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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

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Author contributions

L. Schmidt and F.G. designed research; L. Schmidt, E.H., L. Scheiblecker and F.G. performed experiments and analyzed data; T.E. performed bioinformatic analysis; G.V., J.F., J.G., C.N. and P.V. provided essential material and discussion; L. Schmidt, E.H., L. Scheiblecker and F.G. wrote the manuscript.

Conflict of interest

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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] 10-15% of AML patients harbor mutations in the *CEBPA* gene[2–4], which encodes the transcription factor (TF) CCAAT/enhancer binding protein alpha (C/EBPα). C/EBPα controls self-renewal properties of hematopoietic stem and progenitor cells (HSPCs) as well as critical steps of myeloid differentiation.[5–7] 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]

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 bi-allelic *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.[14–17] 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.[18–20] Other interaction partners such as Menin and Lens epithelium-derived growth factor (LEDGF, PSIP1) mediate chromatin recruitment of SET/MLL complexes.[21–23] 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.

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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) 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. 5 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: * $P < .05$; ** $P < .01$; *** $P < .001$, and **** $P < .0001$.

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 AML24 (*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).

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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% (4 882 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 (Figure 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 (3 549 of 4 882 peaks, Figure 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 (Figure 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 (Figure 1C; supplemental Figure S1G).

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 (Figure 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 (Figure 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).

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 (Figure 2A). In line with previous results,12 mutation of the Wdr5-interacting domain (WIN) of MLL1 strongly impaired proliferation of *Cebpa*$^{p30/p30}$ cells (Figure 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 (Figure 2A-B). In contrast, mutational targeting of the enzymatic SET domain did not affect proliferation (Figure 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 (Figure 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 complex25–27. 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 (Figure 3A-C; supplemental Figure S3A-C). *Cebpa*$^{p30/p30}$ cells showed a 2.4-5.6-fold higher sensitivity towards 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*$^{-/-}$ (Figure 3D, supplemental Figure S3D). Similarly, *Cebpa*$^{p30/p30}$ cells showed hypersensitivity towards MI-463 and MI-503 when compared to a panel of human leukemia cell lines representing different molecular aberrations (Figure 3E, supplemental Figure S3E).

In AML with bi-allelic *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* mutations30 (*Cebpa*$^{p30/C\text{-}mut.}$) was also hypersensitive towards MI-463 and MI-503 treatment (Figure 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 towards MI-463 and MI-503 treatment in colony-formation assays, indicating that inhibitor hypersensitivity was not acquired during the process of cell line establishment (Figure 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).

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 (Figure 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.

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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 up-regulated in response to either MI-463 and/or MI-503 (608 up-regulated genes vs. 212 down-regulated genes; supplemental Figure S5C). Gene set enrichment analysis revealed that inhibitor treatment induced transcriptional changes associated with myeloid differentiation, whereas self-renewal-associated signatures were depleted (Figure 5A, supplemental Figure S5D). MI-463 and MI-503 treatment caused up-regulation 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 (Figure 5B, supplemental Figure S5F).

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 down-regulated 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 (Figure 5C, supplemental Figure S5G). To systematically identify critical effectors of p30- and MLL1-dependent gene regulation, we focused on genes that were up-regulated upon C/EBPα p30 overexpression in myeloid cells.12 13 genes were induced by p30, down-regulated 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 AML31 (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.32–34 *Gata2* expression was up-regulated by p30 and strongly down-regulated upon Menin-MLL inhibitor treatment (Figure 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 (Figure 6B-C). p30 and MLL1 showed extensive co-localization in the *Gata2* promoter, suggesting cooperative regulation of *Gata2* expression (Figure 6D-E). Indeed, MI-463 treatment of *Cebpa*$^{p30/p30}$ cells resulted in a strong decrease in MLL1 occupancy at the *Gata2* locus (Figure 6D,F).

Together, these results show that p30 and the MLL1 complex cooperatively regulate *Gata2* expression and identify GATA2 as an effector of the p30-mediated differentiation block and leukemogenesis.

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Discussion

Here we demonstrate that the p30 C/EBPα 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 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. $Cebpa^{p30/p30}$ 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).22,36–39 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.25–27,29 *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 subtypes26,28, 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 down-regulation 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 models24,42, 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

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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 up-regulated by p30, down-regulated 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*.[34,44–49]

