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# *CEBPA*-mutated leukemia is sensitive to genetic and pharmacological targeting of the MLL1 complex

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## Abstract

The gene encoding the transcription factor C/EBPα is mutated in 10–15% of acute myeloid leukemia (AML) patients. N-terminal *CEBPA* mutations cause ablation of full-length C/EBPα without affecting the expression of a shorter oncogenic isoform, termed p30. The mechanistic basis of p30-induced leukemogenesis is incompletely understood. Here, we demonstrate that the MLL1 histone-methyltransferase complex represents a critical actionable vulnerability in *CEBPA*-mutated AML. Oncogenic C/EBPα p30 and MLL1 show global co-localization on chromatin and p30 exhibits robust physical interaction with the MLL1 complex. CRISPR/Cas9-mediated mutagenesis of MLL1 results in proliferation arrest and myeloid differentiation in C/EBPα p30-expressing cells. In line, *CEBPA*-mutated hematopoietic progenitor cells are hypersensitive to pharmacological targeting of the MLL1 complex. Inhibitor treatment impairs proliferation and restores myeloid differentiation potential in mouse and human AML cells with *CEBPA* mutations. Finally, we identify the transcription factor GATA2 as a direct critical target of the p30-MLL1 interaction. Altogether, we show that C/EBPα p30 requires the MLL1 complex to regulate oncogenic gene expression and that *CEBPA*-mutated AML is hypersensitive to perturbation of the MLL1 complex. These findings identify the MLL1 complex as a potential therapeutic target in AML with *CEBPA* mutations.


## Introduction

Aberrant myeloid homeostasis in Acute myeloid leukemia (AML) leads to an increase in myeloid progenitor cells at the expense of mature blood cells [1]. Ten to fifteen percent~~age~~ of AML patients harbor mutations in the *CEBPA* gene [2, 3, 4], which encodes the transcription factor (TF) CCAAT/enhancer binding protein alpha (C/EBPα). C/EBPα In the PDF document, this word is seperated over the line

break. Please keep as one word _ controls self-renewal properties of hematopoietic stem and progenitor cells (HSPCs) as well as critical steps of myeloid differentiation [5, 6, 7]. Alternative usage of different translation initiation codons results in the expression of full-length (42 kDa) and truncated (30 kDa) isoforms of C/EBPα, termed p42 and p30, respectively [8].

**AQ1**

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In AML, *CEBPA* mutations frequently occur in the N-terminal part of the gene and introduce frameshifts that result in selective ablation of p42. Most AML patients carry biallelic *CEBPA* mutations, combining an N-terminal frameshift with a C-terminal in-frame mutation, which abolishes dimerization and DNA binding [9, 10].

C/EBPα p30 is able to regulate transcriptional processes through recruitment of chromatin-modifying complexes, such as histone methyltransferases [6, 11, 12]. For instance, p30 interacts with WDR5, a component of several protein complexes involved in transcriptional control [13]. These assemblies include SET/MLL complexes which positively regulate gene expression by catalyzing tri-methylation of lysine 4 of histone H3 (H3K4me3) and are crucial for the maintenance of HSPCs [14, 15, 16, 17]. Assembly of different members of the histone-methyltransferase mixed-lineage leukemia family (MLL1-4, also referred to as KMT2A-D) with their conserved binding partners WDR5, RBBP5, ASH2L, and DPY30 is necessary for full enzymatic activity of SET/MLL complexes [18, 19, 20]. Other interaction partners such as Menin and Lens epithelium-derived growth factor (LEDGF, PSIP1) mediate chromatin recruitment of SET/MLL complexes [21, 22, 23]. We hypothesized that p30 and the MLL1 (KMT2A) complex cooperate in the regulation of transcriptional programs that are critical for leukemogenesis.

We used a combination of biochemical, genetic and pharmacological approaches to investigate the role of the MLL1 complex in *CEBPA*-mutated AML. We show that p30 interacts with the MLL1 complex to control gene expression. Genetic and pharmacological targeting of the MLL1 complex caused proliferation arrest

and induced myeloid differentiation in C/EBPα-mutated AML cells. These data identify the transcriptional cooperation between C/EBPα p30 and MLL1 as an actionable vulnerability in *CEBPA*-mutated AML.

# Materials and methods

## CRISPR/Cas9 competition assay

An *SpCas9*-expressing subclone of *Cebpa*$^{p30/p30}$ cells was isolated after lentiviral expression of lenti-Cas9-Blast (Addgene, Cambridge, MA, USA). *SpCas9-Cebpa*$^{p30/p30}$ cells were transduced with sgRNA-containing LentiGuide-Puro-IRES-GFP constructs (Supplemental Table S1) in biological duplicates, obtaining transduction efficiencies of 20–40%. GFP levels were monitored by flow cytometry over time. Data were normalized to values on day 0 (normalized sgRNA = %GFP(dX)/%GFP(d0)) and a non-targeting control sgRNA (Ctrl, (normalized Ctrl/normalized sgRNA) *100%).

## Chromatin immunoprecipitation

*Cebpa*$^{p30/p30}$ cells were crosslinked with 11% formaldehyde (Thermo Fisher Scientific, Waltham, MA, USA) alone ( C/EBPα In the PDF document, this word is seperated over the line break. Please keep as one word _) or with 2 mM disuccinimidyl glutarate (DSG, THP, Vienna, Austria) (MLL1). After quenching, cells were lysed in SDS-containing buffer (Sigma-Aldrich, St. Louis, MO, USA) and incubated with anti-MLL1 (Bethyl Laboratories, Montgomery, TX, USA, A300-086A) and anti-C/EBPα (Santa Cruz, Dallas, Texas, USA, sc-9314) antibodies overnight. After isolating antibody-bound material using protein G-coupled magnetic beads (Dynabeads Protein G, Invitrogen, Camarillo, CA, USA) and de-crosslinking, enrichment of genomic regions was measured by qPCR (Supplemental Table S2).

## Cell viability assay

Cells were seeded in 96-well plates and treated with MI-463 or MI-503 in biological triplicates at indicated concentrations. Five days after treatment, cell viability was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) on a VICTOR X3 luminometer (PerkinElmer, Waltham, MA, USA).

## Statistics

Prism 5.01 software (Graphpad, La Jolla, CA, USA) was used for statistical analyses and data are shown as mean ± SD. Experiments were performed in duplicates/triplicates and/or repeated at least three times. Two-tailed Student's *t*-tests were used for *P* value determination: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, and $****P < 0.0001$.

Additional Materials and methods are described in Supplementary Methods.

