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TO THE EDITOR:

WT1 and *DNMT3A* play essential roles in the growth of certain patient AML cells in mice

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Patients with acute myeloid leukemia (AML) experience poor prognosis, and precision oncology represents an attractive therapeutic option, applying targeted therapies against so-called dependencies.[1-4] Dependencies are essential components required for cell growth and survival; they represent attractive therapeutic targets as their inhibition reduces tumor burden.[1-4]

Many genes recurrently mutated in AML contribute to oncogenesis,[5,6] which may imply a role as dependency and allow precision therapy, based on genetic profiling. Examples already in routine clinical practice include AML with mutated FMS related receptor tyrosine kinase 3 treated with midostaurin and AML with mutated isocitrate dehydrogenase responding to ivosidenib.[2] Herein, we asked whether additional recurrently mutated genes might represent dependencies in established AML.

Previous efforts to identify dependencies used established cell lines, including large-scale functional genomic screens; *WT1* and *DNMT3A* were shown to be dispensable in AML cell lines.[7] As a limitation, cell lines might acquire nonphysiologic alterations, and discrepant results have been described (eg, between cell lines and organoids).[8,9] To approximate the clinical situation, we studied patient-derived xenograft (PDX) models[10,11] and mimicked the complex in vivo situation by performing CRISPR/CRISPR associated protein 9 (Cas9) knockout (KO) studies in mice. Using this highly patient-related in vivo approach, we identified *WT1* and *DNMT3A* as yet unknown dependencies in a subset of patients' AML tumor cells.

From our toolbox of serially transplantable AML xenografts,[12] models derived from 7 patients were selected for the study (supplemental Tables 1-3, available on the *Blood* website). Genetically engineered PDX (GEPDX) models were generated that stably expressed recombinant Cas9 (supplemental Figure 1A).

We had recently established in vivo CRISPR/Cas9 dropout screens in GEPDX models of acute lymphoblastic leukemia[13]; herein, we transferred the technique to AML, which resulted in favorable quality controls (Figure 1A; supplemental Figure 2A). The 34 most frequently mutated genes in AML were studied, restricted to gain-of-function or change-of-function mutations.[5] A library was designed containing 5 single-guide RNAs per target gene, together with positive and negative controls (supplemental Tables 4 and 5); the library was cloned into a lentiviral vector that coexpressed recombinant markers to enrich successfully transduced cells, using our custom library multiplexed cloning (CLUE) technique (supplemental Figures 1 and 2A; supplemental Tables 3 and 4).[14]

A CRISPR/Cas9 dropout screen was performed with 5 GEPDX models. KO resulted in dropout in about half of all genes from the screen, albeit to varying degrees, and most KO induced similar effects across the PDX samples (Figure 1B; supplemental Figure 2B; supplemental Tables 6-8). Confirming the robustness of our technical approach, genes with known common essential function or genes required for the hematopoietic system were strongly depleted in the KO screen. Among them, *NPM1* was a dropout hit and served as a positive control, as it is known to have a broad essential function in malignant cells (Figure 1B).[7] Another expected hit was *KRAS*, which is one of the genes most frequently mutated across all cancers and known to represent a dependency in numerous tumor types, including AML.[15,16]

Hits from dropout screens require validation, and single-KO experiments were performed as competitive in vivo assays where all cell populations are studied under identical conditions within the same mouse, giving robust results at low resources.[18] Recombinant fluorochromes enabled an unbiased differentiation of cell populations by flow cytometry (Figure 1C-D; supplemental Figure 3). For each gene of interest as well as for nontargeting controls, 3 different, highly efficient single-guide RNAs were tested in 3 independent mixtures (supplemental Figures 4 and 5). From the 7 PDX models studied, up to 5 PDX models gave reliable results for each gene.