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.[52–56] 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 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 Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Döhner H, Weisdorf DJ, Bloomfield CD. Acute Myeloid Leukemia. N Engl J Med. 2015; 373(12): 1136–52. [PubMed: 26376137]
2. Zhang Y, Wang F, Chen X, Liu W, Fang J, Wang M, et al. Mutation profiling of 16 candidate genes in de novo acute myeloid leukemia patients. Front Med. 2018:1–9. [PubMed: 29363038]
3. Fasan A, Haferlach C, Alpermann T, Jeromin S, Grossmann V, Eder C, et al. The role of different genetic subtypes of CEBPA mutated AML. Leukemia. 2014; 28(4):794–803. [PubMed: 24056881]
4. Ahn J-S, Kim H-J, Kim Y-K, Lee S-S, Ahn S-Y, Jung S-H, et al. Assessment of a new genomic classification system in acute myeloid leukemia with a normal karyotype. Oncotarget. 2018; 9(4): 4961–8. [PubMed: 29435155]
5. Hasemann MS, Lauridsen FKB, Waage J, Jakobsen JS, Frank AK, Schuster MB, et al. C/EBPα Is Required for Long-Term Self-Renewal and Lineage Priming of Hematopoietic Stem Cells and for the Maintenance of Epigenetic Configurations in Multipotent Progenitors. PLoS Genet. 2014; 10(1)
6. Pabst T, Mueller BU, Zhang P, Radomska HS, Narravula S, Schnittger S, et al. Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. Nat Genet. 2001; 27(3):263–70. [PubMed: 11242107]
7. Zhang P, Iwasaki-Arai J, Iwasaki H, Fenyus ML, Dayaram T, Owens BM, et al. Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBP alpha. Immunity. 2004; 21(6):853–63. [PubMed: 15589173]
8. Lin FT, MacDougald Oa, Diehl aM, Lane MD. A 30-kDa alternative translation product of the CCAAT/enhancer binding protein alpha message: transcriptional activator lacking antimitotic activity. Proc Natl Acad Sci U S A. 1993; 90(20):9606–10. [PubMed: 8415748]
9. Pabst T, Mueller BU. Transcriptional dysregulation during myeloid transformation in AML. Oncogene. 2007; 26(47):6829–37. [PubMed: 17934489]
10. Su L, Tan Y, Lin H, Liu X, Yu L, Yang Y, et al. Mutational spectrum of acute myeloid leukemia patients with double CEBPA mutations based on next-generation sequencing and its prognostic significance. Oncotarget. 2018; 9(38):24970–9. [PubMed: 29861846]
11. Koschmieder S, Halmos B, Levantini E, Tenen DG. Dysregulation of the C/EBPalpha differentiation pathway in human cancer. J Clin Oncol. 2009; 27(4):619–28. [PubMed: 19075268]
12. Grebien F, Vedadi M, Getlik M, Giambruno R, Grover A, Avellino R, et al. Pharmacological targeting of the Wdr5-MLL interaction in C/EBPα N-terminal leukemia. Nat Chem Biol. 2015; 11(8):571–8. [PubMed: 26167872]
13. Guarnaccia, A duPuy; Tansey, WP. Moonlighting with WDR5: A Cellular Multitasker. J Clin Med. 2018; 7(2)
14. Gan T, Jude CD, Zaffuto K, Ernst P. Developmentally induced Mll1 loss reveals defects in postnatal haematopoiesis. Leukemia. 2010; 24(10):1732–41. [PubMed: 20724987]
15. Ernst P, Fisher JK, Avery W, Wade S, Foy D, Korsmeyer SJ. Definitive hematopoiesis requires the mixed-lineage leukemia gene. Dev Cell. 2004; 6(3):437–43. [PubMed: 15030765]
16. Hess JL, Yu BD, Li B, Hanson R, Korsmeyer SJ. Defects in yolk sac hematopoiesis in Mll-null embryos. Blood. 1997; 90(5):1799–806. [PubMed: 9292512]
17. McMahon KA, Hiew SY-L, Hadjur S, Veiga-Fernandes H, Menzel U, Price AJ, et al. Mll has a critical role in fetal and adult hematopoietic stem cell self-renewal. Cell Stem Cell. 2007; 1(3): 338–45. [PubMed: 18371367]
18. van Nuland R, Smits AH, Pallaki P, Jansen PWTC, Vermeulen M, Timmers HTM. Quantitative Dissection and Stoichiometry Determination of the Human SET1/MLL Histone Methyltransferase Complexes. Mol Cell Biol. 2013; 33(10):2067–77. [PubMed: 23508102]
19. Dou Y, Milne TA, Ruthenburg AJ, Lee S, Lee JW, Verdine GL, et al. Regulation of MLL1 H3K4 methyltransferase activity by its core components. Nat Struct Mol Biol. 2006; 13(8):713–9. [PubMed: 16878130]
20. Cao F, Chen Y, Cierpicki T, Liu Y, Basrur V, Lei M, et al. An Ash2L/RbBP5 heterodimer stimulates the MLL1 methyltransferase activity through coordinated substrate interactions with the MLL1 SET domain. PLoS One. 2010; 5(11):1–11.

