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

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Abstract:

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Agreement to Share Publication-Related Data and Data Sharing Statement: Transcriptome data generated in this study are publicly available in Gene Expression Omnibus (GEO) at (XXX ID will be provided before publication). Proteome data generated in this study are publicly available in Proteomics Identification Database (PRIDE) at (XXX ID will be provided before publication). Whole Exome Sequencing raw data generated in this study are not publicly available due to information that could compromise patient privacy or consent but are available upon reasonable request from the corresponding author.

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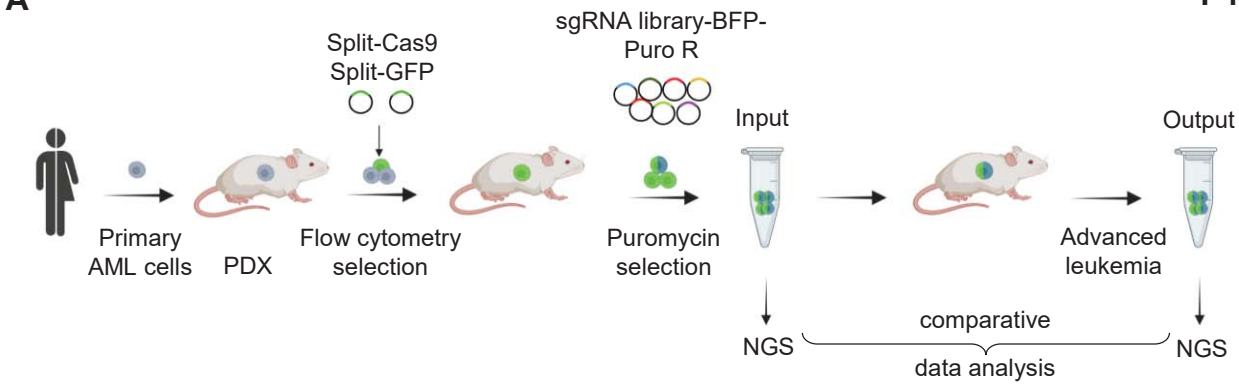
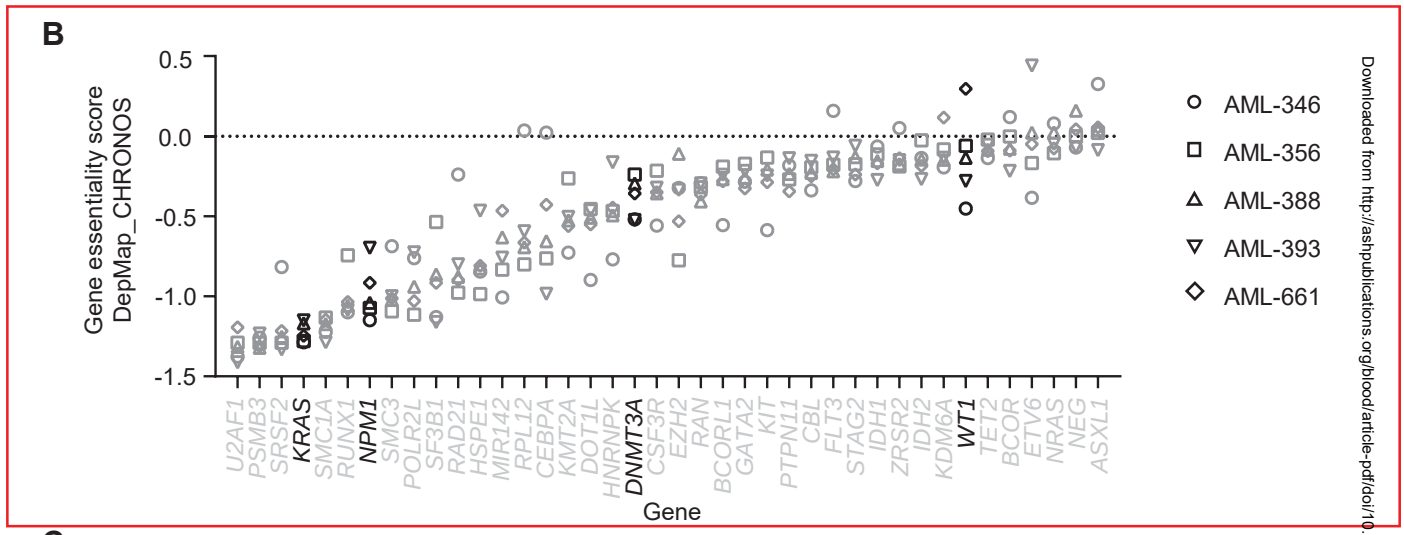
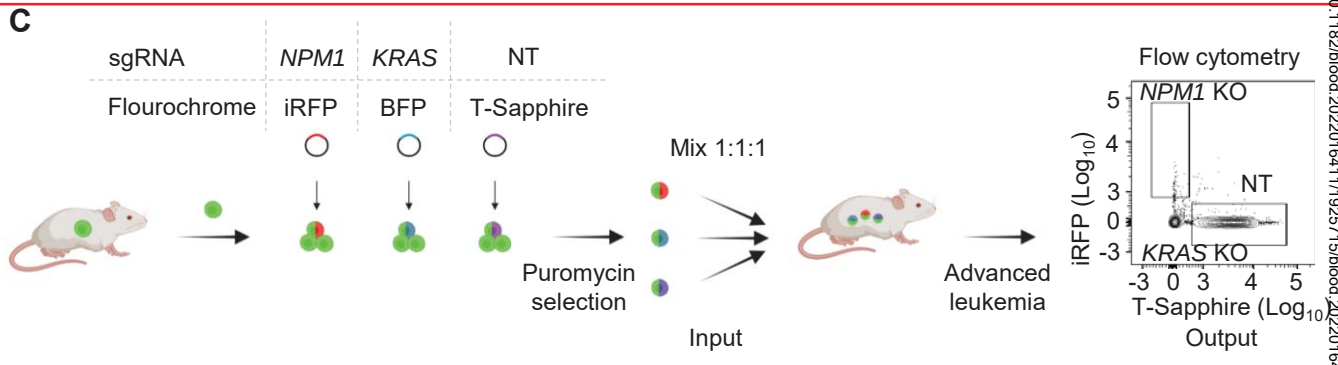
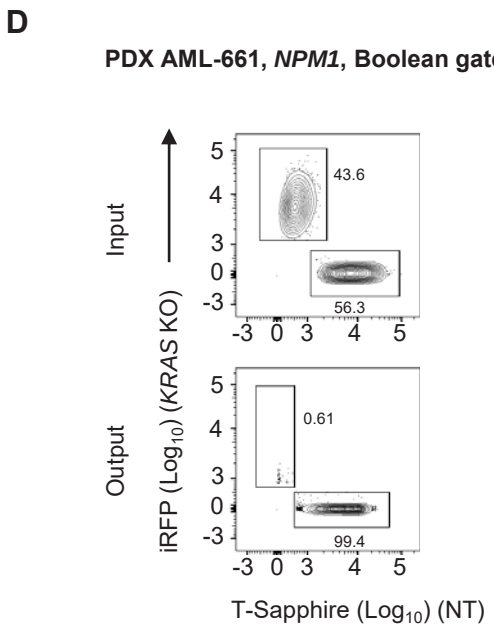
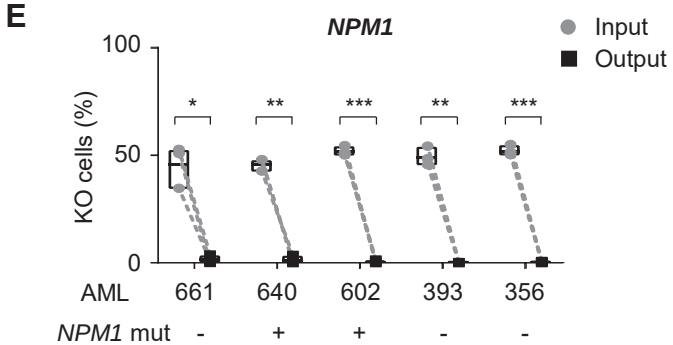
Figure 1.