NPM1 was included as a positive control, and KO of *NPM1* completely eliminated AML GEPDX cells in all GEPDX models tested in vivo (Figure 1E). *KRAS* was studied in PDX models carrying mutant *KRAS* at variant allele frequencies of either 0 or close to 0.5, avoiding intrasample heterogeneity. *KRAS* KO revealed a strong dropout in all GEPDX models studied, which was significantly more pronounced in $KRAS^{mutant}$ PDX models than $KRAS^{wildtype}$ PDX models (Figure 1F; supplemental Figure 6). Thus, our PDX models strengthen previously published data showing that *KRAS* represents a dependency and attractive therapeutic target in AML, especially in tumors carrying a *KRAS* mutation.[16]

Next, we examined 2 genes with poorly defined roles in oncogenes and for which we had suitable PDX models with appropriate variant allele frequencies at hand (supplemental Table 1). Although data on *WT1* as an oncogene are controversial,[19,20] *DNMT3A* mainly represents a tumor suppressor, required for hematopoietic differentiation.[21-24]

Reproducing published data with our own tools,[7] we found no evidence that either *WT1* or *DNMT3A* might play a role as dependencies in AML cell lines, with trends toward slightly increased proliferation rates on gene KO (supplemental Figures 7-9; supplemental Table 1). In contrast and surprisingly, in in vivo GEPDX models, we discovered a pronounced dropout of either of both genes on KO in certain PDX models (Figure 2A). Thus, *WT1* and *DNMT3A* represent dependencies in a subset of PDX AML models in vivo, indicating an obvious discrepancy with their function in cell lines in vitro (Figure 2B), without any meaningful impact on the immunophenotype (supplemental Figure 10). PDX models showed dropout of *WT1* or *DNMT3A* exclusively in the in vivo environment on which PDX cells depend as opposed to cell lines, suggesting that in vivo approaches are required to unmask certain dependencies in AML (Figure 2C). There was no correlation between dependency on *DNMT3A* and presence of a somatic hot spot mutation in *DNMT3A* in the GEPDX models (supplemental Figure 6D). In the transcriptome, KO of *WT1* or *DNMT3A* was accompanied by regulation of biological processes, such as apoptosis and oxidative phosphorylation (Figure 2D; supplemental Figure 11).

When characterizing in vivo essentiality in more detail, we found that KO of *WT1* induced a certain increase in the antitumor effect of cytarabine, an important drug in routine treatment of AML (supplemental Figure 12). *WT1* KO reduced the capacity of AML-346 cells to home to the bone marrow environment on either intrafemural or intravenous cell injection followed by early in vivo growth disadvantage, suggesting an impaired tumor-niche interaction (supplemental Figures 13 and 14). KO of either *WT1* or *DNMT3A* reduced the numbers of leukemia-initiating cells in competitive limiting dilution transplantation assays and prevented reengraftment of AML-346 cells into secondary recipient mice, with and without prior cell enrichment, indicating that stem cell surrogates were depleted on *WT1* or *DNMT3A* KO (Figure 2E; supplemental Figure 15). Taken together, our data reveal that *WT1* and *DNMT3A* represent dependencies in a subset of AML GEPDX models in vivo, suggesting that they might represent therapeutic targets.

Our study identified *WT1* and *DNMT3A* as dependencies in a subset of patient AML PDX samples growing in vivo, although less pronounced and less frequent compared with *KRAS*. KO of *WT1* and *DNMT3A* impaired PDX AML growth in vivo, attenuated the tumor-niche interaction, eradicated AML stem cells, and increased treatment response.

Although cell lines did not reveal the phenotype, PDX models proved valuable tools to identify dependency on *WT1* and *DNMT3* and might more closely resemble patient's tumors.[10,11] Our technique now allows studying gene dependencies in patient PDX models in vivo (eg, to personalize pharmacologic precision therapy). Our data encourage testing additional genes recurrently mutated in AML for their essentiality in PDX models in vivo (eg, additional dropout candidates from our screens).

The essential function of *WT1* identified herein fits with its previously described oncogenic function,[19] whereas different