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21. Yokoyama A, Somervaille TCP, Smith KS, Rozenblatt-Rosen O, Meyerson M, Cleary ML. The menin tumor suppressor protein is an essential oncogenic cofactor for MLL-associated leukemogenesis. Cell. 2005; 123(2):207–18. [PubMed: 16239140]
22. Yokoyama A, Cleary ML. Menin critically links MLL proteins with LEDGF on cancer-associated target genes. Cancer Cell. 2008 Jul 05; 14(1):36–46. [PubMed: 18598942]
23. El Ashkar S, Schwaller J, Pieters T, Goossens S, Demeulemeester J, Christ F, et al. LEDGF/p75 is dispensable for hematopoiesis but essential for MLL-rearranged leukemogenesis. Blood. 2017; 131(1):95–107. [PubMed: 29084774]
24. Kirstetter P, Schuster MB, Bereshchenko O, Moore S, Dvinge H, Kurz E, et al. Modeling of C/EBPα Mutant Acute Myeloid Leukemia Reveals a Common Expression Signature of Committed Myeloid Leukemia-Initiating Cells. Cancer Cell. 2008 Apr.13:299–310. [PubMed: 18394553]
25. He S, Calcagno C, Lobatto ME, Robson PM, Millon A, Grembecka J. Menin-MLL inhibitors block oncogenic transformation by MLL fusion proteins in a fusion independant manner. Leukemia. 2016; 28(10):1304–14.
26. Grembecka J, He S, Shi A, Purohit T, Muntean AG, Sorenson RJ, et al. Menin-MLL inhibitors reverse oncogenic activity of MLL fusion proteins in leukemia. Nat Chem Biol. 2012; 8(3):277–84. [PubMed: 22286128]
27. Shi A, Murai MJ, He S, Lund G, Hartley T, Purohit T, et al. Structural insights into inhibition of the bivalent menin-MLL interaction by small molecules in leukemia. Blood. 2012; 120(23):4461–9. [PubMed: 22936661]
28. Borkin D, He S, Miao H, Kempinska K, Pollock J, Chase J, et al. Pharmacologic inhibition of the menin-MLL interaction blocks progression of MLL leukemia in vivo. Cancer Cell. 2015; 27(4): 589–602. [PubMed: 25817203]
29. Kühn MWM, Song E, Feng Z, Sinha A, Chen C, Deshpande AJ, et al. Targeting Chromatin Regulators Inhibits Leukemogenic Gene Expression in NPM1 Mutant Leukemia. 2016 Oct.
30. Bereshchenko O, Mancini E, Moore S, Bilbao D, Månsson R, Luc S, et al. Hematopoietic Stem Cell Expansion Precedes the Generation of Committed Myeloid Leukemia-Initiating Cells in C/EBPα Mutant AML. Cancer Cell. 2009; 16(5):390–400. [PubMed: 19878871]
31. Zhang H, Alberich-Jorda M, Amabile G, Yang H, Staber PB, Di Ruscio A, et al. Sox4 is a key oncogenic target in C/EBPα mutant acute myeloid leukemia. Cancer Cell. 2013; 24(5):575–88. [PubMed: 24183681]
32. Nandakumar SK, Johnson K, Throm SL, Pestina TI, Neale G, Persons DA. Low-level GATA2 overexpression promotes myeloid progenitor self-renewal and blocks lymphoid differentiation in mice. Exp Hematol. 2015; 43(7):565–77. [PubMed: 25907033]
33. Luesink M, Hollink IHIM, Van Der Velden VHJ, Knops RHJN, Boezeman JBM, Haas D, et al. High GATA2 expression is a poor prognostic marker in pediatric acute myeloid leukemia. 2017; 120(10):2064–76.
34. Johnson KD, Kong G, Gao X, Chang YI, Hewitt KJ, Sanalkumar R, et al. Cis-regulatory mechanisms governing stem and progenitor cell transitions. Sci Adv. 2015; 1(8)
35. Mishra BP, Zaffuto KM, Artinger EL, Org T, Mikkola HKA, Cheng C, et al. The histone methyltransferase activity of MLL1 is dispensable for hematopoiesis and leukemogenesis. Cell Rep. 2014; 7(4):1239–47. [PubMed: 24813891]
36. Lee JH, Voo KS, Skalnik DG. Identification and characterization of the DNA binding domain of CpG-binding protein. J Biol Chem. 2001; 276(48):44669–76. [PubMed: 11572867]
37. Jacobson RH, Ladurner AG, King DS, Tjian R. Structure and function of a human TAFII250 double bromodomain module. Science. 2000; 288(5470):1422–5. [PubMed: 10827952]
38. Chang P-Y, Hom RA, Musselman CA, Zhu L, Kuo A, Gozani O, et al. Binding of the MLL PHD3 finger to histone H3K4me3 is required for MLL-dependent gene transcription. J Mol Biol. 2010; 400(2):137–44. [PubMed: 20452361]
39. Grembecka J, Belcher AM, Hartley T, Cierpicki T. Molecular basis of the mixed lineage leukemia-menin interaction: implications for targeting mixed lineage leukemias. J Biol Chem. 2010; 285(52):40690–8. [PubMed: 20961854]
40. Ayton PM, Cleary ML. Transformation of myeloid progenitors by MLL oncoproteins is dependent on Hoxa7 and Hoxa9. Genes Dev. 2003; 17(18):2298–307. [PubMed: 12952893]