# Results

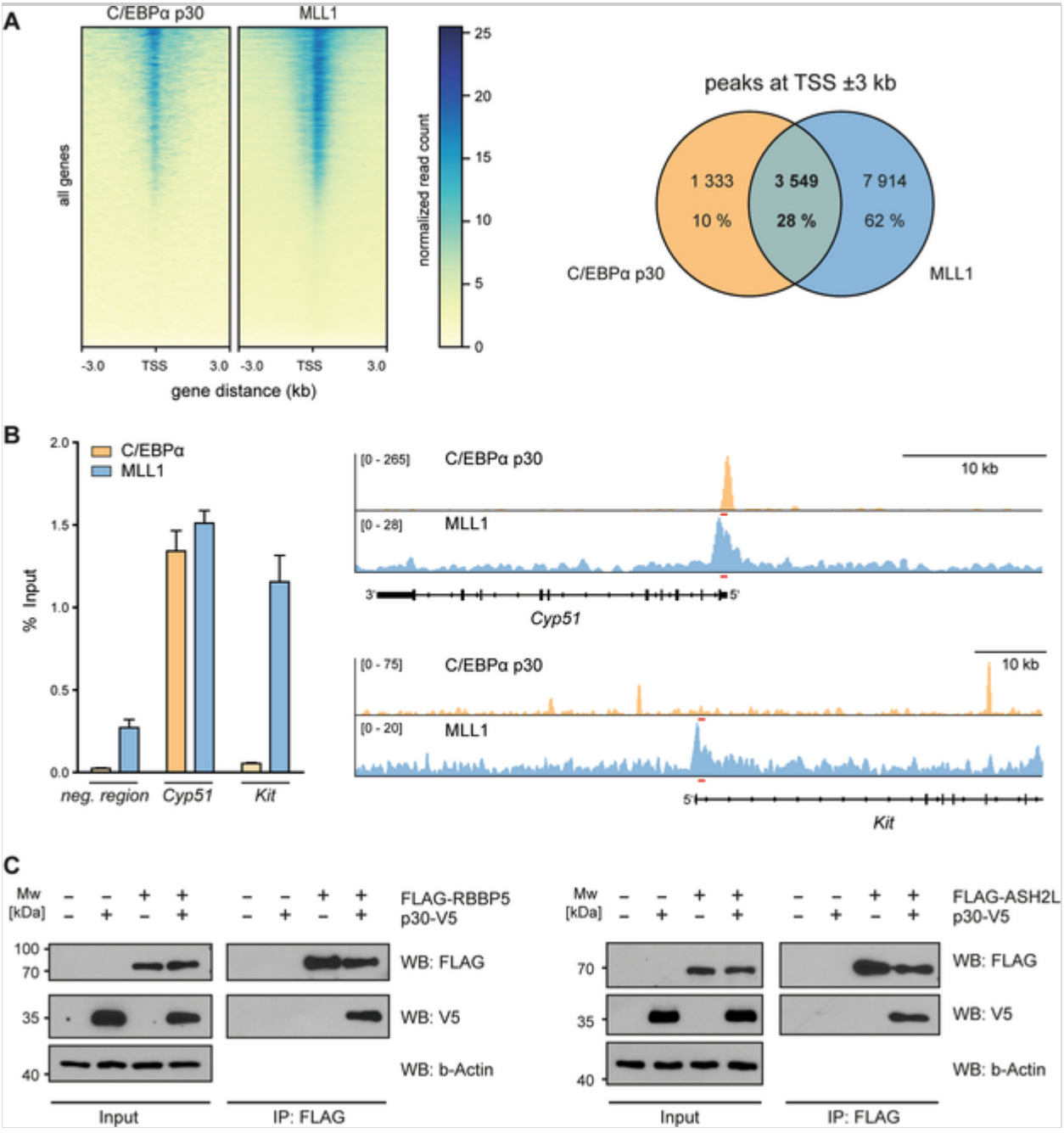
## C/EBPα p30 shows global genomic co-localization with MLL1

To investigate whether p30 cooperates with the MLL1 complex in transcriptional regulation we used a myeloid progenitor cell line derived from a mouse model of *CEBPA*-N-terminal AML [24] ($Cebpa^{p30/p30}$). $Cebpa^{p30/p30}$ cells appear as cytokine-dependent leukemic blasts and co-express c-Kit and Mac-1 (Supplemental Figure S1A-C). These cells were dependent on the original p30 driver lesion for sustained proliferation in culture, as shRNA-mediated knockdown of p30 resulted in immediate growth arrest (Supplemental Figure S1D-E). Chromatin immunoprecipitation followed by sequencing (ChIP-seq) using antibodies recognizing the C/EBPα C-terminus in $Cebpa^{p30/p30}$ cells identified 24,538 genomic binding sites of the p30 protein, of which 19.9% (4882 peaks) were localized within 3 kb of annotated transcription start sites (TSS). ChIP-seq for MLL1 in $Cebpa^{p30/p30}$ cells identified 47,069 peaks of which 24% (11,463 peaks) localized to promoter regions (Fig. 1a, Supplemental Figure S1F). C/EBPα p30 binding showed a strong overlap with global MLL1 occupancy, as 73% of p30-bound promoters were also occupied by MLL1 (3549 of 4882 peaks, Fig. 1a). Consistent with ChIP-seq data, ChIP-qPCR confirmed that p30 and MLL1 binding was detected at the *Cyp51* promoter, but not at the *Kit* promoter (Fig. 1b). Further, p30 co-precipitated with components of the MLL1 core complex, including Wdr5, Rbbp5, and Ash2l, but also with the canonical MLL1 binding partner Menin (Fig. 1c; Supplemental Figure S1G).

**Fig. 1**

Global co-localization of C/EBPα p30 and MLL1 in $Cebpa^{p30/p30}$ cells. **a** Left, heatmap showing p30 and MLL1 chromatin binding (< ± 3 kb of TSS). Right, Venn diagram showing overlapping ChIP-seq peaks within ± 3 kb of TSS between p30

(orange) and MLL1 (blue). **b** qPCR analysis (left), and ChIP-seq tracks (right) showing p30 and MLL1 binding in promoters of indicated genes. Red lines indicate position of ChIP-qPCR products. **c** Western blot (WB) analysis of lysates and FLAG-purifications from extracts of HEK293 cells expressing indicated constructs using indicated antibodies. Mw, molecular weight



