9 weeks continuously), which could potentially be extended to ensure complete clearance of *NPM1c* mutant cells if needed. These data indicate that we can specifically eradicate preleukemic *Npm1c* mutant self-renewing myeloid progenitor cells using targeted epigenetic therapy without having detrimental effects on either normal HSCs or hematopoiesis.

We next wanted to investigate whether *NPM1c* mutant cells remained sensitive to Menin-MLL inhibition after progression to AML. Menin-MLL inhibitors have been shown to be effective targeting *MLL*-fusion leukemias *in vivo*, but whether they will be similarly effective in the more common *NPM1c*-mutant AML was less clear. To this end we used patient derived xenograft (PDX) assays of untreated and relapsed *NPM1c* AML harboring *FLT3, DNMT3a* and *IDH1* co-mutations (Table S5). Inhibiting MLL1-Menin dramatically reduced tumor burden in blood, spleen, and BM of three different PDX models treated for 30–43 days (Fig. S11, A to I). The few detectable human cells expressed high levels of differentiation marker CD11b (Fig. S11, J and K). VTP-50469 treatment significantly

prolonged survival in two independent *NPM1c* PDX models (Fig. 4, C and D, fig. S12 A and B). Gene expression analysis of *NPM1c* PDX cells isolated 10 days post *in vivo* Menin inhibitor treatment confirmed reduced expression of *MEIS1*/*PBX3* as observed in our mouse model, while *HOX* genes were slightly increased (Fig. S12C). Furthermore, Menin inhibition was effective in PDX mice with high tumor burden (40–80% hCD45) (Fig. S13A). A reduction of blood leukemia burden was observed after 3 weeks of VTP-50469 treatment. Except for one mouse that expired after 10 days of treatment, the three remaining VTP-50469 treated mice survived over 150 days post-transplant with hCD45 engraftment <1% (Fig. S13B and C). Our data suggests that Menin-MLL inhibition is highly effective not just in the preleukemic setting, but also in fully developed aggressive human *NPM1c* mutant AMLs.

To determine the feasibility of detecting preleukemic *NPM1* mutant clones in patients, we screened 49 paired MDS and secondary AML (sAML) samples for AML-associated mutations (*NPM1*, *DNMT3A, RUNX1, TP53, NF1, ASXL1, IDH1* and *IDH2*). *NPM1c* was detected in six (12%) MDS and paired sAML samples, while co-occurring signaling mutations *NF1* and *FLT3* were mostly acquired during progression to sAML in these samples (Fig. 4E). Half of these *NPM1c* mutant MDS patient rapidly developed leukemia within 1–2 months, whereas the other group of patients progressed more slowly (5–6.5 month) (Table S6). *NPM1c* can therefore be detected in a preleukemic setting and may act as a marker for progression to AML, making it an ideal target for preventative therapy. In the context of screening and monitoring, this may plausibly be extended to individuals with large *DNMT3A* or *IDH1/2* mutant CHIP clones, which is predictive of high AML risk (9).