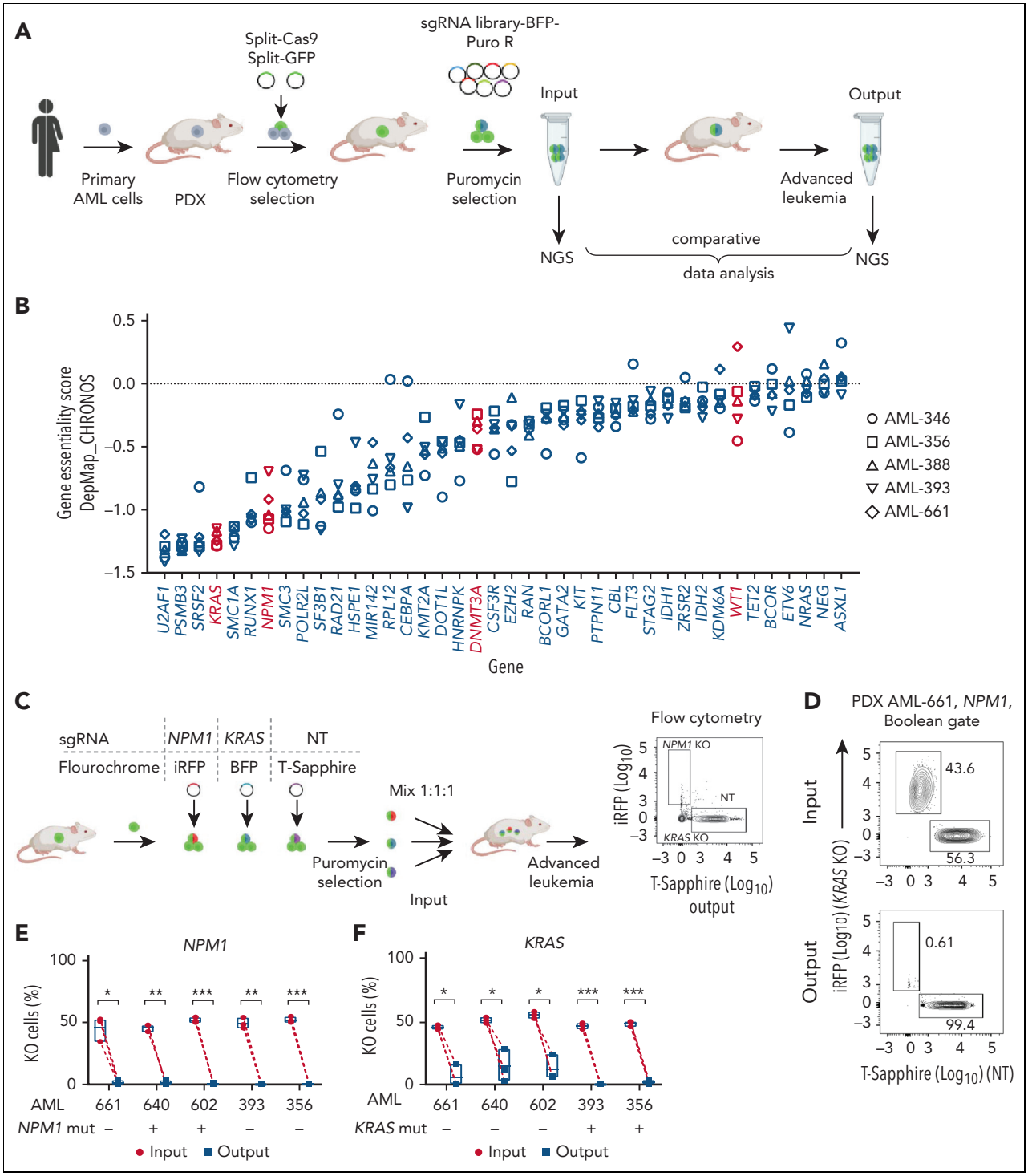

Figure 1. PDX models depend on *KRAS* and *NPM1* for in vivo growth. (A) Experimental procedure for CRISPR/Cas9 in vivo screens performed with PDX models. Serially transplantable AML PDX models were established from primary patient AML cells and lentivirally transduced to express a split version of Cas9 together with a single-guide RNA (sgRNA) library (see supplemental Figure 1 for constructs). Transgenic cells were enriched by flow cytometry (Cas9–green fluorescent protein [GFP]) and puromycin selection (sgRNA library). Except for the input control aliquot, cells were injected into groups of mice and recovered from the mice at advanced leukemia stage (output). Next- generation sequencing (NGS) was performed and analyzed using the DepMap_CHRONOS, Lin et al,[17] MAGeCK algorithm to compare sgRNA distribution between input and output. (B) CRISPR/Cas9 in vivo dropout screens were performed in 5 PDX AML models using the library of 34 genes recurrently mutated in AML; gene essentiality scores were calculated using the DepMap_CHRONOS algorithm (see supplemental Figure 2 for quality controls). (C) Experimental procedure for competitive in vivo assays for single-hit validation. sgRNAs targeting either *KRAS* or *NPM1* or nontargeting (NT) sgRNAs (n = 3 per gene) were cloned into the sgRNA construct together with the appropriate fluorochromes and transduced into Cas9-GFP–expressing PDX cells. After puromycin selection, 3 subpopulations (*KRAS* KO, *NPM1* KO, and NT sgRNA) were mixed at a 1:1:1 ratio as an input. Three replicate mixtures, each containing different sgRNAs, were transplanted into one mouse each (9 different sgRNAs per experiment in 3 replicate mice) and recovered at advanced disease stage (output). The distribution of the subpopulations was analyzed by flow cytometry (see supplemental Figure 3 for the step-by-step analysis and supplemental Figures 4 and 5 for quality controls). Blue fluorescent protein (BFP). (D) Representative flow cytometry plots for *KRAS* KO1 and NT-1 in AML-661, using Boolean gating. (E and F) Quantitative summaries of the knockout effects for *NPM1* (E) and *KRAS* (F) in all PDX models studied. Each dot represents the percentage of gene of interest KO population from a single mouse, with related sgRNAs linked by a dotted line. Bar plots indicate mean, minimum, and maximum. The results of a 2-tailed paired *t*-test are shown if they were significant: $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$.