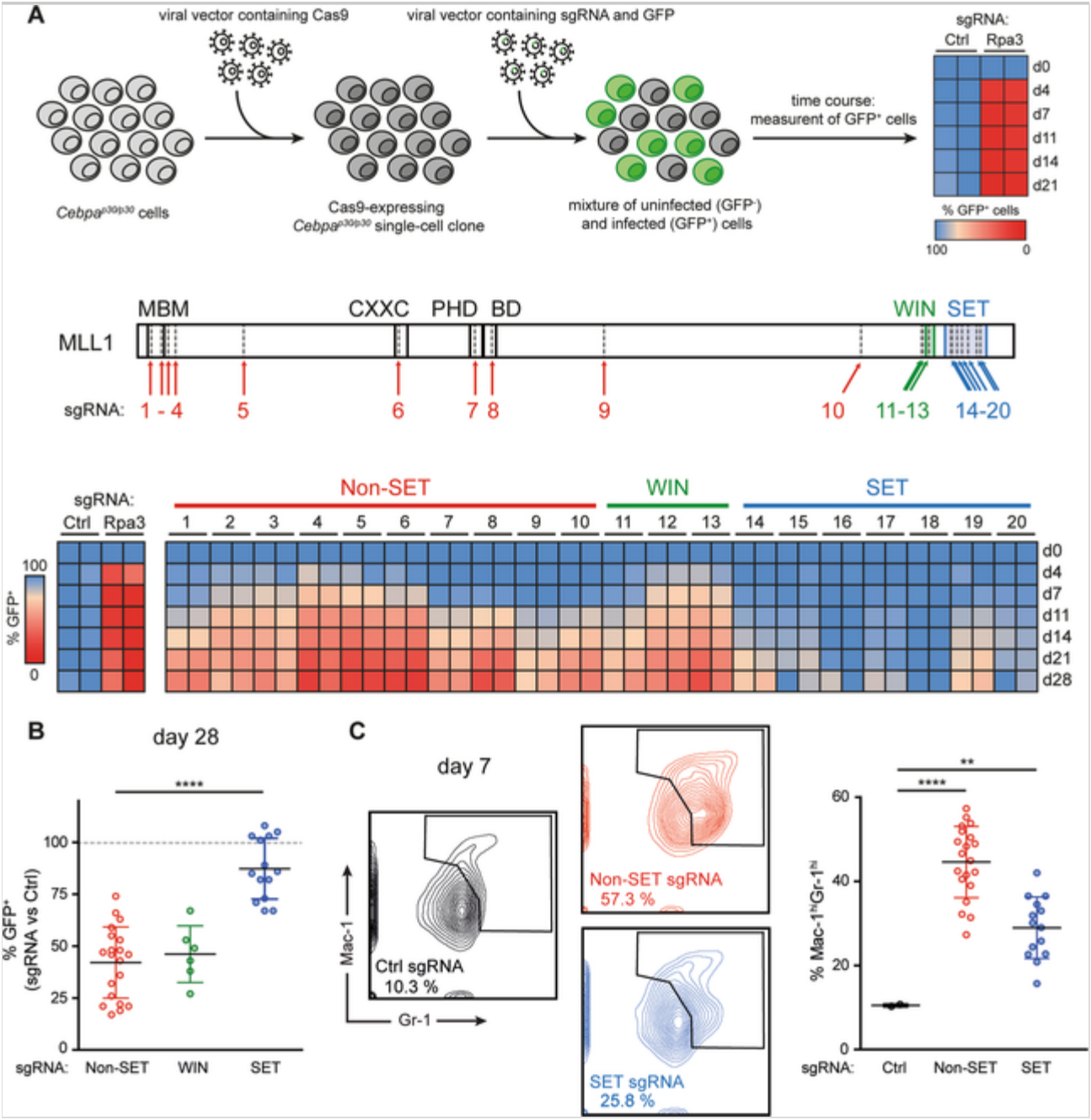

Thus, these results indicate that p30 interacts with canonical MLL1 complexes in *Cebpa*$^{p30/p30}$ cells.

## Proliferation of *Cebpa*$^{p30/p30}$ cells is MLL1-dependent

The prominent genomic co-localization of p30 and MLL1 suggests a functional role of MLL1 in *CEBPA*-N-terminal AML. We isolated and characterized an *SpCas9*-expressing subclone from *Cebpa*$^{p30/p30}$ cells (Supplemental Figure S2A-B) and transduced it with lentiviral vectors enabling sgRNA expression coupled to a GFP reporter gene. This allows dynamic monitoring of competing growth kinetics of sgRNA-expressing GFP-positive (GFP$^{+}$) cells versus uninfected GFP-negative (GFP$^{-}$) cells (Fig. 2a). In contrast to a control sgRNA (Ctrl), targeting of *Rpa3*, a known essential gene for DNA replication, resulted in fast negative selection of GFP$^{+}$ cells (Fig. 2a). Similarly, CRISPR/Cas9-mediated mutagenesis of p30 caused a strong proliferative disadvantage in this assay (Supplemental Figure S2C-D). *Cebpa* targeting resulted in elevated levels of the maturation surface markers Mac-1 and Gr-1 indicating increased signs of myeloid differentiation (Supplemental Figure S2E).

**Fig. 2**

*Cebpa*$^{p30/p30}$ cells are dependent on a functional MLL1 protein. **a** Top, schematic representation of the CRISPR/Cas9-based competition assay. Middle, schematic structure of the MLL1 protein. Positions of sgRNAs are indicated by dashed gray lines and arrows. MBM, Menin-binding motif; CXXC, CXXC motif; PHD, plant homeodomain finger; BD, bromodomain; WIN, WDR5-interacting domain; SET, (enzymatic) Su(var)3-9, Enhancer-of-zeste and Trithorax domain. Bottom, heatmap showing survival of GFP-positive (GFP$^{+}$) sgRNA-expressing cells over time. Ctrl, negative control; Rpa3, positive control. **b** Scatter plot representing percentages of GFP$^{+}$ cells expressing indicated sgRNAs (day 28). Populations were divided into three groups (Non-SET: sgRNAs #1–10, WIN: sgRNAs #11–13, SET: sgRNAs #14–20). The dashed gray line indicates the percentage of GFP$^{+}$ cells in populations expressing a control sgRNA. **c** Flow cytometric analysis of Mac-1 and Gr-1 after *Mll1* targeting with indicated sgRNAs (gated on GFP$^{+}$ cells). Mac-1$^{hi}$Gr-1$^{hi}$ levels are plotted for three groups (Ctrl, Non-SET: sgRNAs #1–10, SET: sgRNAs #14–20)




We investigated the functional role of MLL1 in N-terminal *CEBPA*-mutated AML at domain resolution by CRISPR/Cas9-mediated mutagenesis of different annotated domains in the *Mll1* gene (Fig. 2a). In line with previous results [12], mutation of the Wdr5-interacting domain (WIN) of MLL1 strongly impaired proliferation of *Cebpa*$^{p30/p30}$ In the PDF document, this word is seperated over the line break. Please keep as one word _ cells (Fig. 2a, b). Mutagenesis of MLL1 domains that are important for its integration into the MLL1 multiprotein complex and for its chromatin recruitment, such as the Menin-binding-motif

(MBM, sgRNAs #1–2), the CXXC motif (sgRNA #6), the plant homeodomain (PHD, sgRNA #7), and the bromodomain (sgRNA #8), also caused strong anti-proliferative effects (Fig. 2a, b). In contrast, mutational targeting of the enzymatic SET domain did not affect proliferation (Fig. 2a, b), indicating that the methyltransferase activity of MLL1 is not crucial for the maintenance of *CEBPA*-N-terminal AML. While targeting the amino-terminus as well as functional domains across the *Mll1* gene resulted in increased signs of myeloid differentiation, this effect was much weaker when the MLL1 SET domain was mutated (Fig. 2c).