In summary, this study shows that eliminating preleukemic cells with targeted therapy is a potentially promising approach; specifically, we present evidence in a mouse model of AML that early intervention is possible with molecules that target chromatin regulators. Combined with improved long-term monitoring of patients with high-risk CHIP or MDS for appearance of an *NPM1c* preleukemic clone, disease prevention could become a realistic possibility in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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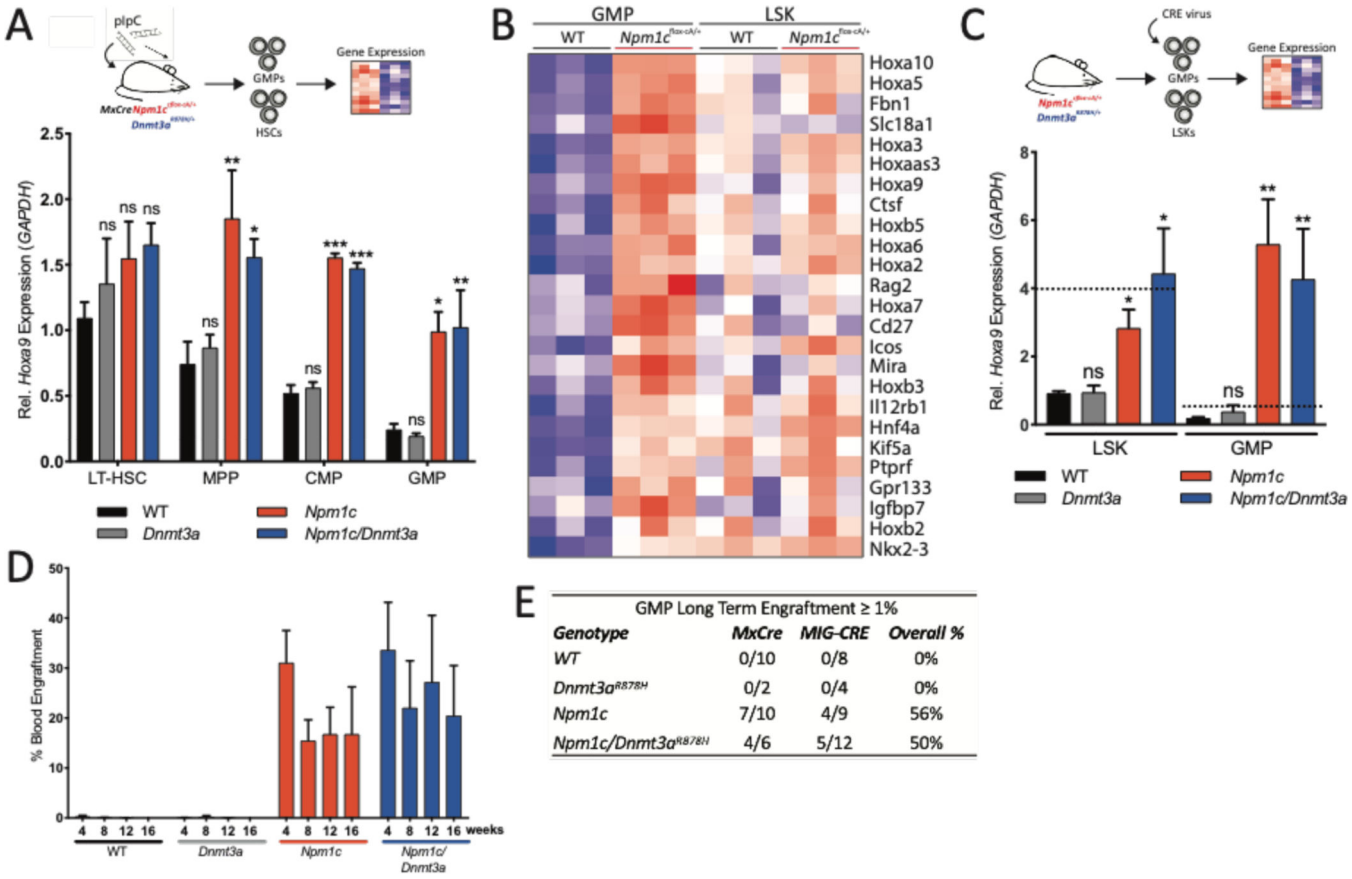
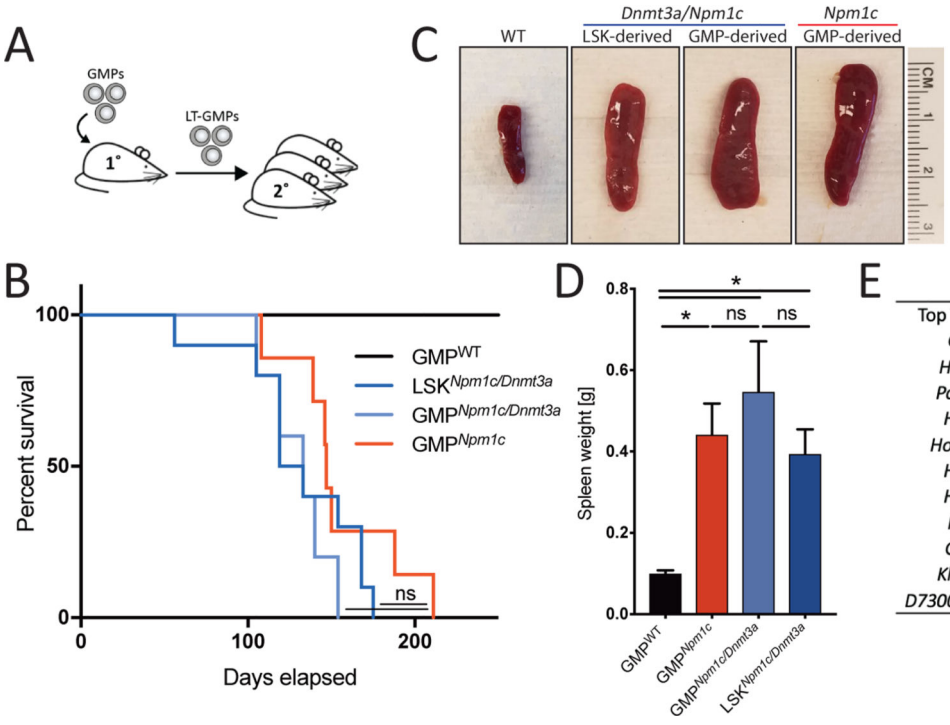


