CANCER

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

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The initiating mutations that contribute to cancer development are sometimes present in premalignant cells. Whether therapies targeting these mutations can eradicate premalignant cells is unclear. Acute myeloid leukemia (AML) is an attractive system for investigating the effect of preventative treatment because this disease is often preceded by a premalignant 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 and self-renewal. We found that this self-renewal can be reversed by oral administration 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.

Nucleophosmin (*NPM1*) mutant acute 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 that includes homeobox cluster A and B (*HOXA/B*) genes and their DNA-binding cofactor 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.

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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 up-regulation in different hematopoietic stem and progenitor populations of *Npm1c*, *Dnmt3a*, and *Npm1c/Dnmt3a* 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 and had normal blood counts, and only the double mutant showed a slight increase in granulocyte-macrophage progenitor (GMP) frequencies (fig. S1, A and B). Sorted wild-type (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). RNA sequencing (RNA-seq) analysis 4 weeks after activation of the *Npm1c* allele revealed that half of the top 20 up-regulated genes in *Npm1c* GMPs were *Hoxa/b* genes. The HSC-enriched Lin$^-$, Sca1$^+$, Kit$^+$ population (LSK) showed much lower fold changes owing 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* mutant AML signatures, which include *Hoxa/b* genes and *Meis1* (fig. S1, C to I). On the basis of 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* and *Meis1* expression. For this, we sorted Cre-negative *Npm1c*, *Dnmt3a*, and *Npm1c/Dnmt3a* 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 up-regulation 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 unit (CFU) assays. *Npm1c* mutant GMPs displayed increased in vitro self-renewal capacity, as shown by their ability to replate up to four rounds in CFU assays (fig. S2, A and B). 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). Although 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 more than 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* single-mutant or *Npm1c/Dnmt3a* double-mutant LT-GMPs developed AML 3 to 5 months after secondary transplant similar to mice that received mutant LSK cells (Fig. 2B). LSK- and GMP-derived secondary transplanted mice presented with high white blood cell 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