Taken together, multiple functional domains of the MLL1 protein are required for proliferation of *Cebpa*$^{p30/p30}$ cells.

## *CEBPA*-mutated AML cells are sensitive to small-molecule-mediated targeting of the MLL1 complex

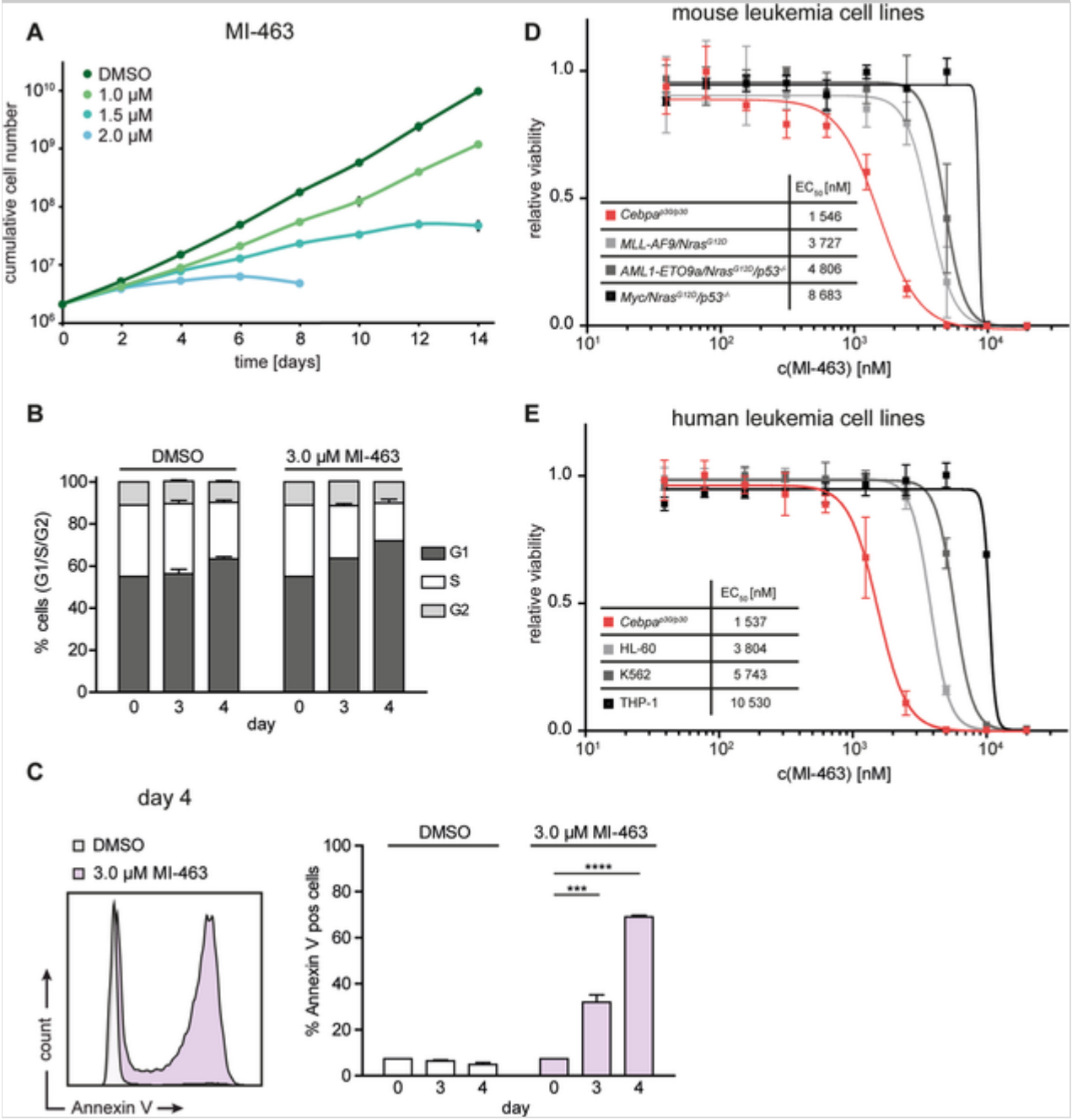
Given the genetic requirement for MLL1 in *CEBPA*-mutated AML, we reasoned that pharmacological perturbation of the MLL1 complex could selectively target p30-dependent cellular functions. MI-463 and MI-503 are potent small-molecule inhibitors that disrupt the Menin-MLL interaction, thereby compromising the integrity and function of the MLL1 complex [25, 26, 27]. Menin-MLL inhibitors were shown to provide survival benefits in AML models with MLL-rearrangements and *NPM1* mutations [26, 28, 29].

Treatment of *Cebpa*$^{p30/p30}$ cells with MI-463 and MI-503 led to time- and dose-dependent impairment of proliferation, induction of cell cycle arrest and apoptosis (Fig. 3a–c; Supplemental Figure S3A-C). *Cebpa*$^{p30/p30}$ cells showed a 2.4–5.6-fold higher sensitivity toward Menin-MLL inhibition than leukemia cell lines derived from different AML mouse models, including *MLL-AF9/Nras*$^{G12D}$, *AML1-ETO9a/Nras*$^{G12D}$*/p53*$^{-/-}$ or *Myc/Nras*$^{G12D}$*/p53*$^{-/-}$ (Fig. 3d, Supplemental Figure S3D). Similarly, *Cebpa*$^{p30/p30}$ cells showed hypersensitivity toward MI-463 and MI-503 when compared to a panel of human leukemia cell lines representing different molecular aberrations (Fig. 3e, Supplemental Figure S3E).