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41. Wong P, Iwasaki M, Somervaille TCP, So CWE, So CWE, Cleary ML. Meis1 is an essential and rate-limiting regulator of MLL leukemia stem cell potential. Genes Dev. 2007; 21(21):2762–74. [PubMed: 17942707]
42. Marcucci G, Maharry K, Radmacher MD, Mrózek K, Vukosavljevic T, Paschka P, et al. Prognostic significance of, and gene and microRNA expression signatures associated with, CEBPA mutations in cytogenetically normal acute myeloid leukemia with high-risk molecular features: a Cancer and Leukemia Group B Study. J Clin Oncol. 2008; 26(31):5078–87. [PubMed: 18809607]
43. Milne TA, Dou Y, Martin ME, Brock HW, Roeder RG, Hess JL. MLL associates specifically with a subset of transcriptionally active target genes. Proc Natl Acad Sci U S A. 2005; 102(41):14765–70. [PubMed: 16199523]
44. Zhou W, Chung YJ, Parrilla Castellar ER, Zheng Y, Chung HJ, Bandle R, et al. Far upstream element binding protein plays a crucial role in embryonic development, hematopoiesis, and stabilizing myc expression levels. Am J Pathol. 2016; 186(3):701–15. [PubMed: 26774856]
45. Buchanan J, Tirado CA. A t(16;21)(p11;q22) in Acute Myeloid Leukemia (AML) Resulting in Fusion of the FUS/TLS and ERG Genes: A Review of the Literature. J Assoc Genet Technol. 2016; 42(1):24–33. [PubMed: 27183148]
46. Nalbant D, Youn H, Nalbant SI, Sharma S, Cobos E, Beale EG, et al. FAM20: an evolutionarily conserved family of secreted proteins expressed in hematopoietic cells. BMC Genomics. 2005; 6:11. [PubMed: 15676076]
47. Tsai FY, Keller G, Kuo FC, Weiss M, Chen J, Rosenblatt M, et al. An early haematopoietic defect in mice lacking the transcription factor GATA-2. Nature. 1994; 371(6494):221–6. [PubMed: 8078582]
48. Rodrigues NP, Janzen V, Forkert R, Dombkowski DM, Boyd AS, Orkin SH, et al. Haploinsufficiency of GATA-2 perturbs adult hematopoietic stem-cell homeostasis. Blood. 2005; 106(2):477–84. [PubMed: 15811962]
49. Rabenhorst U, Thalheimer FB, Gerlach K, Kijonka M, Böhm S, Krause DS, et al. Single-Stranded DNA-Binding Transcriptional Regulator FUBP1 Is Essential for Fetal and Adult Hematopoietic Stem Cell Self-Renewal. Cell Rep. 2015; 11(12):1847–55. [PubMed: 26095368]
50. Minegishi N, Suzuki N, Yokomizo T, Pan X, Fujimoto T, Takahashi S, et al. Expression and domain-specific function of GATA-2 during differentiation of the hematopoietic precursor cells in midgestation mouse embryos. Blood. 2003; 102(3):896–905. [PubMed: 12689939]
51. Persons DA, Allay JA, Allay ER, Ashmun RA, Orlic D, Jane SM, et al. Enforced expression of the GATA-2 transcription factor blocks normal hematopoiesis. Blood. 1999; 93(2):488–99. [PubMed: 9885210]
52. Fujimaki S, Harigae H, Sugawara T, Takasawa N, Sasaki T, Kaku M. Decreased expression of transcription factor GATA-2 in haematopoietic stem cells in patients with aplastic anaemia. Br J Haematol. 2001; 113(1):52–7. [PubMed: 11328281]
53. Xu Y, Takahashi Y, Wang Y, Hama A, Nishio N, Muramatsu H, et al. Downregulation of GATA-2 and overexpression of adipogenic gene-PPARgamma in mesenchymal stem cells from patients with aplastic anemia. Exp Hematol. 2009; 37(12):1393–9. [PubMed: 19772889]
54. Zhang S-J, Ma L-Y, Huang Q-H, Li G, Gu B-W, Gao X-D, et al. Gain-of-function mutation of GATA-2 in acute myeloid transformation of chronic myeloid leukemia. Proc Natl Acad Sci U S A. 2008; 105(6):2076–81. [PubMed: 18250304]
55. Vicente C, Vazquez I, Conchillo A, García-Sánchez MA, Marcotegui N, Fuster O, et al. Overexpression of GATA2 predicts an adverse prognosis for patients with acute myeloid leukemia and it is associated with distinct molecular abnormalities. Leukemia. 2012; 26(3):550–4. [PubMed: 21904383]
56. Nguyen J, Alexander T, Jiang H, Hill N, Abdullaev Z, Pack SD, et al. Melanoma in patients with GATA2 deficiency. Pigment Cell Melanoma Res. 2018; 31(2):337–40. [PubMed: 29156497]
57. Chong C-E, Venugopal P, Stokes PH, Lee YK, Brautigan PJ, Yeung DTO, et al. Differential effects on gene transcription and hematopoietic differentiation correlate with GATA2 mutant disease phenotypes. Leukemia. 2018; 32(1):194–202. [PubMed: 28642594]

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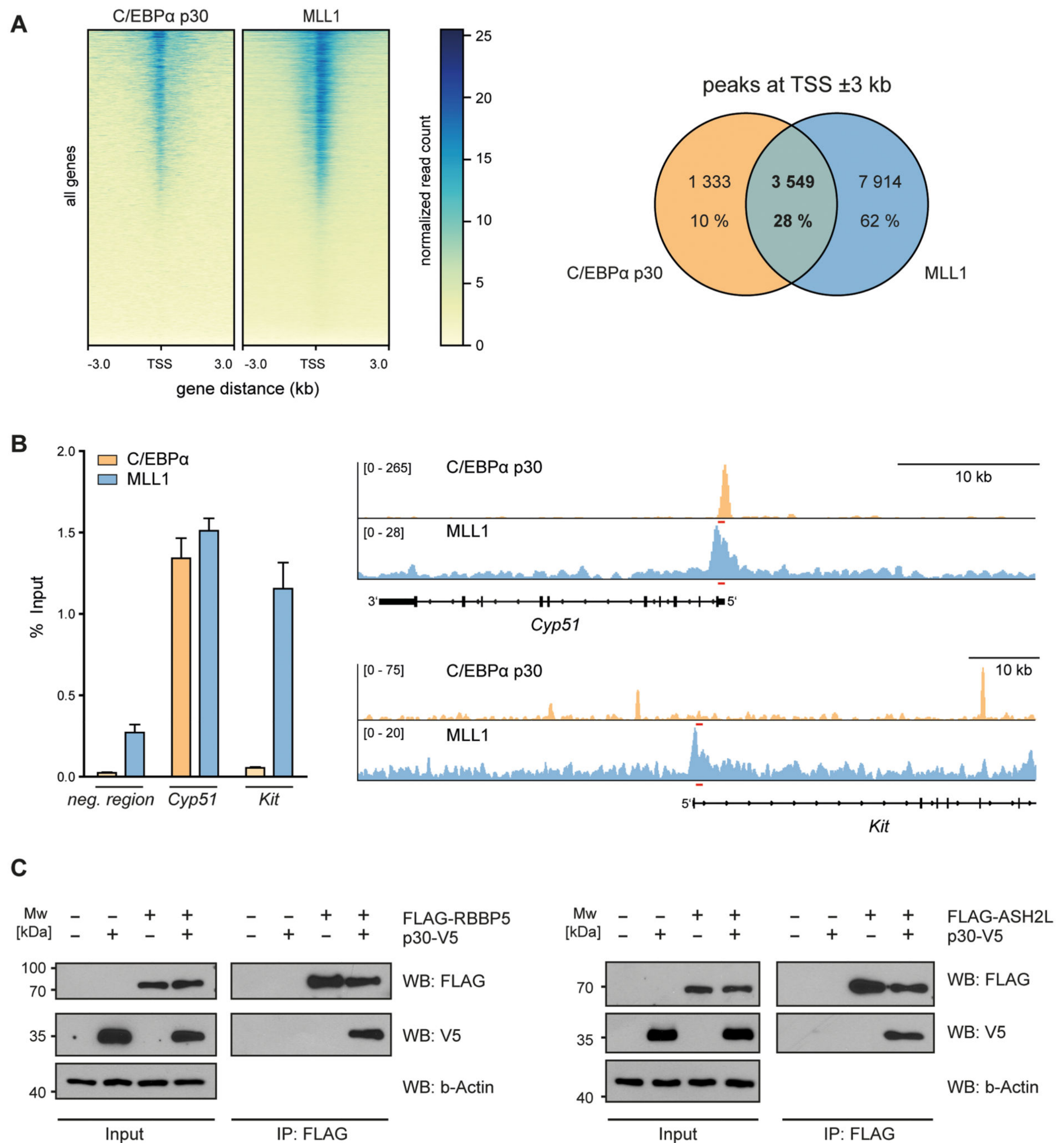

