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Mutant IDH1 blocks neutropoiesis by repressing myeloid progenitor programs

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Abstract:
IDH1 and IDH2 are frequently mutated in various cancers, including acute leukemias. However, the distinct mechanisms by which mutant IDH1 or IDH2 drive hematopoietic neoplasms remain poorly understood. Here, we analyzed DNA methylation in IDH1- and IDH2-mutant AML and found neutrophil lineage-specific epigenetic alterations in IDH1-mutant cases that went along with severely impaired neutrophil differentiation. Transcriptional analysis of normal hematopoiesis in humans and mice revealed a strong physiological upregulation of IDH1/Idh1 in myeloid progenitors. To study the functional effects of Idh1 mutations on hematopoiesis in a pre-leukemic setting, we used a genetically engineered inducible mouse model expressing a heterozygous Idh1 mutation under control of the endogenous promotor. Our study revealed a cell-intrinsic block in neutrophil differentiation caused by repression of myeloid transcription programs in neutrophil progenitors. This included impaired expression of Cebpe, which encodes a key transcription factor regulating neutrophil differentiation. Reactivation of Cebpe expression, by overexpression of its upstream regulator Cebpa or following treatment with hypomethylating agents restored differentiation, indicating that the differentiation block is reversible.In summary, we found a reversible, pre-leukemic impairment of neutrophil differentiation in IDH1-mutant hematopoiesis that correlates with elevated IDH1 expression in myeloid progenitors and likely explains the strong association of IDH1 mutations with myeloid neoplasms.

Conflict of interest: COI declared - see note

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Data Sharing Statement

All sequencing data generated in this study are available at GEO under accession numbers, **GSE330314** (scRNA-seq)**, GSE329858** (ATAC-seq)**, GSE329878** (bulk RNA-seq)**, GSE329880** (Nanopore-seq)**, GSE311328** (scRNA-seq). For access to processed data, please contact the corresponding author (d.lipka@dkfz.de).

Key Points

1. *IDH1*-mutant AML exhibit more severe neutropenia than other AML subtypes and present an epigenetic scar from the neutrophil lineage

2. *IDH1* expression is physiologically upregulated in myeloid progenitors and correlates with a neutrophil differentiation block in *Idh1*-mutant cells mediated by epigenetic repression of *Cebpe*.

Abstract

IDH1 and *IDH2* are frequently mutated in various cancers, including acute leukemias. However, the distinct mechanisms by which mutant *IDH1* or *IDH2* drive hematopoietic neoplasms remain poorly understood. Here, we analyzed DNA methylation in *IDH1*- and *IDH2*-mutant AML and found neutrophil lineage-specific epigenetic alterations in *IDH1*-mutant cases that went along with severely impaired neutrophil differentiation. Transcriptional analysis of normal hematopoiesis in humans and mice revealed a strong physiological upregulation of *IDH1/Idh1* in myeloid progenitors. To study the functional effects of *Idh1* mutations on hematopoiesis in a pre-leukemic setting, we used a genetically engineered inducible mouse model expressing a heterozygous *Idh1* mutation under control of the endogenous promotor. Our study revealed a cell-intrinsic block in neutrophil differentiation caused by repression of myeloid transcription programs in neutrophil progenitors. This included impaired expression of *Cebpe*, which encodes a key transcription factor regulating neutrophil differentiation. Reactivation of *Cebpe* expression, by overexpression of its upstream regulator Cebpa or following treatment with hypomethylating agents restored differentiation, indicating that the differentiation block is reversible.

In summary, we found a reversible, pre-leukemic impairment of neutrophil differentiation in *IDH1*-mutant hematopoiesis that correlates with elevated *IDH1* expression in myeloid progenitors and likely explains the strong association of *IDH1* mutations with myeloid neoplasms.

Introduction

Hematopoiesis relies on an accurately coordinated epigenetic network to maintain the delicate balance between self-renewal and differentiation. Disruption of epigenetic regulation during differentiation increases the risk of hematopoietic malignancies. Mutations in several epigenetic regulators including *TET2*, *DNMT3A* and *IDH1/2*, are frequently observed in acute myeloid leukemia (AML) and myelodysplastic neoplasms (MDS) and are believed to be among the first hits acquired in immature hematopoietic stem and progenitor cells (HSPCs). Moreover, mutations in *TET2*, *DNMT3A*, and *IDH2* are also frequently detected in hematopoietic malignancies of the lymphoid lineage [1-4].

Oncogenic mutations in *IDH1* and *IDH2* confer these enzymes with the neomorphic activity of producing the oncometabolite D-2-hydroxyglutarate (D2HG) [5]. D2HG acts as a competitive inhibitor of α-ketoglutarate-dependent dioxygenases, including TET2 and Jumonji C-domain lysine demethylases [6-8]. Due to the prominent phenotype caused by D2HG, it became widely accepted that mutant IDH1 and IDH2 share biological consequences mediated by D2HG production and accumulation. Accordingly, mutations in *IDH1/2* have been associated with DNA and histone hypermethylation, accumulation of DNA damage and replication fork stalling, which has been associated with defective cell proliferation and differentiation [8-14].

However, to date, there is incomplete understanding of the mechanisms via which mutant *IDH1* and *IDH2* affect cellular and molecular pathways that lead to the development of hematopoietic neoplasms. Due to their shared neomorphic enzymatic activity, other aspects of these isoenzymes, such as their differential involvement in certain biological processes, have been disregarded so far. From a clinical perspective, *IDH1* mutations mainly occur in myeloid neoplasms and are linked to inferior survival in AML as compared to *IDH2*-mutant cases, whereas *IDH2* mutations are also found in lymphoid malignancies and can serve as a prognostic marker in T-ALL [4, 15-19]. In contrast, *IDH1*-mutant glioma and glioblastoma patients exhibit better survival and prognosis than *IDH1*-WT or *IDH2*-mutant patients [20, 21]. Consistent with these clinical findings, molecular studies also highlight distinct biochemical roles of IDH1 and IDH2 [22, 23]. Nevertheless, the mechanisms by which mutant IDH1 preferentially drives myeloid leukemogenesis remain incompletely understood.