**Fig. 3**

Pharmacological targeting of MLL1 function results in decreased self-renewal and induction of apoptosis in *Cebpa*$^{p30/p30}$ cells. **a** Growth curves of *Cebpa*$^{p30/p30}$ cells treated with indicated concentrations of MI-463 or DMSO. **b** Cell cycle analysis of

*Cebpa*$^{p30/p30}$ cells at indicated time points after MI-463 treatment. **c** Flow cytometric analysis of apoptosis at indicated time points after MI-463 treatment. **d**, **e** Representative dose response curves for MI-463 in *Cebpa*$^{p30/p30}$ cells (red) and mouse (**d**) or human (**e**) leukemia cell lines

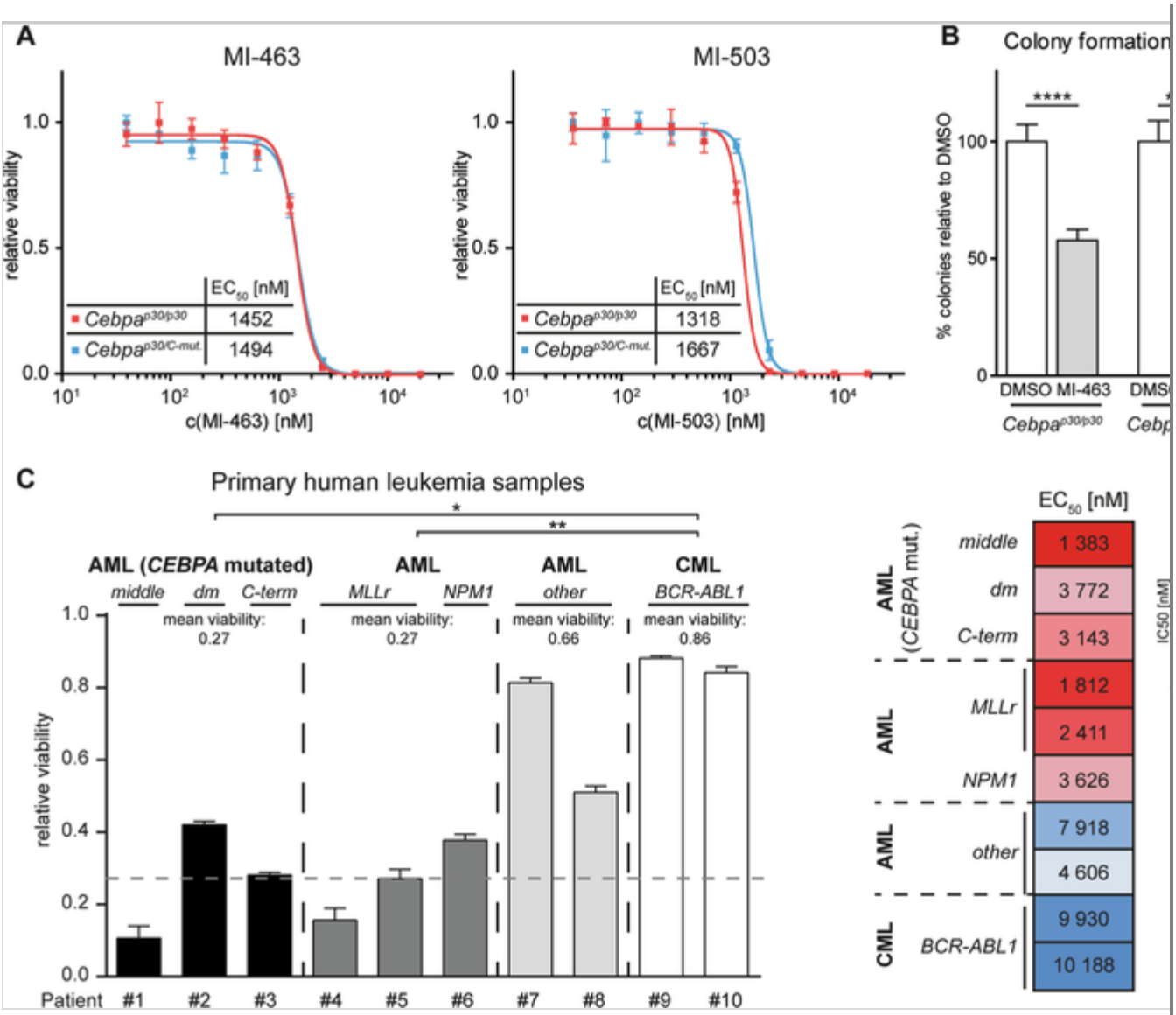



In AML with biallelic *CEBPA* mutations, p30 is the sole functional C/EBPα protein, as C-terminal mutations give rise to dimerization- and DNA binding-deficient proteins. A hematopoietic progenitor cell line derived from an AML mouse model for biallelic *CEBPA* mutations [30] ( *Cebpa*$^{p30/C\text{-}mut.}$ In the PDF

document, this word is seperated over the line break. Please keep as one word _) was also hypersensitive toward MI-463 and MI-503 treatment (Fig. 4a). Treatment of *Cebpa*$^{p30/C\text{-}mut.}$ cells resulted in time- and dose-dependent impairment of proliferation and induction of apoptosis (Supplemental Figure S4A-B). Primary fetal liver-derived hematopoietic progenitor cells from both *Cebpa*-mutated AML mouse models were highly sensitive toward MI-463 and MI-503 treatment in colony-formation assays, indicating that inhibitor hypersensitivity was not acquired during the process of cell line establishment (Fig. 4b, Supplemental Figure S4C). MLL1 complex perturbation caused loss of compact colony formation and induced the formation of small, dispersed colonies reminiscent of mature myeloid cell clusters (Supplemental Figure S4D).

**Fig. 4**

*Cebpa*$^{p30/C\text{-}mut.}$ cells and primary human AML cells with *CEBPA* mutations are sensitive to pharmacological targeting of the MLL1 complex. **a** Dose response curves for MI-463 (left) and MI-503 (right) in *Cebpa*$^{p30/p30}$ (red) and *Cebpa*$^{p30/C\text{-}mut.}$ cells (blue). **b** Colony-formation assay of primary mouse *Cebpa*$^{p30/p30}$ and *Cebpa*$^{p30/C\text{-}mut.}$ cells in the presence of MI-463 (4 µM) or DMSO. Counts were normalized to colonies in the DMSO samples for each genotype.) **c** Left, viability of primary human leukemia cells after MI-463 treatment (5 days, 4 µM). Data were normalized to DMSO controls for each patient sample. The dashed line indicates mean viability of *CEBPA*-mutant AML samples. Right, Heatmap representation of half-maximal effective concentrations ($EC_{50}$) of MI-463 in primary human leukemia cells. C-term, C-terminal; dm, double-mutated; MLLr, MLL-rearranged; AML (other): *CEBPA*-, *NPM1*- and MLLr-negative




MI-463 and MI-503 caused a significant decrease in cellular viability and half-maximal responses in primary human *CEBPA*-mutated AML samples as well as in primary human AML cells with MLL-rearrangements and *NPM1* mutations, while it had much weaker effects on the viability of other AML samples negative for these mutations and on cells from BCR-ABL1-positive chronic myeloid leukemia (CML) patients (Fig. 4c, Supplemental Figure S4E-F, Supplemental Table S3).