Figure 1. Global co-localization of C/EBPα p30 and MLL1 in *Cebpa*$^{p30/p30}$ cells.

(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.

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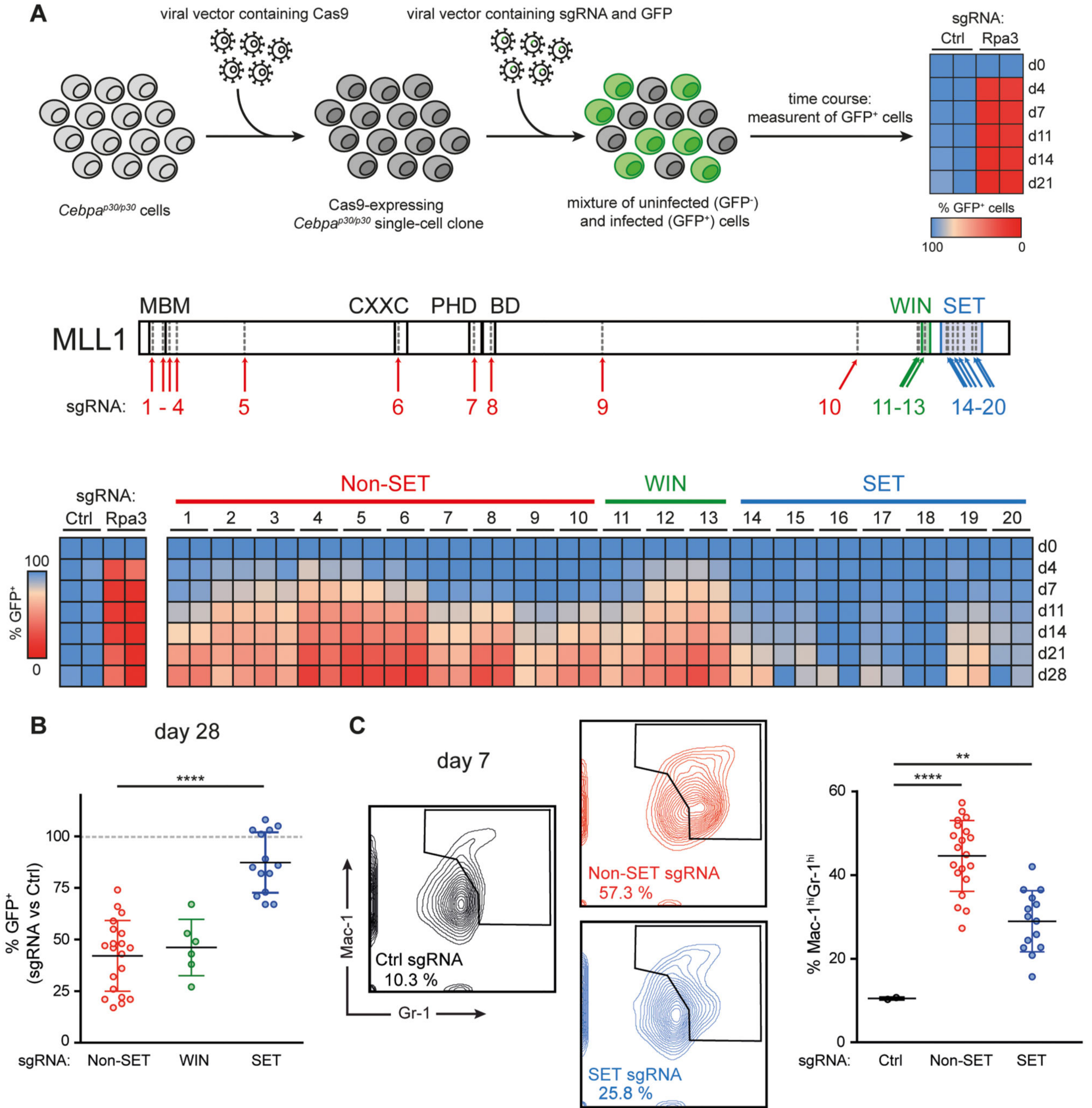

Figure 2. *Cebpa$^{p30/p30}$* cells are dependent on a functional MLL1 protein.