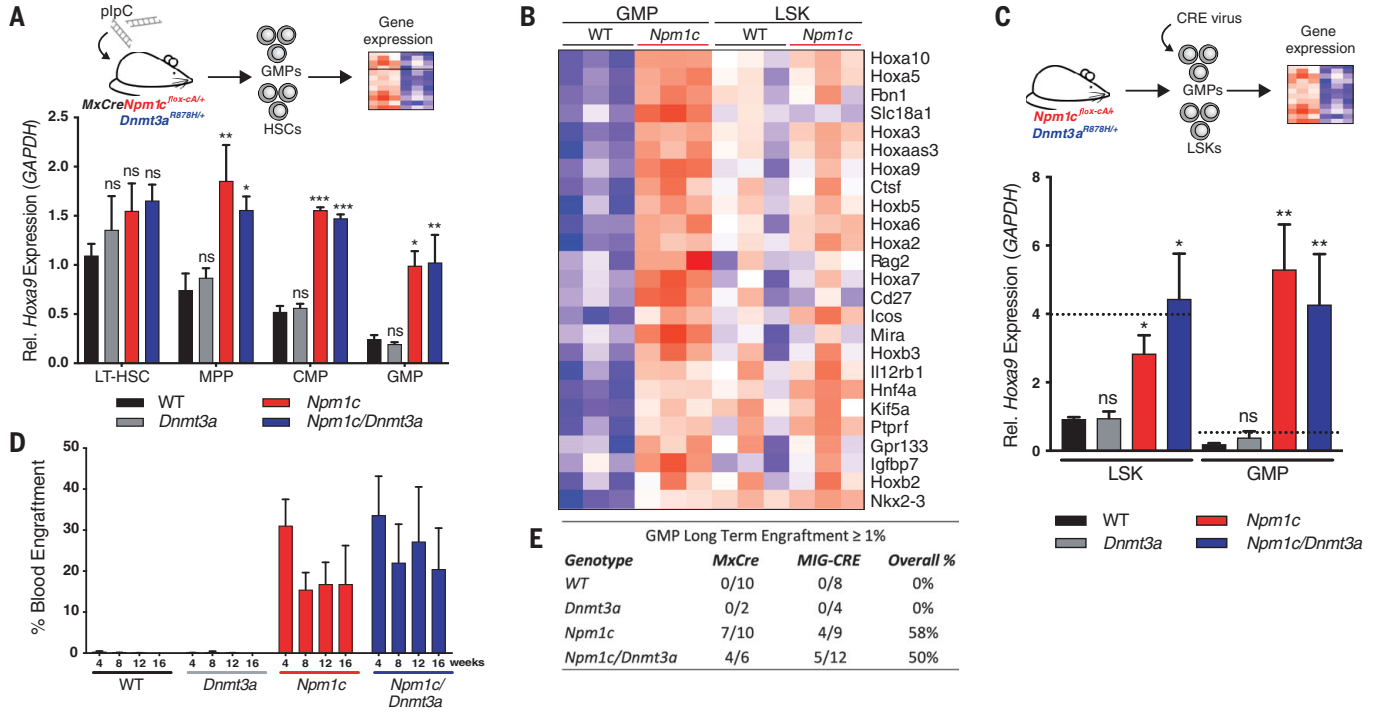

Fig. 1. *Npm1c* induces self-renewal properties in myeloid progenitor cells. (**A**) *Hoxa9* gene expression in *Npm1c*, *Dnmt3a*, and *Npm1c/Dnmt3a* mutant LT-HSCs, multipotential progenitors (MPPs), common myeloid progenitors (CMPs), and GMPs 16 weeks after pIpC injection ($n \geq 3$ mice per group; error bars indicate mean ± SD). Rel., relative; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. (**B**) Heatmap showing the top 25 up-regulated genes in *Npm1c* versus WT LSK cells and GMPs, 4 weeks after pIpC treatment ($n = 3$ mice per group). (**C**) Relative expression of *Hoxa9* 3 days after in vitro *Cre* transduction ($n \geq 4$ 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. *MIG-CRE*, MSCV-CRE-IRES-GFP retrovirus. ns, not significant; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

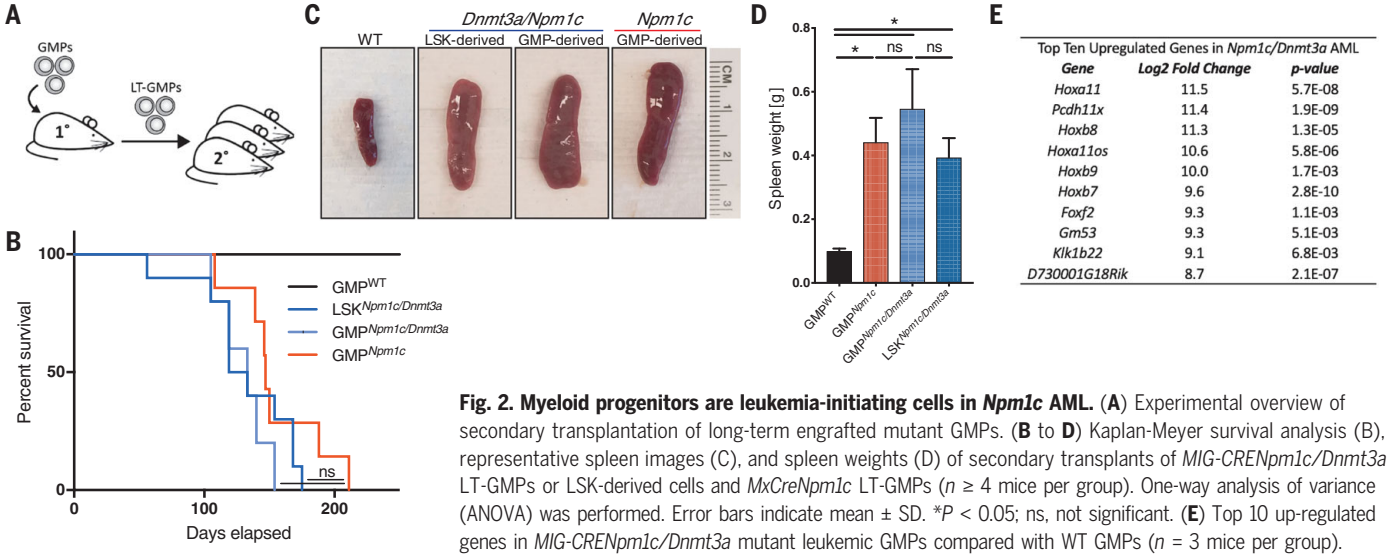

Fig. 2. Myeloid progenitors are leukemia-initiating cells in *Npm1c* AML. (**A**) Experimental overview of secondary transplantation of long-term engrafted mutant GMPs. (**B** to **D**) Kaplan-Meyer survival analysis (B), representative spleen images (C), and spleen weights (D) of secondary transplants 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).