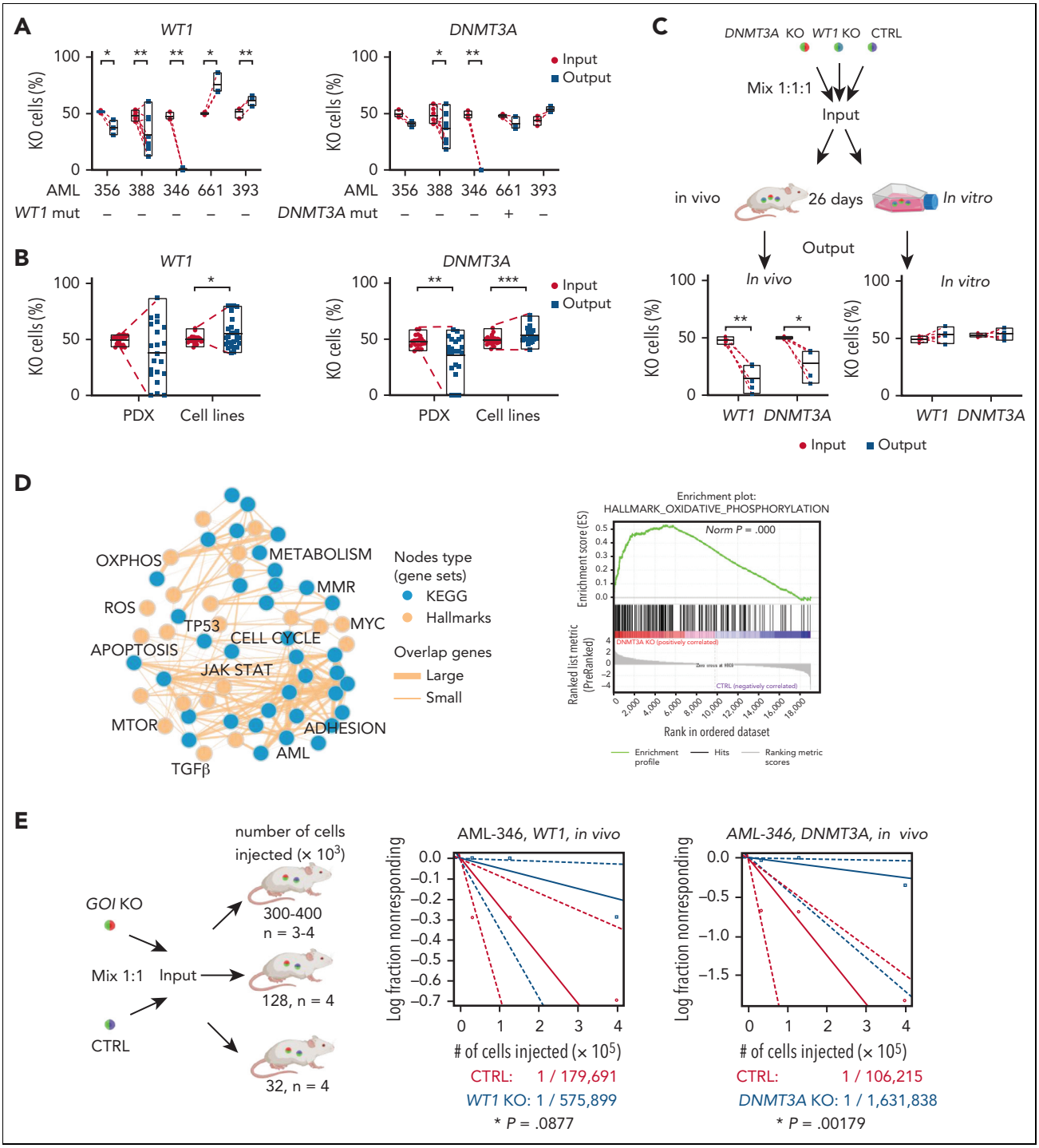

Figure 2. Certain PDX models depend on *WT1* and *DNMT3A* for in vivo growth. (A) Competitive in vivo assays were performed, analyzed, and depicted as in Figure 1C,D, except that *WT1* and *DNMT3A* were studied (see supplemental Figure 6 for quality controls). (B) Comparing gene dependency in PDX models vs cell lines. Raw data from Figure 2A and supplemental Figures 8 and 9 are summarized using a single dot for each single KO of each PDX model or cell line. For each PDX model or cell line, 3 single-guide RNAs (sgRNAs) per gene were studied. Results of an unpaired *t*-test are shown if they were significant (*P < .05, **P < .01, and ***P < .001). (C) Comparing behavior of PDX cells with KO in vitro vs in vivo. Experiment with AML-346 cells was performed, analyzed, and depicted as in Figure 2A, except that the incubation time was 26 days and an aliquot of cells was kept in vitro (*P < .05, **P < .01, and ***P < .001). (D) Transcriptomes of AML-356, AML-388, AML-661, and AML-346 cells with *DNMT3A* knockout were compared with nontargeting (NT) control (raw and complementary data in supplemental Figure 10). Gene enrichment map shows gene overlap (lines) in gene sets of hallmarks (orange nodes) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (blue nodes) pathways. Node size is proportional to the number of genes in each set; the proportion of shared genes between gene sets is depicted by the thickness of the line between nodes. Enrichment plot shows the genes differentially regulated in the hallmark oxidative phosphorylation on KO of *DNMT3A* (normalized enrichment score [NES] = 2.1537; P < .001; adjusted P [false discovery rate q-value] < 0.001). (E) Limiting dilution transplantation assay. PDX AML-346 cells were transduced with sgRNAs against *WT1* or *DNMT3A* or control (CTRL), enriched, mixed in a 1:1 ratio for *WT1*:CTRL or *DNMT3A*:CTRL, and injected into 4 mice each at 400 000, 128 000, or 32 000 cells per mouse (WT1, n = 12; and DNMT3A, n = 11 mice). After 14 weeks, bone marrow was analyzed by flow cytometry, and data were analyzed using the ELDA software. Mean (solid lines) and 95% confidence interval (CI; dashed line) are depicted.