(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 grey 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 3 groups (Non-SET: sgRNAs #1-10, WIN: sgRNAs #11-13, SET: sgRNAs #14-20). The dashed grey 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). $Mac\text{-}1^{hi}Gr\text{-}1^{hi}$ levels are plotted for 3 groups (Ctrl, Non-SET: sgRNAs #1-10, SET: sgRNAs #14-20).

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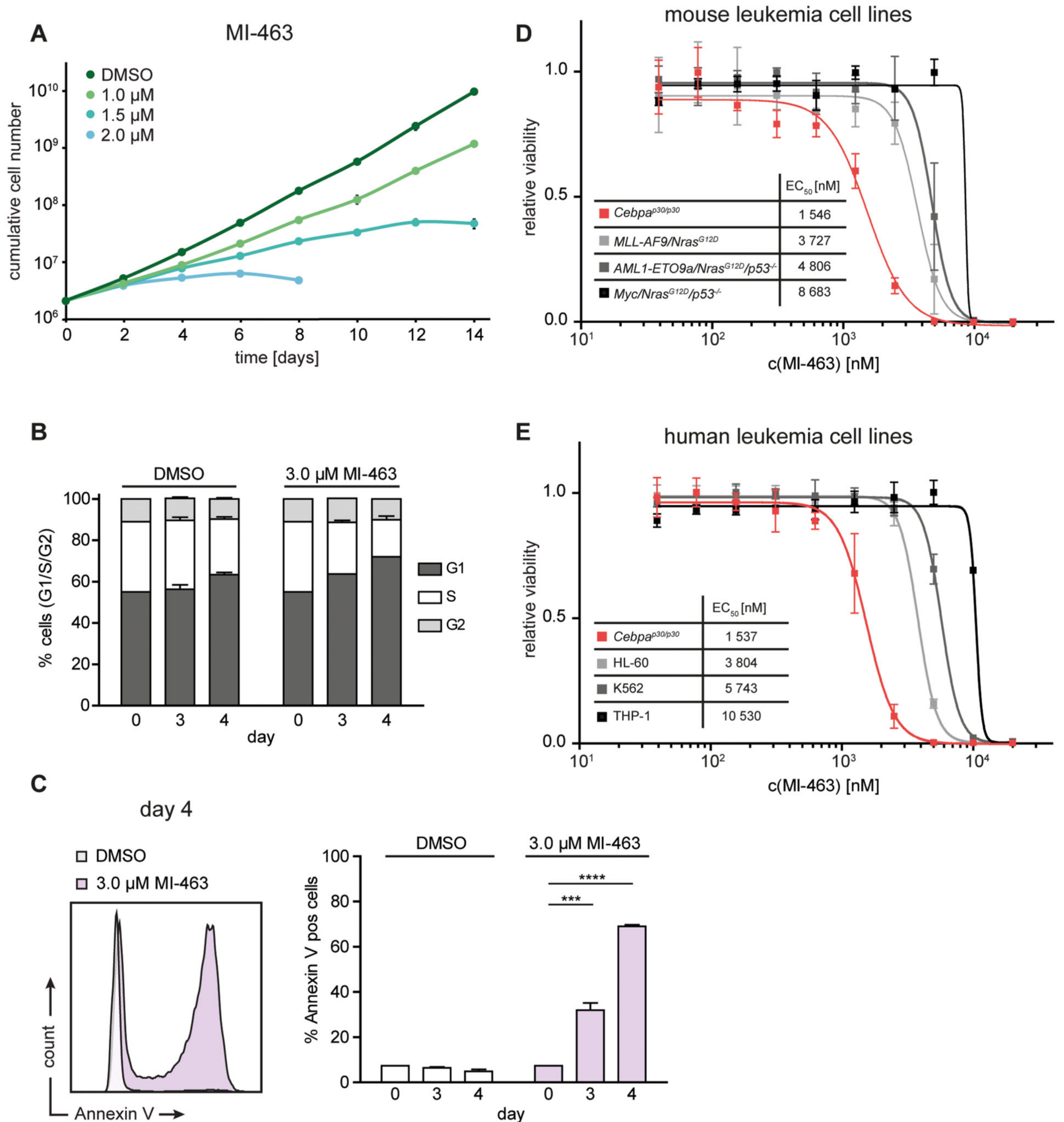

Figure 3. Pharmacological targeting of MLL1 function results in decreased self-renewal and induction of apoptosis in *Cebpa*$^{p30/p30}$ cells.

(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.