E

GMP Long Term Engraftment ≥ 1%			
Genotype	***MxCre***	***MIG-CRE***	***Overall %***
WT	0/10	0/8	0%
Dnmt3a^{R878H}	0/2	0/4	0%
Npm1c	7/10	4/9	56%
Npm1c/Dnmt3a^{R878H}	4/6	5/12	50%

Fig. 1. *Npm1c* induces self–renewal properties in myeloid progenitor cells.

(A) *Hoxa9* gene expression in *Npm1c, Dnmt3a* and *Npm1c/Dnmt3a* mutant LT-HSCs,multipotent progenitors (MPPs), common myeloid progenitors (CMPs) and GMPs 16 weeks after pIpC injection (n≥3 mice per group; error bars indicate mean ± SD). Rel., relative;GAPDH, glyceraldehyde 3-phosphate dehydrogenase. (B) Heatmap showing top 25 up-regulated genes in *Npm1c* versus WT LSK and GMPs, 4 weeks after pIpC treatment (n=3 mice per group).(C) Relative expression of *Hoxa9* 3 days after in vitro Cre transduction (n ≥ 4 mice per group;error bars indicate mean ± SEM). The dotted lines indicate Hoxa9 expression level from freshly isolated LSK cells and GMPs. (D) Peripheral blood percentage CD45.2 engraftment of WT and Npm1c, Dnmt3a, and Npm1c/Dnmt3a mutant GMPs sorted 4 weeks after pIpC induction, transplanted into lethally irradiated recipients. Error bars indicate mean ± SEM. (E) Summary table of GMP-transplanted mouse numbers and percentages engrafted ≥1% for >12 weeks. MIGCRE,MSCV-CRE-IRES-GFP retrovirus. ns, not significant; *P < 0.05; **P < 0.01; ***P <0.001.



E

Top Ten Upregulated Genes in *Npm1c/Dnmt3a* AML

Gene	Log2 Fold Change	p-value
Hoxa11	11.5	5.7E-08
Pcdh11x	11.4	1.9E-09
Hoxb8	11.3	1.3E-05
Hoxa11os	10.6	5.8E-06
Hoxb9	10.0	1.7E-03
Hoxb7	9.6	2.8E-10
Foxf2	9.3	1.1E-03
Gm53	9.3	5.1E-03
Klk1b22	9.1	6.8E-03
D730001G18Rik	8.7	2.1E-07

Fig. 2. Myeloid progenitors are leukemia-initiating cells in Npm1c AML.

(A) Experimental overview of secondary transplantation of long-term engrafted mutant GMPs. (B to D) Kaplan-Meyer survival analysis (B), representative spleen images (C), and spleen weights (D) of secondary transplants of MIG-CRENpm1c/Dnmt3a LT-GMPs or LSK-derived cells and MxCreNpm1c LT-GMPs ($n \geq 4$ mice per group). One-way analysis of variance (ANOVA) was performed. Error bars indicate mean ± SD. *$P < 0.05$; ns, not significant. (E) Top 10 up-regulated genes in MIG-CRENpm1c/Dnmt3a mutant leukemic GMPs compared with WT GMPs ($n = 3$ mice per group).