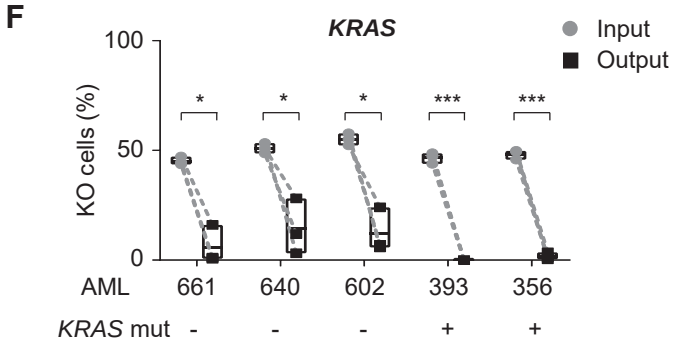
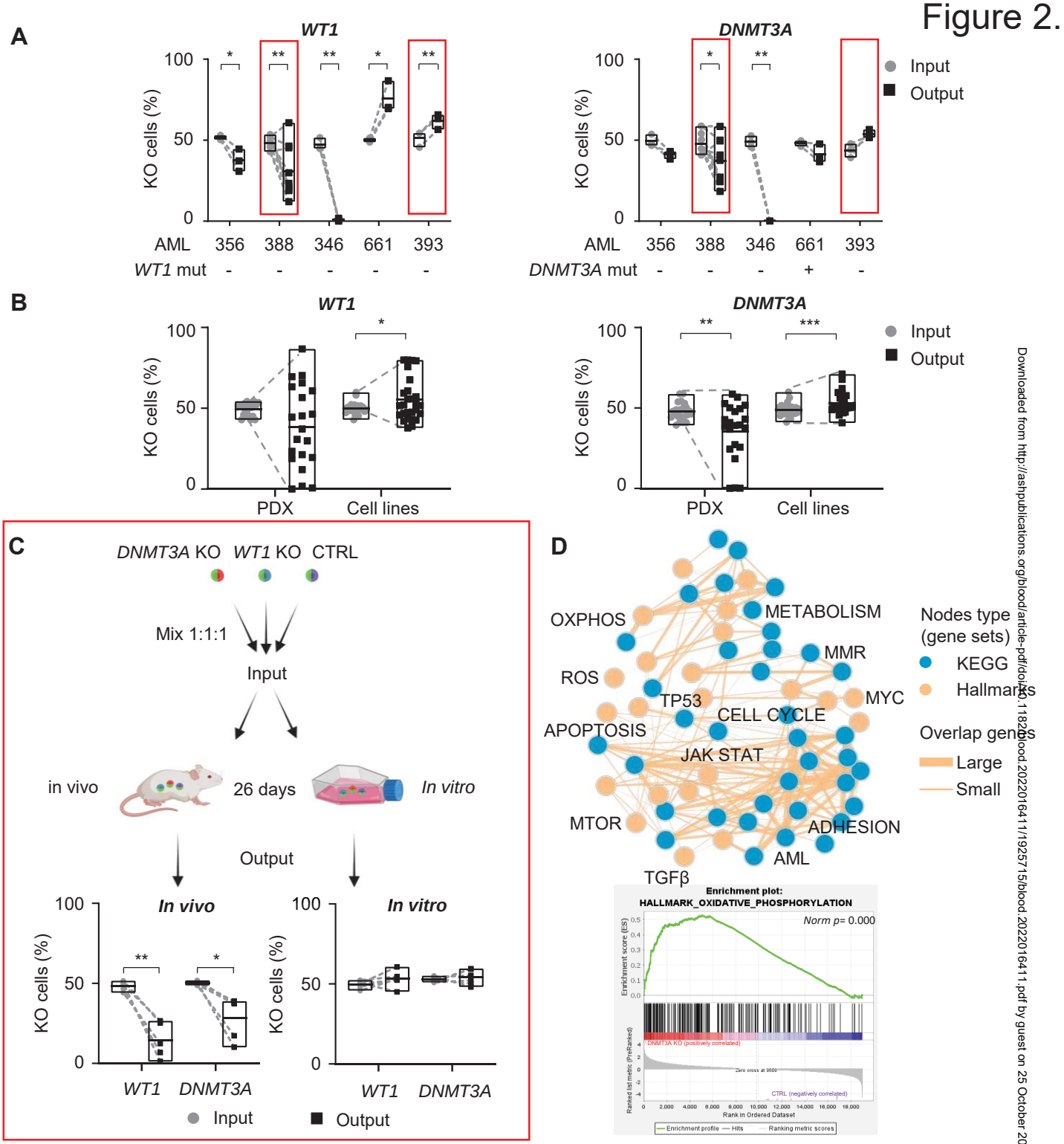