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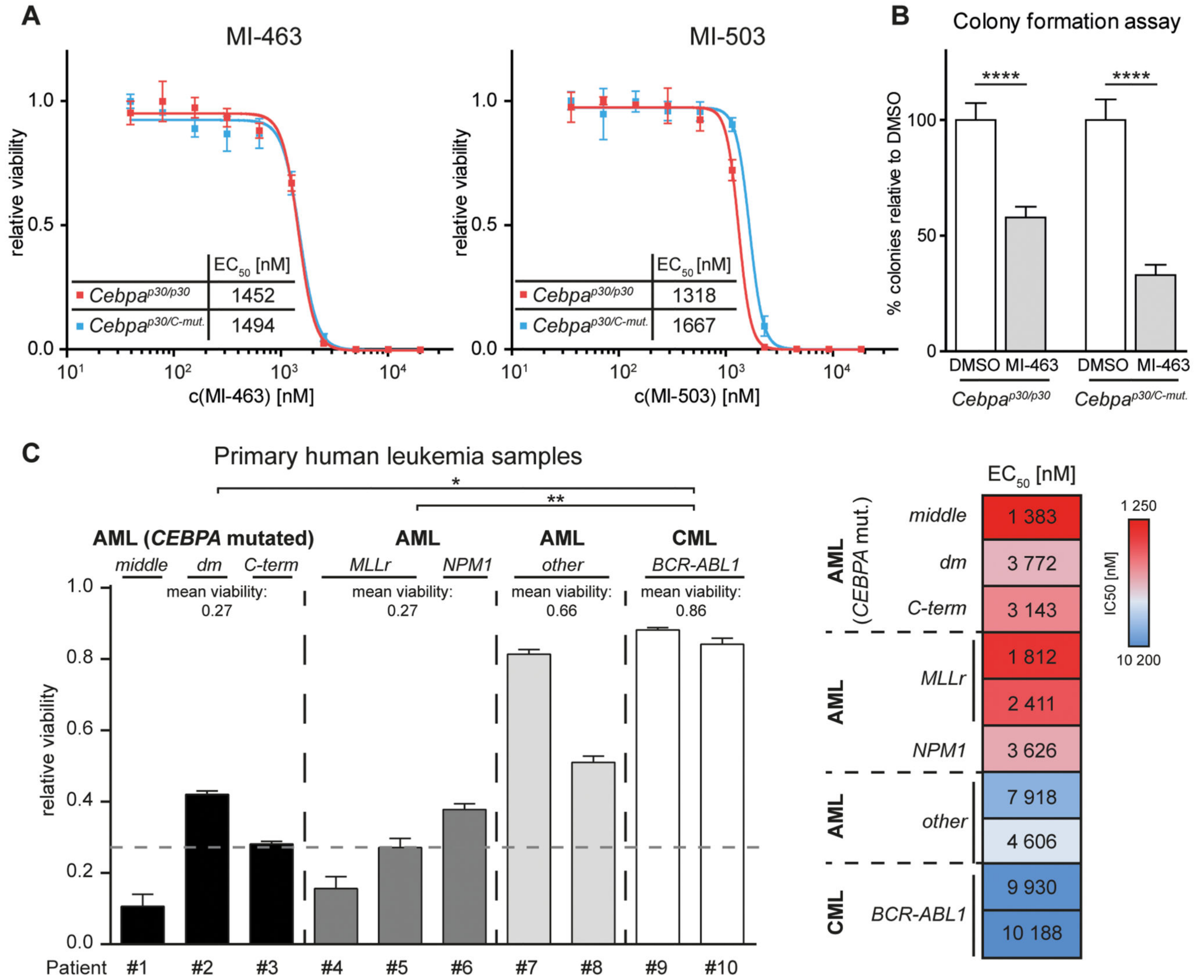

Figure 4. ***Cebpa*** $^{p30/C\text{-}mut.}$ **cells and primary human AML cells with *CEBPA* mutations are sensitive to pharmacological targeting of the MLL1 complex.**

(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.

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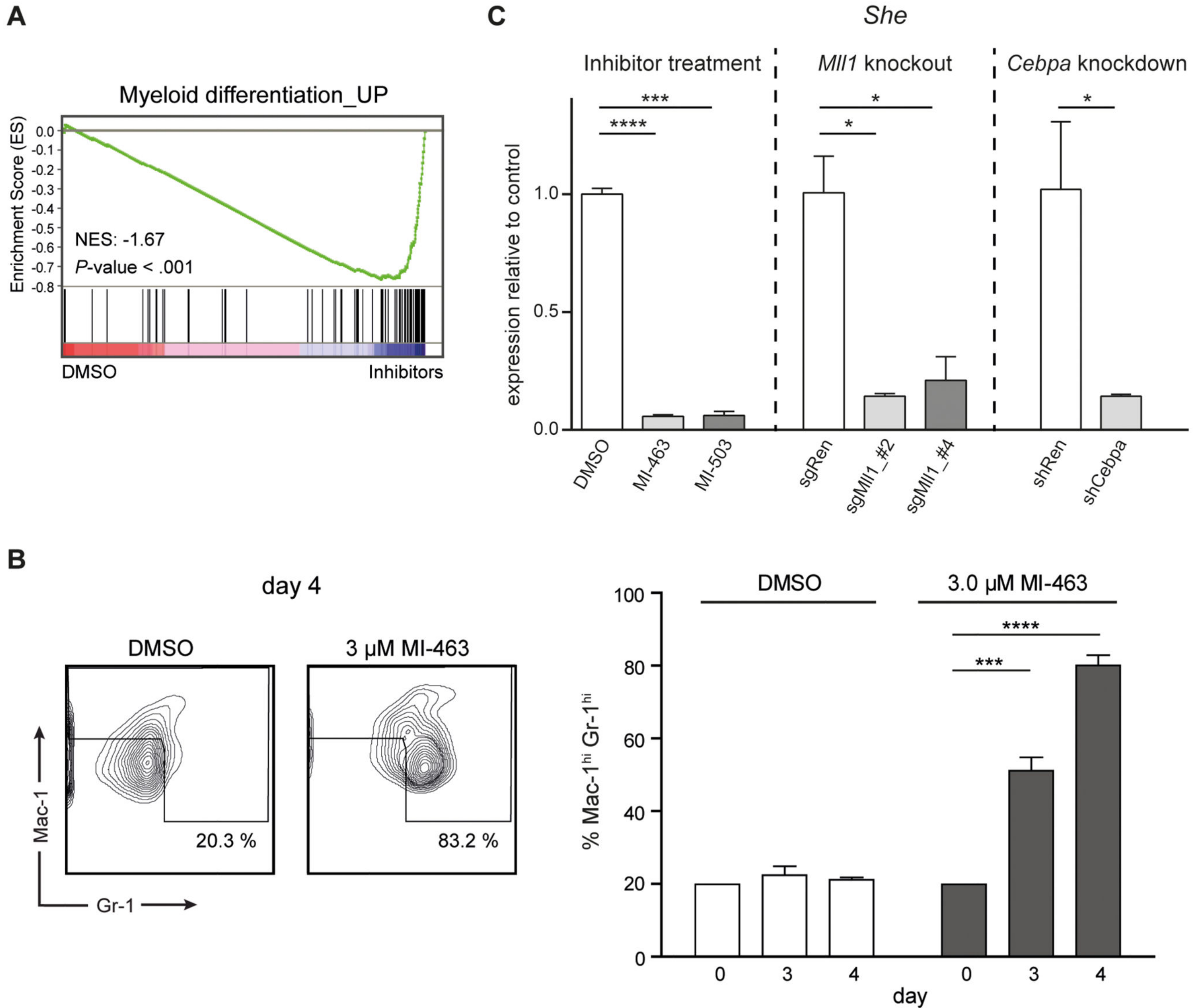