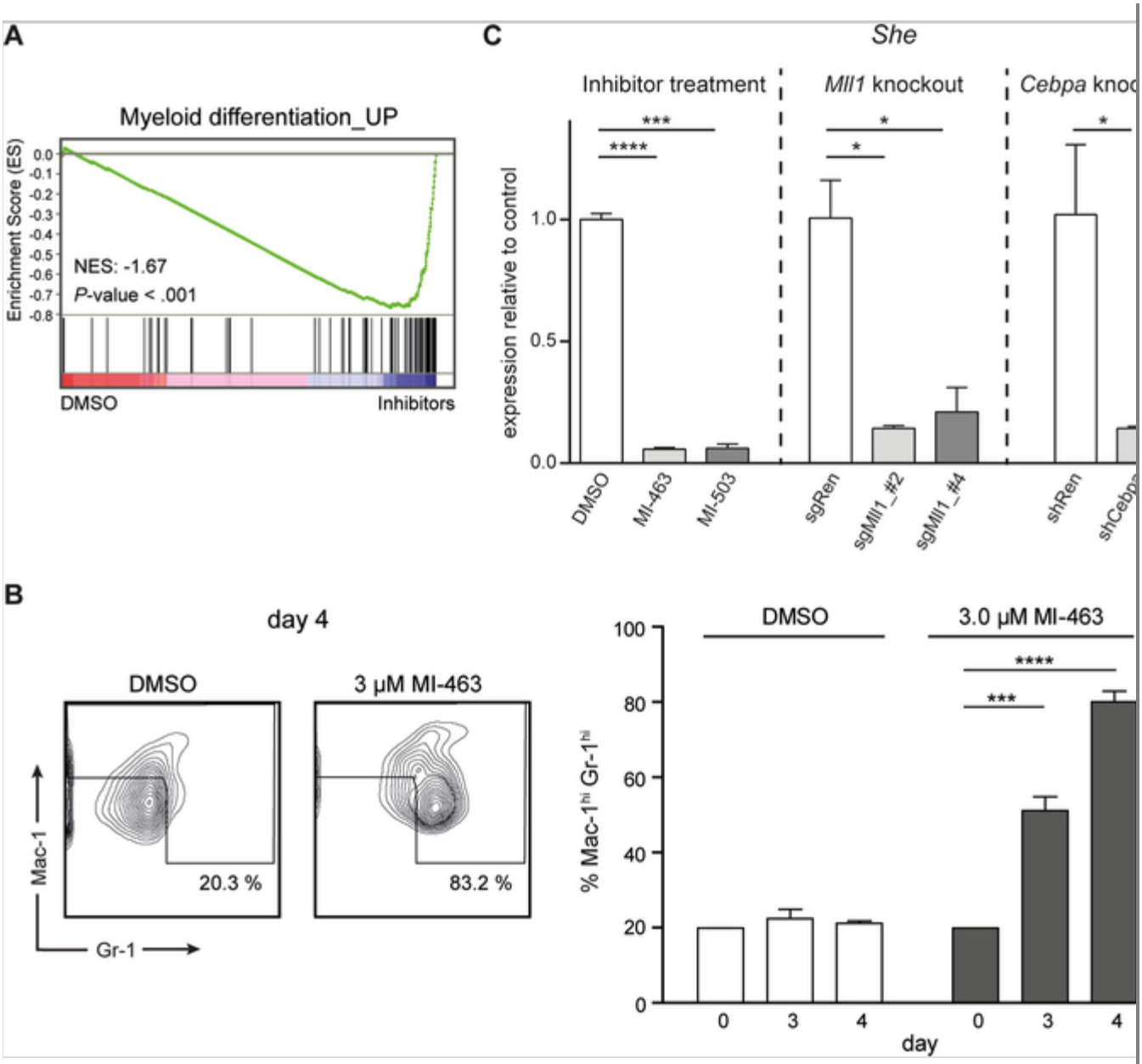
Taken together, these data reveal that *CEBPA*-mutated AML is highly sensitive to pharmacological targeting of the MLL1 complex.

## Menin-MLL inhibition induces terminal myeloid differentiation of C/EBPα p30-expressing cells

To investigate the molecular consequences of MLL1 complex perturbation in *CEBPA*-mutated AML, we performed RNA sequencing (RNA-seq) upon inhibitor treatment of *Cebpa*$^{p30/p30}$ cells. Both inhibitors had comparable and strongly overlapping effects on global gene expression changes (Supplemental Figure S5A-B). The majority of genes was upregulated in response to either MI-463 and/or MI-503 (608 upregulated genes vs. 212 downregulated genes; Supplemental Figure S5C). Gene set enrichment analysis (GSEA) revealed that inhibitor treatment induced transcriptional changes associated with myeloid differentiation, whereas self-renewal-associated signatures were depleted (Fig. 5a, Supplemental Figure S5D). MI-463 and MI-503 treatment caused upregulation of the myeloid marker gene *Lyz2* (Supplemental Figure S5E) and a time- and dose-dependent increase in surface expression of the myeloid differentiation markers Mac-1 and Gr-1 (Fig. 5b, Supplemental Figure S5F).

**Fig. 5**

Loss of MLL1 function restores terminal myeloid differentiation potential of *Cebpa*$^{p30/p30}$ cells. **a** GSEA showing global upregulation of genes associated with myeloid differentiation in *Cebpa*$^{p30/p30}$ cells upon inhibitor treatment. NES, normalized enrichment score. **b** Flow cytometric analysis of Mac-1 and Gr-1 surface expression upon MI-463 treatment at indicated time points. **c** qRT-PCR analysis of *She2* in *Cebpa*$^{p30/p30}$ cells upon inhibitor treatment (day 2), CRISPR/Cas9-mediated *Mll1* mutagenesis (day 14) or shRNA-mediated *Cebpa* knockdown (day 9) relative to control. MI-463: 3.0 µM; MI-503: 2.5 µM




These results indicate that pharmacological perturbation of the MLL1 complex can overcome the differentiation block of *Cebpa*$^{p30/p30}$ cells, restoring terminal myeloid differentiation potential.