Figure 2.



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

Running Title: *WT1* and *DNMT3A* are essential in AML PDX *in vivo*

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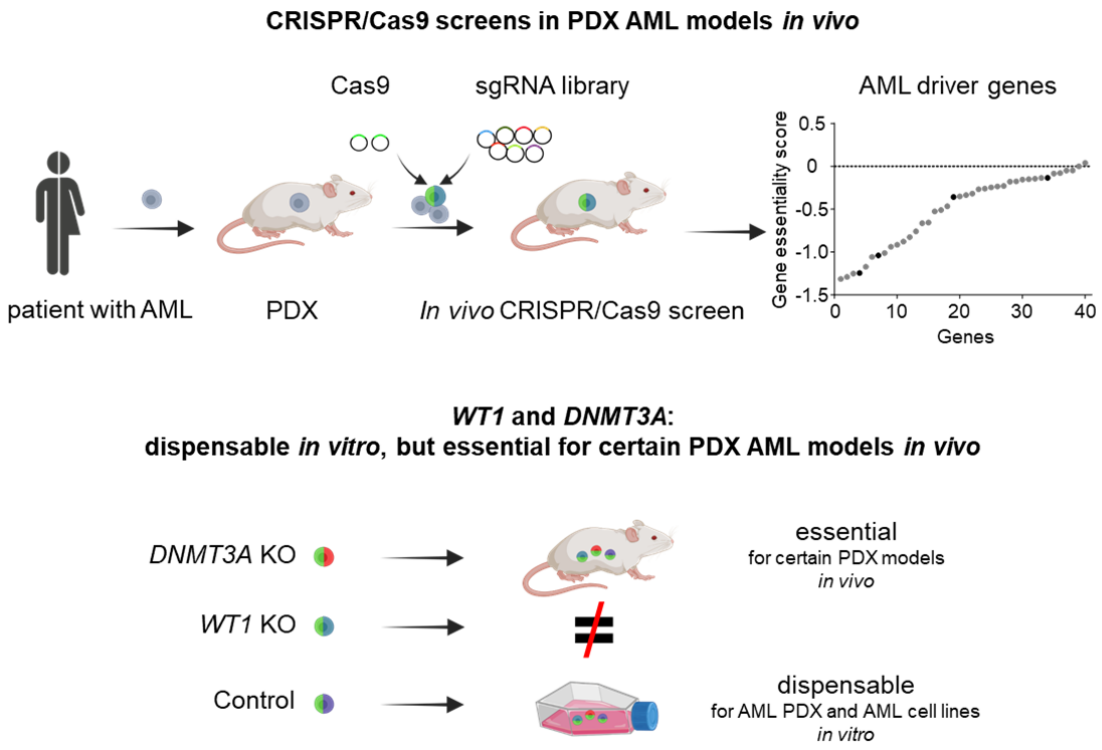
Visual abstract



To the Editor

Patients with acute myeloid leukemia (AML) suffer from 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 FLT3 kinase treated with Midostaurin and AML with mutated isocitrate dehydrogenase (IDH) responding to Ivosidenib[2]. Here 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 limitation, cell lines might acquire non physiologic alterations and discrepant results have been described, e.g., 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/Cas9 knockout 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 (Tables S1-S3). Genetically engineered PDX (GEPDX) models were generated that stably expressed recombinant Cas9 (Figure S1A).

We had recently established *in vivo* CRISPR/Cas9 dropout screens in GEPDX models of acute lymphoblastic leukemia[13]; here, we transferred the technique to AML which resulted in favourable quality controls (Figures 1A and S2A). 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 sgRNAs per target gene, together with positive and negative controls (Table S4 and S5); the

library was cloned into a lentiviral vector that co-expressed recombinant markers to enrich successfully transduced cells, using our CLUE technique (Figures S1 and S2A; Tables S3 and S4)[14].

A CRISPR/Cas9 dropout screen was performed with five 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 (Figures 1B and S2B; Tables S6-S8). 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 knockout 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-knockout 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[17]. Recombinant fluorochromes enabled an unbiased differentiation of cell populations by flow cytometry (Figures 1C-D and S3). For each gene of interest as well as for non-targeting (NT) controls, three different, highly efficient sgRNAs were tested in three independent mixtures (Figures S4 and S5). 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 knockout of *NPM1* completely eliminated AML GEPDX cells in all GEPDX models tested *in vivo* (Figures 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* knockout revealed a strong dropout in all GEPDX models studied, which was significantly more pronounced in $KRAS^{mutant}$ PDX models than $KRAS^{wildtype}$ PDX models (Figures 1F and S6). 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 two genes with poorly defined roles in oncogenes and for which we had suitable PDX models with appropriate variant allele frequencies (VAFs) at

hand (Table S1). While data on *WT1* as an oncogene is controversial[18,19], *DNMT3A* mainly represents a tumor suppressor, required for hematopoietic differentiation[20-23]. 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 upon gene knockout (Figures S7-S9; Table S1). In contrast and surprisingly, in *in vivo* GEPDX models, we discovered a pronounced dropout of either of both genes upon knockout 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 (Figure S10). 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 (Figure S6D). In the transcriptome, knockout of *WT1* or *DNMT3A* was accompanied by regulation of biological processes such as apoptosis and oxidative phosphorylation (Figures 2D and S11).