Here, we used publicly available DNA methylation data to investigate, whether AMLs expressing mutant *IDH1* differ from those expressing mutant *IDH2*. We observed epigenetic scars indicating defective neutrophil differentiation in *IDH1*-mutant cases and found that *IDH1*-mutant AMLs have impaired neutropoiesis. We employed a genetically engineered mouse model to study the cellular and molecular basis of the neutrophil differentiation defect in pre-leukemic *Idh1*-mutant hematopoiesis.

Methods

A detailed description of all materials and methods used in this manuscript can be found in Supplementary Materials and Methods.

Computational methods

All data analyzes and visualizations were performed using RStudio. All single-cell RNA sequencing (scRNA-seq) analyses were performed with *Seurat.* Pseudotime and trajectory analysis were performed using *destiny and Slingshot.* DNA methylation was analyzed using *RnBeads.* Bulk RNA-sequencing was analyzed using *DESeq2*.

Clinical data

Bone marrow samples were analyzed by cytomorphology [24]. The percentage of bone marrow granulopoiesis was defined as the sum of the percentage of promyelocytes, myelocytes, metamyelocytes and neutrophil granulocytes cytomorphologically determined on May-Grünwald-Giemsa (MGG) stains. *IDH1* and *IDH2* mutations were analyzed using targeted panel next generation sequencing with a limit of detection of 3% variant allele fraction (VAF) [25].

Animal models

Idh1-R132H, *Scl*-CreER^{T} and *Rosa26*-EYFP mice used in this study were crossed with B6.SJL-Ptprc^{a} Pepc^{b}/Boy mice to generate animals with homozygous expression of CD45.1. *Idh1*$^{R132H/R132H}$ mice were bred with *Scl*-CreER$^{T+/d}$ *Rosa26*-EYFP$^{d/d}$ mice to

obtain *Idh1*$^{+/R132H}$ Scl-CreER$^{T+/d}$ *Rosa26*-EYFP$^{+/d}$ mice which were used in all mouse experiments, while *Idh1*$^{+/+}$ *Scl*-CreER$^{T+/d}$ *Rosa26*-EYFP$^{+/d}$ mice were used as controls. For all experiments, both male and female mice were used. Recombination was induced by tamoxifen administration, resulting in activation of heterozygous *Idh1*-R132H together with EYFP expression (or EYFP alone in wildtype mice). Two weeks after induction, EYFP^{+}Lin^{-} bone marrow cells were isolated and transplanted into lethally irradiated recipient mice without supportive bone marrow. This strategy generated fully chimeric *Idh1*-WT or *Idh1*-R132H hematopoiesis derived exclusively from recombined donor cells. All subsequent experiments were performed using these fully reconstituted chimeric mice. All mouse experiments were approved by the local authorities (Regierungspräsidium Karlsruhe, Germany).

Differentiation models

For adoptive transfer, recipient C57BL/6J mice were transplanted without prior irradiation, and bone marrow was collected 5 – 7 days later. For *ex vivo* differentiation, 5000 cells were plated in differentiation medium for four days. To differentiate 32D cells, IL-3 was exchanged with G-CSF for six days.

Results

Myeloid neoplasms with mutant IDH1 present severe neutropenia

To study potential differences in DNA methylation signatures of *IDH1*- and *IDH2*-mut AML patients, we performed a combined analysis using publicly available data from three different studies (TCGA: 194 pts., BEAT-AML: 224 pts. and Augsburg: 55 pts; **Suppl. Fig. 1A**) [15, 26-28]. The combined cohort encompassed 37 *IDH1*- and 48 *IDH2*-mut patients (**Suppl. Fig. 1A,B**). Among these, 16 patients were identified to have *IDH1*- and 29 patients to have *IDH2*-mutations as the sole epigenetic regulator aberration (further referred to as *IDH1*-mut and *IDH2*-mut AML, respectively; **Suppl. Fig. 1C**). AML patients without mutations in *IDH1*, *IDH2, TET2, DNMT3A* or Ras-pathway genes served as a control AML group. Both, *IDH1*- and *IDH2*-mut AML showed the previously described hypermethylation phenotype, with *IDH1*-mut AML being slightly more methylated than IDH2-mut AML (**Suppl. Fig. 1B,D,E**).

To study *IDH1*- and *IDH2*-mutant AMLs in the context of healthy hematopoiesis, we integrated the AML data with DNA methylation data spanning normal hematopoietic differentiation from HSPCs to terminally differentiated cells [29-31] (**Suppl. Fig. 1F**, **Figure 1A-D**). When focusing on myelopoiesis, principal component analysis (PCA) demonstrated that both *IDH1*- and *IDH2*-mutant AMLs grouped together with HSPCs (**Figure 1A-B**). However, in principal component 3, which accounts for about 7% of the overall variance, *IDH1*-mutant AMLs also grouped close to neutrophils (**Figure 1C**), which was not observed in *IDH2*-mutant AML (**Figure 1D**). This suggested that *IDH1*-mutant AML might present an epigenetic scar from neutrophil differentiation. Therefore, we hypothesized that *IDH1*-mutant AMLs might originate from myeloid progenitors which failed to differentiate into mature neutrophils. To assess the clinical

relevance of our finding, we analyzed cell counts in peripheral blood and bone marrow from AML patients. *IDH1*-mutant AML patients presented strong and significant reduction of bone marrow granulopoiesis and of absolute neutrophil counts (ANC) in peripheral blood as compared to both *IDH2*-mutant and *IDH1/2*-wildtype (IDH-WT) AML patients (**Figure 1E-H**). Importantly, these differences could not be attributed to other parameters, such as blast counts, bone marrow erythropoiesis, hemoglobin levels or platelet counts, indicating a specific impairment of granulopoiesis in AML in the presence of an *IDH1* mutation [32] (**Suppl. Table 1, Suppl. Fig. 1G-J)**. In summary, these molecular and clinical data suggest that *IDH1*-mutant AMLs show a specific differentiation defect in the neutrophil lineage.