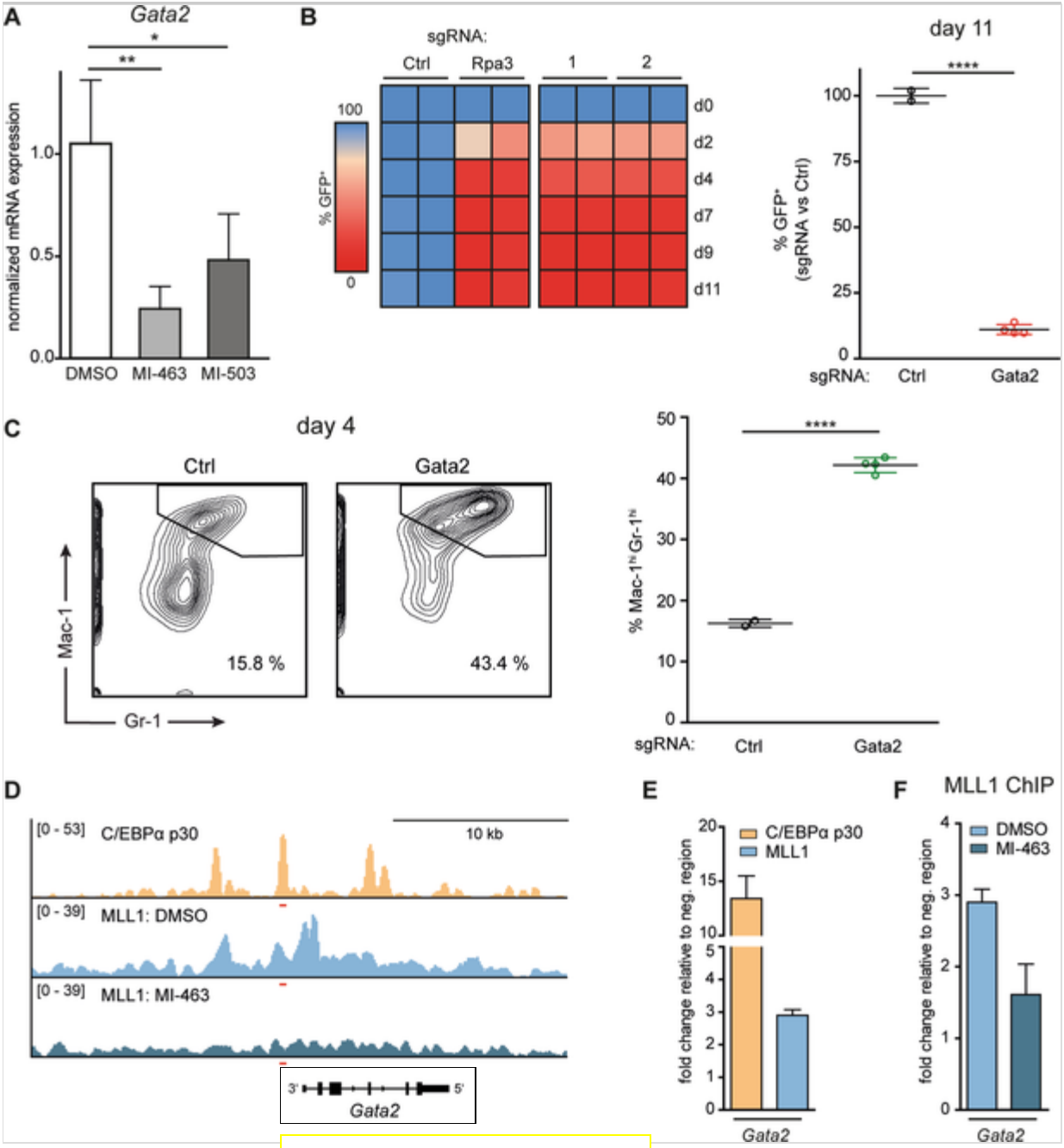
## GATA2 is a critical effector of the p30-MLL1 axis

Genes with strongly downregulated expression upon Menin-MLL inhibition showed comparable responses after CRISPR/Cas9-mediated *Mll1* targeting and shRNA-mediated *Cebpa* knockdown, indicating cooperative gene regulation by C/EBPα p30 and MLL1 (Fig. 5c, Supplemental Figure S5G). To systematically identify critical effectors of p30- and MLL1-dependent gene regulation, we

focused on genes that were upregulated upon C/EBPα p30 overexpression in myeloid cells [12]. Thirteen genes were induced by p30, downregulated upon Menin-MLL inhibition and showed robust p30 binding within 10 kb upstream of their TSS, implying direct, positive transcriptional regulation that depends on p30 and MLL1 (Supplemental Figure S6A). This list was highly enriched for factors regulating normal and aberrant hematopoiesis (Supplemental Figure S6B). In addition to *Sox4*, a known oncogenic target in *CEBPA*-mutated AML [31] (Supplemental Figure S6C-D), *Gata2* fulfilled all criteria for a potential effector of the p30-MLL1 axis. GATA2, is a zinc-finger TF that is frequently de-regulated or mutated in leukemia [32, 33, 34]. *Gata2* expression was upregulated by p30 and strongly downregulated upon Menin-MLL inhibitor treatment (Fig. 6a, Supplemental Figure S6E). CRISPR/Cas9-mediated mutagenesis of *Gata2* (Supplemental Figure S6F) caused a rapid decrease of $GFP^+$ sgRNA-expressing cells and induced terminal myeloid differentiation (Fig. 6b, c). p30 and MLL1 showed extensive co-localization in the *Gata2* promoter, suggesting cooperative regulation of *Gata2* expression (Fig. 6d, e). Indeed, MI-463 treatment of *Cebpa*$^{p30/p30}$ In the PDF document, this word is seperated over the line break. Please keep as one word cells resulted in a strong decrease in MLL1 occupancy at the *Gata2* locus (Fig. 6d, f).

**Fig. 6**

GATA2 is a critical effector of the p30-MLL1 axis. **a** qRT-PCR analysis of *Gata2* in *Cebpa*$^{p30/p30}$ cells treated with MI-463 (3.0 µM) or MI-503 (2.5 µM) for 2 days, normalized to DMSO treatment. **b** Left, heatmap showing survival of $GFP^+$ sgGata2-expressing cells (#1–2) over time. Ctrl, negative control; Rpa3, positive control. Right, scatter plot representing the percentage of $GFP^+$ cells (day 11). **c** Flow cytometric analysis of Mac-1 and Gr-1 surface expression after *Gata2* targeting. **d** ChIP-seq tracks for p30 (orange) and MLL1 (treated with DMSO (blue) or 3.0 µM MI-463 (petrol)) at the *Gata2* promoter in *Cebpa*$^{p30/p30}$ cells. Red lines indicate positions of ChIP-qPCR products. **e** ChIP-qPCR analysis of p30 and MLL1 binding to the *Gata2* promoter in *Cebpa*$^{p30/p30}$ cells. **f** ChIP-qPCR analysis of MLL1 binding to the *Gata2* promoter in *Cebpa*$^{p30/p30}$ cells treated with DMSO or 3.0 µM MI-463

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Together, these resul LL1 complex cooperatively regulate *Gata2* expre s an effector of the p30-mediated differentiation block and leukemogenesis.