phenotypes between different PDX models might mirror conflicting data on *WT1* obtained during leukemogenesis.[19,20]

For *DNMT3A*, a prevailing tumor suppressor function was described,[21-23] making a dependency function unlikely. Amid complexity, a tumor-supportive function of mutant *DNMT3A* was reported in specific AML subsets (eg, AML driven by a partial tandem duplication in KMT2A).[25] AML-388 harbors a KMT2A-AFDN translocation (supplemental Table 2), indicating that KMT2A-driven AML might preferably depend on *DNMT3A*.

Taken together, our molecular PDX AML in vivo studies allowed identifying *WT1* and *DNMT3A* as dependencies and putative therapeutic targets in defined subsets of AML, warranting further evaluation.

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Authorship

Contribution: M.G. and Y.G. designed and performed experiments and designed figures; D.A. performed CLUE cloning; G.K. and M.P.M. analyzed DepMap data; B.V. established patient-derived xenograft models and in vivo chemotherapy protocols; K.S. provided primary acute myeloid leukemia samples; A.M. and M.S. performed immunophenotype assay; M.R.-T. and K.H.M. performed panel sequencing; E.B. and V.J. analyzed the single-cell RNA barcoding and sequencing data; and I.J. designed the study, guided the experiments, and wrote the manuscript, with the help of all authors.

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Footnotes

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Transcriptome data generated in this study are publicly available in Gene Expression Omnibus at (GSE215836). Whole exome sequencing raw data generated in this study are not publicly available because of information that could compromise patient privacy or consent but are available on reasonable request from the corresponding author.

The online version of this article contains a data supplement.

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