IDH1 is specifically upregulated in myeloid progenitor cells

To understand the potential association of mutant *IDH1* and impaired neutropoiesis, we analyzed the expression levels of *IDH1* in normal human hematopoiesis using single-cell RNA-sequencing (scRNA-seq) data from the Human Cell Atlas project [33] (**Figure 2A**). We observed that *IDH1* transcripts are expressed at rather low levels in most hematopoietic cells but become strongly upregulated in myeloid progenitors (**Figure 2B**). This *Idh1* expression pattern was recapitulated in scRNA-seq data from mouse hematopoiesis (**Figure 2C,D; Supp. Fig. 2A,B**), while other members of the *Idh* gene family did not show lineage-specific expression patterns (**Supp. Fig. 2A,C)**. These findings were additionally validated in publicly available bulk RNA-sequencing data [34] (**Suppl. Fig. 2D,E)**. To test whether *Idh1* expression might be regulated epigenetically, we examined DNA methylation in the *Idh1* promoter region in normal mouse hematopoiesis (**Figure 2E**) [35, 36]. The murine genome encodes two *Idh1* transcript variants (TVs), both of which share the same open reading frame (ORF) but differ in their 5'-untranslated region (5'-UTR). Specifically, the TSS of *Idh1*-TV2 showed high levels of DNA methylation in all hematopoietic lineages but revealed a progressive loss in the myeloid lineage starting from $CD55^{neg}$ CMPs (**Figure 2E**). To test the contribution of *Idh1*-TV2 to the transcriptional upregulation of *Idh1* in myeloid progenitors, we performed TV-specific quantitative real-time PCR (qRT-PCR; **Supp. Fig. 3**). Little to no expression differences were observed for *Idh1*-TV1 (**Figure 2F**), while TV2 was strongly upregulated in the myeloid lineage with the strongest increase found in $Ly6C^{neg}$ and $Ly6C^{pos}$ GMPs (**Figure 2G**). Together, our data show that *Idh1* expression is specifically upregulated in myeloid progenitor cells and correlates with DNA methylation changes at the TSS of the predominantly expressed *Idh1* transcript variant.

Mutant *IDH1* primarily affects the myeloid lineage

To examine the effect of mutant *Idh1* on hematopoietic differentiation, we generated an inducible, heterozygous pre-clinical mouse model of *Idh1*-mutant hematopoiesis [37-40] (**Suppl. Fig. 4A**). Tamoxifen-induced *Scl*-$CreER^T$ activation triggered expression of heterozygous *Idh1*-R132H from the endogenous locus, with EYFP marking recombined cells. (**Suppl. Fig. 4B-E**). To generate fully chimeric mice, lineage-negative, $EYFP^{pos}$ cells were isolated two weeks after tamoxifen injection and

transplanted into lethally irradiated recipient mice without supportive bone marrow (**Figure 3A, Suppl. Figure 4G**). Starting from week 16 post-transplantation, quantitative and qualitative analyses of *Idh1*-mut mediated effects were performed. *Idh1*-mut mice presented high serum levels of the oncometabolite D2HG (**Suppl Fig. 4F**). Flow cytometric analysis of bone marrow revealed no differences in the frequencies of LSK cells, LT- and ST-HSCs, MPP2, MPP3+4, MPP5 and MEPs (**Suppl Fig. 5A,B**). While overall frequencies of CMPs and GMPs also did not differ between *Idh1*-WT and *Idh1*-R132H mice, more detailed analyses revealed a significant increase in myeloid-enriched $CD55^{neg}$ CMPs (**Figure 3B**) at the expense of erythroid-megakaryocyte-like $CD55^{pos}$ CMPs (**Figure 3C**) in *Idh1*-R132H mice. Similarly, *Idh1*-R132H mice increased $Ly6C^{pos}$ GMP proportions at the expense of $Ly6C^{neg}$ GMPs (**Figure 3D,E**). In the peripheral blood, *Idh1*-mut mice showed a significant reduction in $CD11b^{pos}$ myeloid cells and mature neutrophils while the frequencies of monocytes, B and T cells as well as other parameters such as white blood cell counts, platelets, red blood cells and hemoglobin remained unchanged (**Figure 3F-H**, **Suppl. Figure 5C-H**). Taken together, our data show that *Idh1*-R132H leads to a myeloid differentiation defect that occurs in parallel to the observed upregulation of *Idh1* expression, suggesting that elevated activity of mutant *Idh1* might be involved in mediating this defect.