# Discussion

Here we demonstrate that the ~~p30~~ C/EBPα p30 isoform and the MLL1 complex co-localize on chromatin, enabling the cooperative regulation of specific target genes. *CEBPA*-mutated AML depends on a functional MLL1 complex, as genetic

and pharmacological perturbation of its function blocks proliferation and induces myeloid differentiation in leukemia cells. Thus, this work validates the MLL1 complex as a potential target for therapeutic intervention in *CEBPA*-mutated AML.

Our ChIP-seq studies of p30- and MLL1-binding in myeloid progenitors from a *Cebpa*$^{p30/p30}$ AML mouse model allow for the first time to unambiguously identify genomic targets of the ~~p30~~ C/EBPα p30 isoform. This analysis also identified a high overlap of p30 with MLL1-binding in promoter-proximal regions. The genomic interaction of p30 with the MLL1 complex was confirmed by co-immunoprecipitation studies, validating the interaction of p30 with WDR5, RBBP5, ASH2L, and Menin, which are important factors in regulating assembly, enzymatic activity, and chromatin-anchoring of the MLL1 complex [19, 22].

CRISPR/Cas9-mediated screening of functional domains of the *Mll1* gene revealed that the enzymatic SET domain appears to be dispensable for N-terminal *CEBPA*-mutated AML. Similar findings have been reported in other systems [35], suggesting significant redundancy among H3K4me3-catalyzing enzymes in the regulation of normal and aberrant hematopoiesis. C/EBPα p30-expressing cells were hypersensitive to mutagenesis of the WIN domain, which is required for the MLL-WDR5 interaction, supporting the important role of WDR5 in *CEBPA*-mutated AML in the context of the MLL1 complex [12]. *Cebpa*$^{p30/p30}$ cells were also particularly sensitive to disruption of domains with known functions in chromatin binding of MLL1, such as the CXXC motif, the PHD finger, the bromodomain, and the Menin-binding motif (MBM) [22, 36, 37, 38, 39]. Small-molecule-mediated inhibition of the Menin-MLL interaction was reported to compromise the integrity of the MLL1 complex, resulting in decreased H3K4me3 levels on MLL1 target genes [25, 26, 27, 29]. In vivo, pharmacological targeting of the MLL1 complex induced survival benefits in models of MLL-rearranged and *NPM1*-mutated AML [28, 29].

*CEBPA*-mutated AML cells require a functional MLL1 complex, as its pharmacological perturbation resulted in impaired proliferation, cell cycle arrest and differentiation. In contrast to other AML subtypes [26, 28], however, apoptosis appears to be an early event in response to inhibitor treatment in *CEBPA*-mutated AML.

In MLL-rearranged and *NPM1*-mutated AML, MLL1 complex inhibitors block

leukemia progression via downregulation of the critical target genes *HOXA9* and *MEIS1* [26, 27, 29, 40, 41]. However, *HOXA9* and *MEIS1* do not contribute to leukemogenesis in *CEBPA*-mutated AML patient samples and mouse models [24, 42], indicating that MLL1 inhibitor treatment impairs leukemic self-renewal through different mechanisms in this disease context. In line with this, MLL1 is able to regulate subsets of transcriptionally active promoters that can strongly differ in various cell types and diseases [43]. It was proposed that this specificity is mediated via interactions with cell-type-specific TFs and histone modifications that shape the epigenetic landscape and regulate MLL1 recruitment to specific target genes [43]. Thus, it is possible that p30 and MLL1 co-regulate the expression of specific gene sets that are not shared with other AML subtypes.

To identify potential effector genes of the p30-MLL1 axis that are involved in aberrant self-renewal of *Cebpa*$^{p30/p30}$ cells, we focused on genes that are upregulated by p30, downregulated upon Menin-MLL inhibition and exhibit robust p30 chromatin binding in their promoter regions. This filtering strategy identified *Sox4*, a known oncogenic target of *CEBPA*-mutated AML [31]. Other genes with known roles in normal and aberrant hematopoiesis or leukemogenesis were also identified, including *Fam20a*, *Fubp1*, and the TFs *Erg* and *Gata2* [34, 44, 45, 46, 47, 48, 49].

Balanced GATA2 expression levels are critical for normal blood cell differentiation and development [50, 51]. *GATA2* is often found mutated and/or de-regulated in various malignancies [52, 53, 54, 55, 56]. Whereas gain-of-function mutations in *GATA2* were detected in CML [54], AML patients often display increased levels of GATA2 expression, which correlates with poor prognosis [33, 55]. Interestingly, heterozygous deletion [57], but also low-level overexpression of GATA2 [32] was linked to accelerated leukemogenesis and increased progenitor self-renewal, highlighting the detrimental consequences of any de-regulation of GATA2 expression.

We found that *Cebpa*$^{p30/p30}$ cells are highly dependent on GATA2 function, as CRISPR/Cas9-mediated *Gata2* mutagenesis led to a strong reduction of proliferative capacity and a substantial induction of myeloid differentiation. C/EBPα In the PDF document, this word is seperated over the line break. Please keep as one word p30 and MLL1 co-localized on the *Gata2* promoter, implying cooperative regulation of *Gata2* expression. Treatment of *Cebpa*$^{p30/p30}$ cells with

Menin-MLL inhibitors resulted in a decrease in MLL1 occupancy at the *Gata2* promoter, indicating a direct role of MLL1 in the regulation of *Gata2* expression in *CEBPA*-mutated AML.

In summary, our results identify a critical link between the C/EBPα p30 variant and the MLL1 complex and show that perturbation of the MLL1 complex could represent a novel therapeutic strategy for patients with *CEBPA*-mutated AML.

## Supplementary information

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## Compliance with ethical standards

*Conflict of interest* P.V. received honoraria from Novartis, Incyte, Celgene, and Pfizer and a research grant from Incyte. J.G. receives research support from Kura Oncology, Inc and has an equity ownership in the company. Other

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## Supplementary information

Supplemental Methods, Tables and Figures

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