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 showed highly up-regulated *Hoxa/b* expression, which resembled expression patterns observed in human *NPM1c* AML and other *HOX*-associated AMLs such as *MLL*-AF9 AML (Fig. 2E and fig. S3, C and D). These results confirm that pre-leukemic *Npm1c* mutant LT-GMPs 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 the *NPM1c* AML cell line OCI-AML3 (*13*). Menin-MLL interaction inhibitors were originally developed

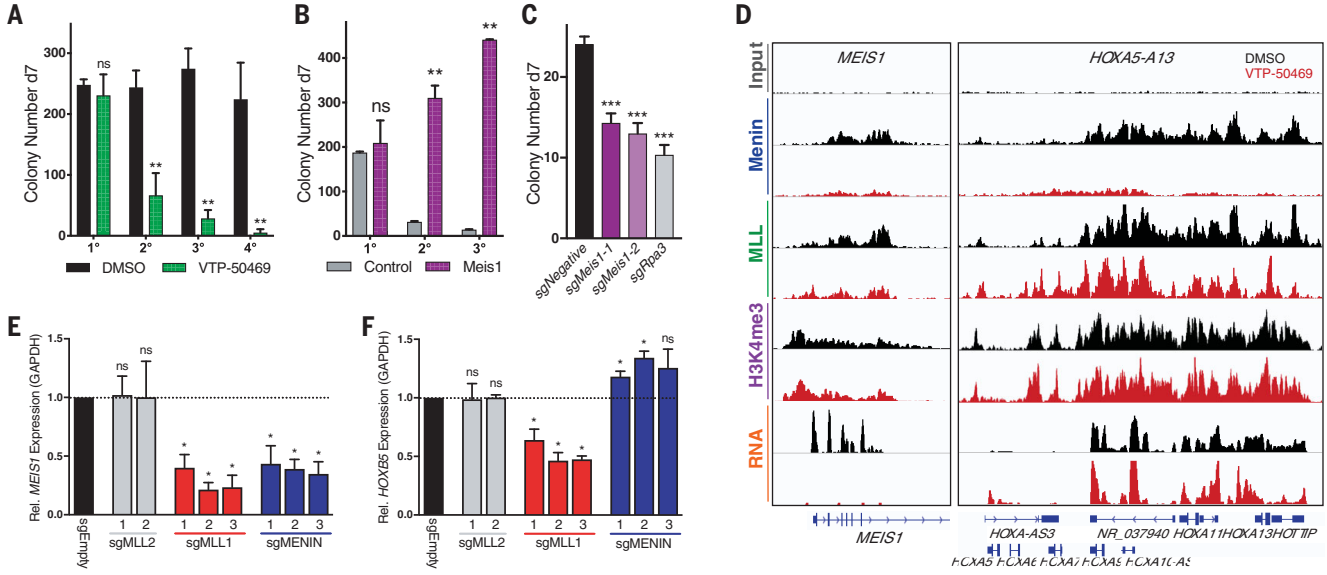

Fig. 3. Meis1, Menin, and MLL1 are essential for maintaining self-renewal program. (**A**) CFU serial replating assay of mouse *MIG-CRENpm1c/Dnmt3a* mutant cell line (SIIIL12) with dimethyl sulfoxide (DMSO) or VTP-50469. Data represent the mean of three independent experiments. d7, day 7. (**B**) CFU 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 immunoprecipitation 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; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