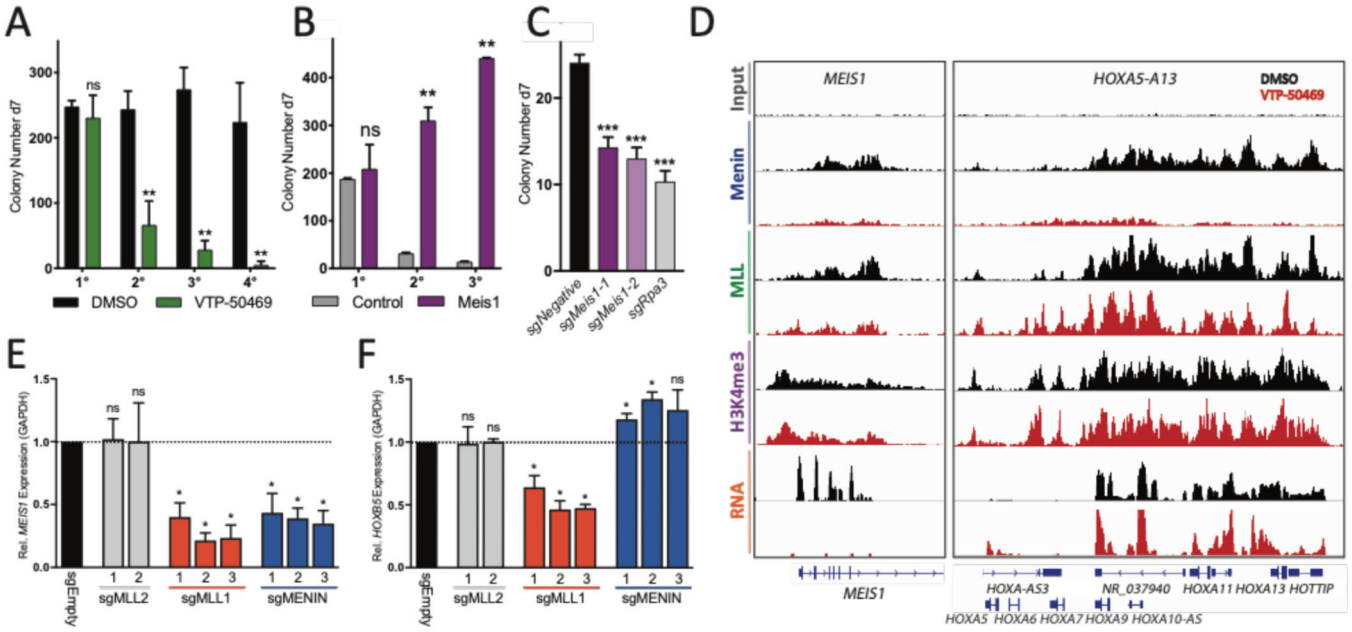


Fig. 3. Meis1, Menin and MLL1 are essential for maintaining self-renewal program.

(A) CFU serial replating assay of mouse MIG-CRENpm1c/Dnmt3a mutant cell line (SIIIL12) with dimethyl sulfoxide (DMSO) or VTP-50469. Data represent the mean of three independent experiments. d7, day 7. (B) CFU assay of mouse SIIIL12 cells transduced with MSCV-PURO control (left) or Meis1-PURO (right) virus and grown in the presence of 10 nM VTP-50469. Data represent the mean of three independent experiments. (C) CFU assay of SIIIL12 cells electroporated with control or Meis1- or Rpa3-targeting single guide RNAs (sgRNAs) for Cas9-mediated KO. Data represent the mean of three independent experiments. (D) Chromatin immuno- precipitation sequencing (ChIP-seq) density plots showing changes in chromatin occupancy of Menin, MLL, and H3K4me3 and changes in mRNA expression in response to VTP-50469 in OCI-AML3 cells at the MEIS1 and HOXA loci. (E and F) MEIS1 (E) and HOXB5 (F) gene expression in OCI-AML3 cells transduced with control, sgMLL1, sgMLL2, and sgMenin. Data represent the mean of three independent experiments. One-way ANOVA was performed. Error bars indicate mean ± SD. ns, not significant; *P < 0.05; **P < 0.01; ***P <0.001.