Idh1-R132H myeloid progenitors exhibit a cell-intrinsic neutrophil differentiation block

Next, we sought to investigate whether the reduction of mature neutrophils in *Idh1*-R132H mice is due to a cell-intrinsic differentiation defect of myeloid progenitor cells. Therefore, we performed adoptive transfer of $EYFP^{pos}CD55^{neg}$ CMPs into non-irradiated secondary recipient mice (**Figure 4A**). Assessment of the $EYFP^{pos}$ compartment at day 7 revealed that *Idh1*-R132H progenitors gave rise to significantly fewer neutrophils (**Figure 4B,C**), while the production of monocytes was not impaired (**Figure 4D**). Adoptive transfer of *Idh1*-mutant $EYFP^{pos}Ly6C^{neg}$ GMPs presented a similar differentiation defect (**Suppl. Fig. 6A-D**). These data suggest a selective cell-intrinsic differentiation defect towards the neutrophilic lineage. To further test this observation, we expressed *Idh1*-R132H in the murine myeloid progenitor cell line 32Dcl3 (32D cells) (**Suppl. Fig. 6E**). 32D cells expressing *Idh1*-R132H (32D-R132H) produced high levels of the oncometabolite D2HG and exhibited a substantial impairment of cell proliferation both in steady-state culture (**Suppl. Fig. 6F,G**). Upon differentiation with granulocyte colony-stimulating factor (G-CSF) 32D-R132H cells continued to proliferate significantly less than 32D empty vector (32D-EV) control cells and exhibited a significant reduction in the frequency of $CD11b^{high}$ cells and mature neutrophils (**Figure 4E-H**, **Suppl. Fig. 6H**). This neutrophil differentiation defect persisted in the presence of an oncogenic co-mutation (**Suppl. Fig. 6I**). In summary, these experiments confirm a cell-intrinsic neutrophil differentiation defect in *Idh1*-mutant myeloid progenitors *in vivo* and *in vitro*.

Cebpe expression is impaired in the presence of *Idh1*-R132H

Normal *Idh1* expression was most notably upregulated in Ly6C^{neg} and Ly6C^{pos} GMPs, while *Idh1*-R132H Ly6C^{neg} GMPs exhibited a pronounced cell-intrinsic block in granulopoiesis (**Figure 2G, Suppl. Fig. 6A-D**). Bulk transcriptome analysis revealed elevated myeloid progenitor marker gene expression in *Idh1*-mutant as compared to *Idh1*-wt Ly6C^{pos} GMPs, whereas expression of several neutrophil lineage-associated genes was strongly reduced (**Suppl. Figure 7A**). To study aberrant myeloid progenitor differentiation in *Idh1*-mut hematopoiesis in more detail, we performed scRNA-sequencing **(Figure 5A-B**, **Suppl. Fig. 7B**). Both, *Idh1*-mutant early (ENPs) and late (LNPs) neutrophil progenitors showed a significant reduction in the expression of GMP and neutrophil progenitor (NeutroP) expression scores [41], suggesting impaired expression of neutrophil lineage genes (**Figure 5C-F**).

To achieve a more profound understanding of the gene expression dynamics during neutrophil differentiation, the top 50 cell type-specific marker genes were calculated using our WT scRNA-sequencing data (**Figure 2C**). Analysis of marker gene expression along the neutrophil differentiation pseudotime revealed that *Idh1*-R132H ENPs and LNPs failed to upregulate several marker genes to the same extent as was observed in their *Idh1*-WT counterparts **(Figure 5G-H).** Quantification of key affected genes, including *Elane, Gsr*, *Fcy-receptor III* and *Cebpe*, confirmed significantly lower expression in *Idh1*-R132H LNPs compared to their WT counterparts (**Figure 5I-L**). In *Idh1*-R132H LNPs, impaired expression of *Cebpe* and other marker genes was more pronounced compared to ENPs, suggesting that the transition from ENPs to LNPs could be specifically defective at the molecular level (**Suppl. Fig. 7G**). Trajectory analysis (*slingshot*) confirmed enrichment of ENPs and a depletion of LNPs and mature neutrophils in *Idh1*-R132H hematopoiesis (**Figure 5M-N**). Gene expression analysis along the neutrophil trajectory revealed comparable *Cebpe* expression between *Idh1*-WT and *Idh1*-R132H cells during early stages of differentiation, while at later stages *Idh1*-R132H progenitors failed to upregulate *Cebpe* to the same extent as *Idh1*-WT cells (**Suppl. Fig. 7H**, **Figure 5O**). Using qRT-PCR, we identified a similar impairment of *Cebpe* expression in our 32D-R132H cell line model (**Figure 5P**). In summary, our data demonstrate that *Idh1*-mut myeloid progenitors fail to progress appropriately along the neutrophil differentiation trajectory. Our molecular analyses suggest inadequate upregulation of the gene encoding the neutrophil transcription factor *Cebpe* as a potential major contributing factor to this phenotype.

Idh1-R132H cells are responsive to G-CSF

We next assessed whether the neutrophil differentiation defect in *Idh1*-R132H hematopoiesis could be reversed via G-CSF treatment. *Idh1*-R132H or -WT EYFP^{pos}CD55^{neg} CMPs were cultured *ex vivo* either in basal medium, or in the presence of M-CSF or G-CSF (**Figure 6A–D**). In the absence of instructive cytokines (i.e. basal medium), frequencies of CD11b^{pos} myeloid cells were comparable, although *Idh1*-mut progenitors exhibited a trend toward reduced myeloid and neutrophil output combined with an increased monocyte output (**Figure 6B**). M-CSF promoted

differentiation towards mature monocytes, with a more pronounced effect in *Idh1*-R132H cells, without altering CD11b^{pos} myeloid cell proportions (**Figure 6C**). G-CSF robustly induced neutrophil differentiation in both genotypes; however, *Idh1*-R132H progenitors generated significantly fewer CD11b^{pos} myeloid cells and mature neutrophils compared to WT controls and showed impaired expression of *Cebpe*, indicating that the differentiation block persists despite stimulation with instructive cytokines (**Figure 6D, Suppl. Figure 7I**).