to target the oncogenic MLL-fusion complexes by directly disrupting the oncogene complex from assembling on chromatin. Our findings, however, suggest that the WT 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 cell acute lymphoblastic leukemia [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 up-regulation of myeloid differentiation marker CD11b with no significant increase in apoptosis (fig. S4, B and C). Gene expression analysis of *Npm1c/Dnmt3a* mutant mouse cells after Menin inhibition revealed a rapid repression of stem cell genes, including *Meis1* and *Pbx3* (fig. S5A, left). Meis1 and Pbx3 are important cofactors 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 cofactors 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 median inhibitory concentration (IC_{50}) values significantly (Fig. 3B and fig. S5B). Whereas 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 knockout (KO) 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* mutant 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 cell line OCI-AML3 also responds to VTP-50469. OCI-AML3 cells were highly sensitive to Menin-MLL inhibition, as demonstrated by their low IC_{50} value (3 nM on day 6) and rapid down-regulation 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 (fig. S6, B and C). In mouse cells, a modest repressive effect on some *Hox* genes was observed, whereas others were up-regulated (figs. 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 (TSSs), whereas MLL1 binding at *HOXA/B* TSSs was retained in regions where Menin was depleted (Fig. 3D, fig. S6E, and table S2). Globally, Menin chromatin occupancy was decreased, whereas MLL1 and trimethylated histone H3 lysine 4 (H3K4me3) were lost only at specific sites that were highly enriched for genes down-regulated in response to Menin inhibition (figs. S6, F to H, and S7, A to C). To verify that MLL1 loss is responsible for the observed loss of stem cell–associated gene expression, we generated Cas9-mediated OCI-AML3 KO cell lines of *MLL1*, *MLL2*, and *Menin* (fig. S8, A to C, and table S4). *Menin* KO mimicked the expression changes observed upon VTP-50469 treatment, with reduced *MEIS1* and *PBX3* expression and up-regulation of *HOXB5* and *HOXA5* (Fig. 3, E and F, and fig. S8, D and E). Loss of *MLL1*, however, also resulted in a reduction in *HOX* expression, whereas *MLL2*

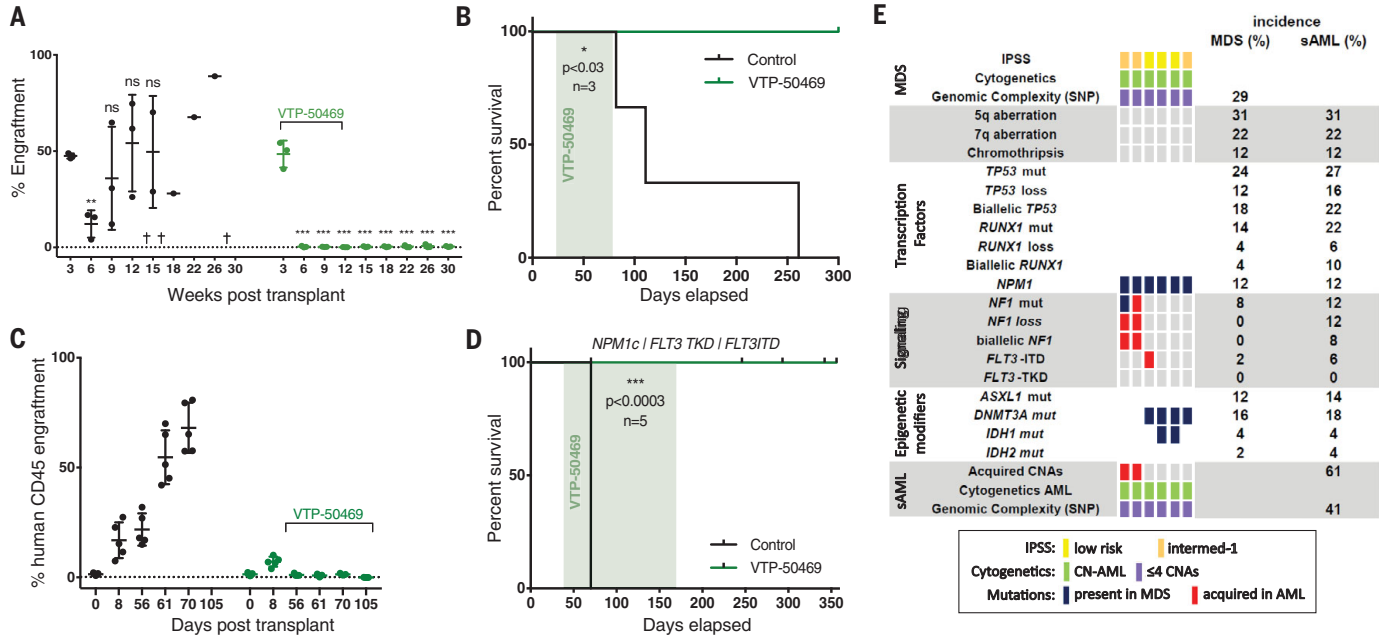

Fig. 4. Preleukemic *Npm1c* LT-GMPs and human AML cells can be eradicated by Menin inhibition. (**A** and **B**) Percent engraftment of CD45.2 in peripheral blood (A) and Kaplan-Meier survival analysis (B) of mice transplanted with *Npm1c/Dnmt3a* LT-GMPs receiving control or 0.1% VTP-50469–spiked chow 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 Kaplan-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; $^{**}P < 0.01$; $^{***}P < 0.001$.