Figure 5. Loss of MLL1 function restores terminal myeloid differentiation potential of *Cebpa*$^{p30/p30}$ cells.

(A) GSEA showing global up-regulation 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.

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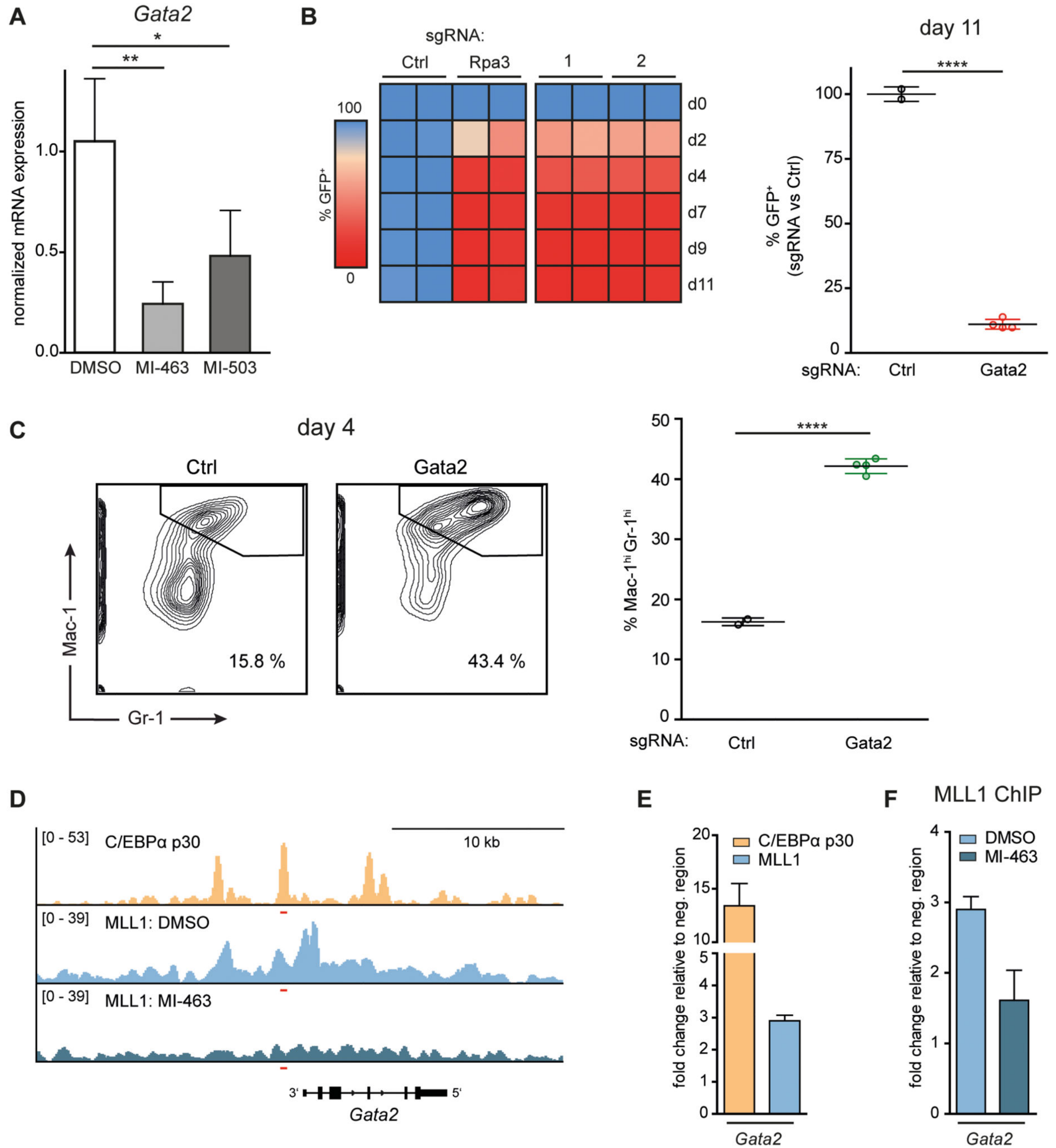

Figure 6. GATA2 is a critical effector of the p30-MLL1 axis.

(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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