Restoration of *Cebpe* expression rescues neutrophil differentiation of *Idh1*-R132H myeloid progenitors

To study whether inhibition of mutant IDH1 enzymatic activity would restore neutrophil differentiation, we treated 32D-R132H cells with the IDH1-inhibitor AGI-5198. This resulted in complete depletion of D2HG and differentiation in the presence of G-CSF recovered cell proliferation and restored myeloid differentiation (**Figure 7A-C**). However, expression of *Cebpe* and differentiation towards mature neutrophils remained impaired (**Figure 7D,E**). Therefore, we tested whether recovery of *Cebpe* expression would be sufficient to overcome the neutrophil differentiation block. To this end, we used a construct containing *CEBPA* fused to an estrogen receptor domain (ER) [34, 42] (**Figure 7F**). This construct, *CEBPA-ER,* has been shown to induce myeloid differentiation and upregulation of *Cebpe* expression in the presence of hydroxytamoxifen (OH-TAM) and G-CSF [34, 42]. Importantly, the 32D-R132H-CEBPA^{ER} cells did not show spontaneous differentiation, nor did they upregulate *Cebpe* expression in the absence of OH-TAM (**Figure 7G)**. Upon OH-TAM exposure, G-CSF treatment led to a strong increase in *Cebpe* expression, and to full recovery of the production of both CD11b^{pos} myeloid cells and of mature neutrophils in 32D-R132H-CEBPA^{ER} cells (**Figure 7H-J**).

Next, we examined the underlying epigenetic restriction in *Idh1*-R132H cells that limits their ability to upregulate *Cebpe* expression. We performed ATAC-seq in 32D-EV and 32D-R132H cells and observed reduced chromatin accessibility in 32D-R132H cells upstream of the *Cebpe* transcription start site (TSS; **Suppl. Figure 8A-H**, **Figure 7K**, upper panel). To determine whether this reduced accessibility is associated with aberrant DNA methylation, we performed long-read Oxford Nanopore sequencing, including a 5-day treatment with AGI-5198 or with the hypomethylating agent 5-azacitidine (Aza, 500 nM) (**Suppl. Figure 8I**). We detected increased DNA methylation upstream of the *Cebpe* TSS overlapping with one of the differential ATAC-seq peaks in untreated 32D-R132H cells compared to 32D-EV controls (**Figure 7K**, lower panel). While AGI-5198 partially reduced aberrant DNA methylation, Aza treatment completely erased aberrant DNA methylation marks at the *Cebpe* locus (**Figure 7K**, lower panel). Consistently, Aza treatment alone restored *Cebpe* expression (**Figure 7L**), and combined treatment with AGI-5198 fully normalized neutrophil differentiation in 32D-R132H cells (**Figure 7M**), indicating that aberrant DNA methylation at the *Cebpe* locus may contribute to impaired expression of this key transcription factor.

In summary, these data provide experimental evidence that epigenetic repression of *Cebpe* represents a central molecular mechanism underlying the neutrophil differentiation block in *Idh1*-mutant hematopoiesis.

Discussion

Despite major advancements in understanding the biology of IDH-mutations and the successful application of small molecule inhibitors in AML patients with mutant IDH1 or IDH2, the mechanisms underlying the leukemogenic potential of these mutations remain unclear. In the past decade, *IDH1* and *IDH2* mutations have been perceived as interchangeable genetic aberrations due to their shared neomorphic enzymatic activity [6, 7, 9, 11, 14, 43]. Despite their shared DNA hypermethylation signature, we sought to investigate whether *IDH1*- or *IDH2*-mutant AMLs retain residual DNA methylation patterns reflecting their cell-of-origin and developmental history [26, 44, 45]. Our integrative DNA methylation analyses demonstrated that *IDH1*-mutated AML patients exhibit an epigenetic scar that originates from the neutrophil differentiation program. This methylation signature was accompanied by impaired granulopoiesis in AML patients, suggesting that mutant *IDH1* enforces a lineage-specific differentiation block. These findings could provide a biological explanation for the early neutrophil burst observed in *IDH1*-mutant AML patients treated with IDH1-inhibitors, as well as other neutrophil-associated phenomena in *IDH1*-mutant AML [46-50].

Our analyses further revealed a specific and strong upregulation of *IDH1* expression in myeloid progenitor cells, both in human and murine bone marrow. Importantly, we demonstrated progressive loss of DNA methylation in the vicinity of the TSS of the murine *Idh1* transcript variant 2, indicating a possible specific role of IDH1 in myeloid progenitors. In contrast, both murine and human *IDH2* were generally expressed more uniformly across different hematopoietic lineages and cell types. Although causality cannot be formally established, the gene expression differences observed here mirror the broader disease distribution, with *IDH2* mutations occurring in both myeloid and lymphoid malignancies, whereas *IDH1* mutations are largely confined to myeloid neoplasms. [4, 17, 18, 51].

To understand the contribution of mutant IDH1 to pre-leukemic hematopoiesis, we generated an inducible, pre-clinical mouse model of *Idh1*-mutant hematopoiesis. Our model allowed us to assess the effects of a heterozygous *Idh1* mutation on adult hematopoietic differentiation while maintaining both alleles under the control of the endogenous promoter. Our detailed characterization of these mice revealed an intrinsic blockade in neutrophil maturation, characterized by the accumulation of immature myeloid progenitors and reduced formation of terminally differentiated neutrophils. Impaired neutrophil maturation was caused by the inability of *Idh1*-mutant myeloid progenitors to upregulate *Cebpe* expression even upon inhibition of mutant IDH1. Enforced activity of CEBPA restored *Cebpe* expression and rescued neutrophil differentiation. Furthermore, the *Cebpe* locus in *Idh1*-mutant cells exhibited reduced chromatin accessibility and aberrant DNA methylation. Treatment with the clinically

relevant combination of IDH1 and DNMT inhibitors did not only restore *Cebpe* expression but also fully rescued neutrophil differentiation of *Idh1*-mutant cells. These observations are consistent with recent clinical trials in IDH1-mutant AML showing that combined treatment with ivosidenib and azacitidine leads to an early and strong increase in neutrophil counts. This combination therapy was further associated with higher objective response rates, prolonged overall survival, and fewer infectious complications compared with azacitidine monotherapy [46, 52].