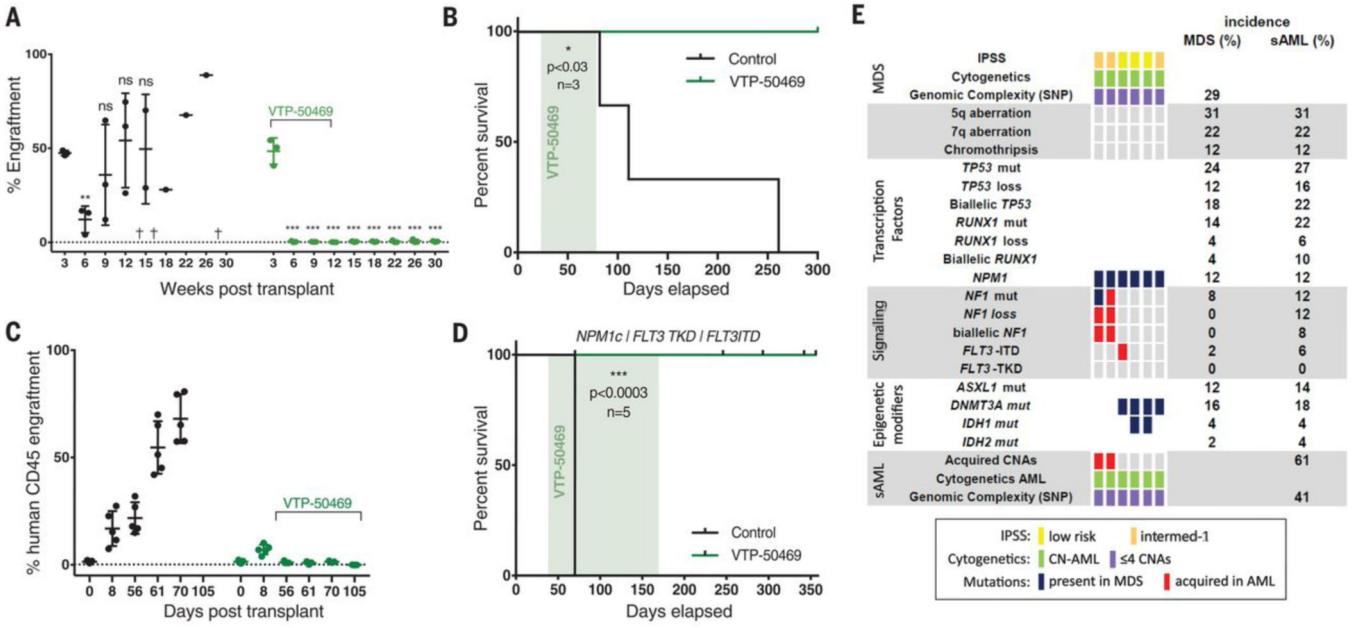


Fig. 4. Preleukemic *Npm1c* LT-GMPs and human AML cells can be eradicated by Menin inhibition.

(A and B) Percent engraftment of CD45.2 in peripheral blood (A) and Kaplan-Meier survival analysis (B) of mice transplanted with Npm1c/Dnmt3a LT-GMPs receiving control or 0.1% VTP-50469–spiked chow for 9 weeks (one-way ANOVA; n = 3 mice per group; error bars indicate mean ± SD). (C and D) Percent engraftment of hCD45 in peripheral blood (C) and aplan-Meier survival analysis (D) of NPM1c,FLT3ITD,FLT3TKD-transplanted PDX mice receiving control or 0.1% VTP-50469–spiked chow for 129 days (patient 1, table S5; n = 5 mice per group; error bars indicate mean ± SD). (E) Mutational screening of 49 paired MDS and sAML patient samples for RUNX1, TP53, NPM1, FLT3, ASXL1, DNMT3A, IDH1, and IDH2 mutations revealed six patients with persistent NPM1 mutations detected in MDS samples before AML development. IPSS, International Prognostic Scoring System; SNP, single- nucleotide polymorphism; CN-AML, cytogenetically normal AML; CNAs, copy number alterations. ns, not significant; **P < 0.01; ***P < 0.001.