disruption showed no or only minor effects on *HOX* and *MEIS1* (Fig. 3F and fig. S8E). In agreement with this, *Menin* and *MLL1* KO cells were rapidly depleted in competition assays, whereas *MLL2* KO cells were not (fig. S8F). Our findings confirm *MLL1* as the main driver of oncogenic *HOX* and *MEIS1* gene expression in *NPM1c* mutant 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 by means of eradication of *Npm1c* mutant preleukemic clones. We evaluated the in vivo efficacy of VTP-50469 using secondary transplants of *Npm1c* single-mutant and *Npm1c/Dnmt3a* double-mutant LT-GMPs (fig. S9A). Engraftment was confirmed 3 weeks after 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 LT-GMP engraftment and eventually mice succumbed to AML (Fig. 4, A and B, and fig. S9D). After 3 weeks, Menin inhibitor–treated preleukemic mice showed a rapid decrease in engraftment (<1%) (Fig. 4A and fig. S9D). Notably, 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 more than 9 months versus an average of 5 months in the untreated groups (Fig. 4B and fig. S9E). Furthermore, when VTP-50469–treated mice were sacrificed 300 days after transplant, no *Npm1c* mutant cells were detected in bone marrow, spleen, or liver (fig. S9, F to H). WT stem cell self-renewal was not affected by VTP-50469 treatment, as demonstrated by stable engraftment of WT HSCs (fig. S9I). Repression of *Meis1* and *Pbx3* and other stem cell–associated genes was validated by RNA-seq analysis of sorted *Npm1c/Dnmt3a* LT-GMPs after 5 days of 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 investigated 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* comutations (table S5). Inhibiting MLL1-Menin dramatically reduced tumor burden in blood, spleen, and bone marrow of three different PDX models treated for 30 to 43 days (fig. S11, A to I). The few detectable human cells expressed high levels of the 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, and fig. S12, A and B). Gene expression analysis of *NPM1c* PDX cells isolated 10 days after in vivo Menin inhibitor treatment confirmed reduced expression of *MEIS1* and *PBX3*, as observed in our mouse model, whereas *HOX* genes were slightly increased (fig. S12C). Furthermore, Menin inhibition was effective in PDX mice with high tumor burden [40 to 80% human CD45

(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 more than 150 days after transplant with hCD45 engraftment of <1% (fig. S13, B and C). Our data suggest 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 myelodysplastic syndrome (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%) of MDS and paired sAML samples, whereas 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 patients rapidly developed leukemia within 1 to 2 months, whereas the remaining three patients (or the other half of patients) progressed more slowly (5 to 6.5 months) (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.

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SUPPLEMENTARY MATERIALS

science.sciencemag.org/content/367/6477/586/suppl/DC1
Materials and Methods
Figs. S1 to S13
Tables S1 to S6
References (*25*–*32*)

View/request a protocol for this paper from *Bio-protocol*.

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Science

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

Hannah J. Uckelmann, Stephanie M. Kim, Eric M. Wong, Charles Hatton, Hugh Giovinazzo, Jayant Y. Gadrey, Andrei V. Krivtsov, Frank G. Rücker, Konstanze Döhner, Gerard M. McGeehan, Ross L. Levine, Lars Bullinger, George S. Vassiliou and Scott A. Armstrong

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Taking preventive measures

Recent technological advances have made it possible to detect, in healthy individuals, premalignant blood cells that are likely to progress to hematologic cancer. These advances in early detection have fueled interest in "cancer interception," the idea that drugs designed to treat advanced cancer might also be useful for cancer prevention. Uckelmann *et al.* now provide support for this concept in a study of mice genetically predisposed to develop acute myeloid leukemia. Early administration of an epigenetic therapy that had previously been shown to have anticancer activity in advanced leukemia models was able to eliminate preleukemia cells and extend survival of the mice.

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