Clinically and experimentally, lack of response to ivosidenib or relapse have been observed previously [47, 53]. In this study, we observed that 32D-R132H cells treated with the IDH1-inhibitor tool-compound AGI-5198 were fully depleted from D2HG. This resulted in near complete normalization of cell proliferation and partial recovery of myeloid differentiation while neutrophil differentiation remained impaired. Similarly, IDH1-mutant patients not responding to ivosidenib may have D2HG-levels below the detection limit [47]. Further, impaired *Cebpe* expression in *Idh1*-mutant myeloid progenitors could not be rescued even upon IDH1-inhibitor treatment. This suggested that reduced *Cebpe* expression might underlie the persistent neutrophil differentiation defect as CEBPE plays a central role in terminal neutrophil differentiation [54-57] [34, 58, 59]. Genetic loss of *Cebpe* results in enrichment of early neutrophil progenitors at the expense of mature neutrophils [58, 59], resembling the defects observed in our *Idh1*-R132H mice. Inhibition of differentiation pathways by mutant IDH1 has already been demonstrated in other organ contexts. For example, *IDH1*- or *IDH2*-mutated hepatoblasts exhibited decreased HNF-4α expression, resulting in impaired hepatocyte differentiation [60]. Similarly, accumulation of D2HG has been linked to repressed expression of lineage-specific genes *in vitro* in adipocyte and myogenic differentiation systems [7, 14]. Taken together, mutant IDH1 may suppress activation of specific lineage programs leading to inhibition of cell differentiation and maturation.

Previously, different mouse models of mutant *IDH1* have been employed which resulted in phenotypes that are partially different from those observed in the present study. Sasaki and colleagues established the first conditional *Idh1*-R132H mouse model driven by Cre-expression either from the *Vav1*- or *LysM*-promoter [61]. Their study presented mild changes in *Idh1*-mutant hematopoiesis characterized by anemia and splenomegaly in older animals. While we observed neither, it has to be mentioned that in *Vav1*- and *LysM*-driven mouse models, animals are born with active *Idh1*-R132H resulting in constitutively elevated D2HG serum levels. Similar to our study, Sasaki and colleagues did not observe any differences in overall GMP and CMP frequency. However, we observed an enrichment of $Ly6C^+$ GMPs and $CD55^-$ CMPs at the expense of $Ly6C^-$ GMPs and $CD55^+$ CMPs. The relative reduction of $CD55^+$ CMPs with erythroid potential in *Idh1*-R132H mice was the only hint for a potential erythropoietic defect in our mouse model, as MEP frequency, RBC counts as well as hemoglobin levels remained unchanged during the observation period of 16 weeks.

Alternative experimental systems have examined mutant *IDH1* under non-physiological expression contexts. Gruber and colleagues studied *IDH1*-R132H

hematopoiesis by overexpressing mutant *IDH1* together with mutant *DNMT3A* and *NRAS* in murine fetal liver HSPCs which led to strong enrichment of immature neutrophils in triple-mutated leukemic mice [54]. In addition, Landberg and colleagues recently expressed *IDH1*-R132H in human $CD34^+$ HSPCs from the AAVS1 locus under an SFFV promoter, thereby bypassing endogenous regulation [62]. In their model, *IDH1*-mutant HSPCs retained multilineage engraftment capacity in NSG mice with impaired differentiation across multiple hematopoietic lineages. In contrast, using our inducible *Scl*-$CreER^T$ model that preserves endogenous, cell type-specific regulation of *Idh1* expression, we observed a lineage-restricted phenotype predominantly affecting neutrophil differentiation. Together, these findings emphasize that the level and context of *IDH1* expression are likely critical determinants of the resulting hematopoietic phenotype and may explain differences observed between constitutive overexpression models and models preserving endogenous regulation.

In summary, our findings reveal critical differences between mutant *IDH1* and *IDH2* in normal hematopoiesis and in AML patients, particularly regarding their effects on neutrophil differentiation. In the presence of mutant IDH1, the physiological upregulation of *IDH1* expression in myeloid progenitors is tightly linked to impaired activation of *Cebpe* expression which causes a block in neutrophil differentiation. These results suggest a unique, lineage-specific role for mutant IDH1 in myeloid neoplasms and our findings reveal the mechanisms underlying the observed hematopoietic defects. Furthermore, our data indicate that mutant IDH1 has D2HG independent effects on hematopoietic differentiation. Together, these insights could lead to novel therapeutic approaches that increase the efficacy of IDH1 inhibition and to biomarkers that predict how IDH1-mutant AML patients respond to therapy.

Supplementary Data

See supplementary file

Code availability

No original code was created for this study.