When characterizing *in vivo* essentiality in more detail, we found that knockout of *WT1* induced a certain increase in the anti-tumor effect of Cytarabine, an important drug in routine treatment of AML (Figure S12). *WT1* knockout reduced the capacity of AML-346 cells to home to the bone marrow environment upon either intrafemural or intravenous cell injection followed by early in vivo growth disadvantage, suggesting an impaired tumor-niche interaction (Figure S13 and S14). Knockout of either *WT1* or *DNMT3A* reduced the numbers of leukemia-initiating cells in competitive limiting dilution transplantation assays and prevented re-engraftment of AML-346 into secondary recipient mice, with and without prior cell enrichment, indicating that stem cell surrogates were depleted upon *WT1* or *DNMT3A* KO (Figures 2E and S15). 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

to *KRAS*. Knockout of *WT1* and *DNMT3A* impaired PDX AML growth *in vivo*, attenuated the tumor-niche interaction, eradicated AML stem cells and increased treatment response.

While 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, e.g., to personalize pharmacologic precision therapy. Our data encourage testing additional genes recurrently mutated in AML for their essentiality in PDX models *in vivo*, e.g., additional dropout candidates from our screens.

The essential function of WT1 identified here fits with its previously described oncogenic function[18], while different phenotypes between different PDX models might mirror conflicting data on *WT1* obtained during leukemogenesis[18,19].

For DNMT3A, a prevailing tumor suppressor function was described[20-22], making a dependency function unlikely. Amid complexity, a tumor-supportive function of mutant *DNMT3A* was reported in specific AML subsets, e.g., AML driven by a partial tandem duplication in KMT2A[24]. AML-388 harbors a KMT2A-AFDN translocation (Table S2), 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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Authors' contributions

M.G. and Y.G. designed and performed experiments and designed figures; D.A. performed CLUE cloning; G.K. and M.M. analyzed DepMap data; B.V. established PDX models and *in vivo* chemotherapy protocols; K.S. provided primary AML 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 scrb seq data; and I.J. designed the study, guided the experiments and wrote the manuscript, with the help of all authors.

Conflict of Interests

M.M. is a former employee at AstraZeneca, academically collaborates with AstraZeneca, GSK and Roche, and receives funding from GSK and Roche.

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Figure Legends

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 sgRNA library (see Figure S1 for constructs). Transgenic cells were enriched by flow cytometry (Cas9-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., 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 Figure S2 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* knockout (*KRAS* KO), *NPM1* knockout (*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 Figure S3 for the step-by-step analysis and Figures S4 and S5 for quality controls).

D Representative flow cytometry plots for *KRAS* KO1 and NT-1 in AML-661, using Boolean gating.

E, F Quantitative summaries of the knockout effects for *NPM1* (**E**) and *KRAS* (**F**) in all PDX models studied. Each dot represents the percentage of GOI 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 two-tailed paired t-test are shown if they were significant; *p<0.05, **p<0.01 and ***p<0.001.

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 1CD, except that *WT1* and *DNMT3A* were studied (see Figure S6 for quality controls).

B Comparing gene dependency in PDX models versus cell lines. Raw data from Figures 2A, S8 and S9 are summarized using a single dot for each single KO of each PDX model or cell line. For each PDX model or cell line, three sgRNAs per gene were studied. Results of an unpaired t-test are shown if they were significant (*p<0.05, **p<0.01 and ***p<0.001).

C Comparing behavior of PDX cells with KO *in vitro* versus *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<0.05, **p<0.01 and ***p<0.001).

D Transcriptome of AML-356, AML-388, AML-661 and AML-346 cells with *DNMT3A* knockout were compared to NT control (raw and complementary data in Figure S10). Gene enrichment map shows gene overlap (lines) in gene sets of hallmarks (orange nodes) and 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 upon KO of *DNMT3A* (Normalized enrichment score (NES) = 2.1537, p-value < 0.001, adjusted p-value (FDR q-value) < 0.001).

E Limiting dilution transplantation assay. PDX AML-346 cells were transduced with sgRNAs against *WT1* or *DNMT3A* or 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, BM was analyzed by flow cytometry and data analyzed using the ELDA software. Mean (solid lines) and 95% confidence interval (CI, dashed line) are depicted.