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Authorship contributions

M.H., and D.B.L. conceived the study and designed experiments.
M.H., J.L., E.K., M.S. and S.S. performed all mouse experiments.
M.H., J.L., M.J.R.M., E.K., M.S., M. Hartmann, H.R., J.W., M.T. and Y.P. performed experiments.
M.H. performed all bioinformatic analyses, M.S. provided bioinformatic support.
R.C., L.B., C.C.O, K.Z., M.C., U.P., N.K., S.R., U.G., G.H. collected or/and provided patient data.
M.H., M. Hartmann, S.H., K.R., M. Hartmann designed and established single-cell RNA sequencing experiments.
M.H., J.L., M.S., M. Hartmann, N.K., S.R., U.G., S.F., S.P., C.P., M.D.M., D.B.L interpreted the data.
D.B.L. acquired funding and supervised the project.
M.H. and D.B.L. wrote the manuscript.

Conflicts of interest

D.B.L. received honoraria from Infectopharm GmbH.
S.F. reports consultancy fees from Illumina.
G.H. is an employee of MLL Munich Leukemia Laboratory GmbH.
N.K. is an employee of Servier Deutschland GmbH.

All other authors declare no conflicts of interest.

References

1. Gozdecka, M., et al., *Genetic Vulnerabilities of DNMT3AR882H in Myeloid Malignancies.* Blood, 2019. **134**(Supplement_1): p. 111-111.
2. Ley, T.J., et al., *DNMT3A mutations in acute myeloid leukemia.* N Engl J Med, 2010. **363**(25): p. 2424-33.
3. Miles, L.A., et al., *Single-cell mutation analysis of clonal evolution in myeloid malignancies.* Nature, 2020. **587**(7834): p. 477-482.
4. Simonin, M., et al., *Oncogenetic landscape and clinical impact of IDH1 and IDH2 mutations in T-ALL.* J Hematol Oncol, 2021. **14**(1): p. 74.
5. Dang, L., et al., *Cancer-associated IDH1 mutations produce 2-hydroxyglutarate.* Nature, 2009. **462**(7274): p. 739-44.
6. Kusi, M., et al., *2-Hydroxyglutarate destabilizes chromatin regulatory landscape and lineage fidelity to promote cellular heterogeneity.* Cell Rep, 2022. **38**(2): p. 110220.
7. Lu, C., et al., *IDH mutation impairs histone demethylation and results in a block to cell differentiation.* Nature, 2012. **483**(7390): p. 474-8.
8. Turcan, S., et al., *Mutant-IDH1-dependent chromatin state reprogramming, reversibility, and persistence.* Nat Genet, 2018. **50**(1): p. 62-72.
9. Figueroa, M.E., et al., *Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation.* Cancer Cell, 2010. **18**(6): p. 553-67.
10. Gaidzik, V.I., et al., *TET2 mutations in acute myeloid leukemia (AML): results from a comprehensive genetic and clinical analysis of the AML study group.* J Clin Oncol, 2012. **30**(12): p. 1350-7.
11. Chowdhury, R., et al., *The oncometabolite 2-hydroxyglutarate inhibits histone lysine demethylases.* EMBO Rep, 2011. **12**(5): p. 463-9.
12. Xu, W., et al., *Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases.* Cancer Cell, 2011. **19**(1): p. 17-30.
13. Schvartzman, J.M., et al., *Oncogenic IDH mutations increase heterochromatin-related replication stress without impacting homologous recombination.* Mol Cell, 2023.
14. Schvartzman, J.M., et al., *2-hydroxyglutarate inhibits MyoD-mediated differentiation by preventing H3K9 demethylation.* Proc Natl Acad Sci U S A, 2019. **116**(26): p. 12851-12856.
15. Cancer Genome Atlas Research, N., et al., *Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia.* N Engl J Med, 2013. **368**(22): p. 2059-74.
16. Schnittger, S., et al., *IDH1 mutations are detected in 6.6% of 1414 AML patients and are associated with intermediate risk karyotype and unfavorable prognosis in adults younger than 60 years and unmutated NPM1 status.* Blood, 2010. **116**(25): p. 5486-96.
17. Cairns, R.A., et al., *IDH2 mutations are frequent in angioimmunoblastic T-cell lymphoma.* Blood, 2012. **119**(8): p. 1901-3.
18. Hetzel, S., et al., *Acute lymphoblastic leukemia displays a distinct highly methylated genome.* Nat Cancer, 2022. **3**(6): p. 768-782.
19. Xu, Q., et al., *Correlation Between Isocitrate Dehydrogenase Gene Aberrations and Prognosis of Patients with Acute Myeloid Leukemia: A Systematic Review and Meta-Analysis.* Clin Cancer Res, 2017. **23**(15): p. 4511-4522.

20. Hartmann, C., et al., *Patients with IDH1 wild type anaplastic astrocytomas exhibit worse prognosis than IDH1-mutated glioblastomas, and IDH1 mutation status accounts for the unfavorable prognostic effect of higher age: implications for classification of gliomas.* Acta Neuropathol, 2010. **120**(6): p. 707-18.
21. Wang, H.Y., et al., *The comparison of clinical and biological characteristics between IDH1 and IDH2 mutations in gliomas.* J Exp Clin Cancer Res, 2016. **35**: p. 86.
22. Badur, M.G., et al., *Oncogenic R132 IDH1 Mutations Limit NADPH for De Novo Lipogenesis through (D)2-Hydroxyglutarate Production in Fibrosarcoma Cells.* Cell Rep, 2018. **25**(6): p. 1680.
23. Thomas, D., et al., *Dysregulated Lipid Synthesis by Oncogenic IDH1 Mutation Is a Targetable Synthetic Lethal Vulnerability.* Cancer Discov, 2023. **13**(2): p. 496-515.
24. Haferlach, T., et al., *Morphologic dysplasia in de novo acute myeloid leukemia (AML) is related to unfavorable cytogenetics but has no independent prognostic relevance under the conditions of intensive induction therapy: results of a multiparameter analysis from the German AML Cooperative Group studies.* J Clin Oncol, 2003. **21**(2): p. 256-65.
25. Maierhofer, A., et al., *The clinical and genomic landscape of patients with DDX41 variants identified during diagnostic sequencing.* Blood Adv, 2023. **7**(23): p. 7346-7357.
26. Giacopelli, B., et al., *DNA methylation epitypes highlight underlying developmental and disease pathways in acute myeloid leukemia.* Genome Res, 2021. **31**(5): p. 747-761.
27. Tyner, J.W., et al., *Functional genomic landscape of acute myeloid leukaemia.* Nature, 2018. **562**(7728): p. 526-531.
28. Schmutz, M., et al., *Differential DNA Methylation Predicts Response To Combined Treatment Regimens With a DNA Methyltransferase Inhibitor In Acute Myeloid Leukemia (AML).* Blood, 2013. **122**(21): p. 2539-2539.
29. Lipka, D.B., et al., *RAS-pathway mutation patterns define epigenetic subclasses in juvenile myelomonocytic leukemia.* Nat Commun, 2017. **8**(1): p. 2126.
30. Reinius, L.E., et al., *Differential DNA methylation in purified human blood cells: implications for cell lineage and studies on disease susceptibility.* PLoS One, 2012. **7**(7): p. e41361.
31. Jung, N., et al., *An LSC epigenetic signature is largely mutation independent and implicates the HOXA cluster in AML pathogenesis.* Nat Commun, 2015. **6**: p. 8489.
32. Issa, G.C. and C.D. DiNardo, *Acute myeloid leukemia with IDH1 and IDH2 mutations: 2021 treatment algorithm.* Blood Cancer J, 2021. **11**(6): p. 107.
33. Regev, A., et al., *The Human Cell Atlas.* Elife, 2017. **6**.
34. Theilgaard-Monch, K., et al., *Transcription factor-driven coordination of cell cycle exit and lineage-specification in vivo during granulocytic differentiation : In memoriam Professor Niels Borregaard.* Nat Commun, 2022. **13**(1): p. 3595.
35. Stäble, S., *Deconvolution of Hematopoietic Commitment Decisions by Genome-wide Analysis of Progressive DNA Methylation Changes.* Doctoral Thesis at the Faculty of Biosciences, Ruprecht-Karls-University Heidelberg, 2019.
36. Krämer, S., *Uncovering the mechanisms and information content of CpG-resolved DNA methylation programming during hematopoietic differentiation.*

Doctoral Thesis at the Faculty of Biosciences, Ruprecht-Karls-University Heidelberg, 2023.
37. Sanchez, M., et al., *An SCL 3' enhancer targets developing endothelium together with embryonic and adult haematopoietic progenitors.* Development, 1999. **126**(17): p. 3891-904.
38. Gottgens, B., et al., *Establishing the transcriptional programme for blood: the SCL stem cell enhancer is regulated by a multiprotein complex containing Ets and GATA factors.* EMBO J, 2002. **21**(12): p. 3039-50.
39. Gothert, J.R., et al., *In vivo fate-tracing studies using the Scl stem cell enhancer: embryonic hematopoietic stem cells significantly contribute to adult hematopoiesis.* Blood, 2005. **105**(7): p. 2724-32.
40. Sanchez, M.J., et al., *Selective rescue of early haematopoietic progenitors in Scl(-/-) mice by expressing Scl under the control of a stem cell enhancer.* Development, 2001. **128**(23): p. 4815-27.
41. Baccin, C., et al., *Combined single-cell and spatial transcriptomics reveal the molecular, cellular and spatial bone marrow niche organization.* Nat Cell Biol, 2020. **22**(1): p. 38-48.
42. Theilgaard-Monch, K., et al., *Haptoglobin is synthesized during granulocyte differentiation, stored in specific granules, and released by neutrophils in response to activation.* Blood, 2006. **108**(1): p. 353-61.
43. Flavahan, W.A., et al., *Insulator dysfunction and oncogene activation in IDH mutant gliomas.* Nature, 2016. **529**(7584): p. 110-4.
44. Hartmann, M., et al., *Molecular Plasticity Results in Oncofetal Reprogramming and Therapeutic Vulnerabilities in Juvenile Myelomonocytic Leukemia.* Blood Cancer Discov, 2025.
45. Schönung, M., et al., *Dynamic DNA methylation reveals novel cis-regulatory elements in mouse hematopoiesis.* Exp Hematol, 2023. **117**: p. 24-42 e7.
46. Montesinos, P., et al., *Ivosidenib and Azacitidine in IDH1-Mutated Acute Myeloid Leukemia.* N Engl J Med, 2022. **386**(16): p. 1519-1531.
47. DiNardo, C.D., et al., *Durable Remissions with Ivosidenib in IDH1-Mutated Relapsed or Refractory AML.* N Engl J Med, 2018. **378**(25): p. 2386-2398.
48. Birendra, K.C. and C.D. DiNardo, *Evidence for Clinical Differentiation and Differentiation Syndrome in Patients With Acute Myeloid Leukemia and IDH1 Mutations Treated With the Targeted Mutant IDH1 Inhibitor, AG-120.* Clin Lymphoma Myeloma Leuk, 2016. **16**(8): p. 460-5.
49. Dunn-Valadez, S., et al., *IDH1 inhibitor-induced neutrophilic dermatosis in a patient with acute myeloid leukemia.* Cancer Treat Res Commun, 2022. **31**: p. 100560.
50. Norsworthy, K.J., et al., *Differentiation Syndrome with Ivosidenib and Enasidenib Treatment in Patients with Relapsed or Refractory IDH-Mutated AML: A U.S. Food and Drug Administration Systematic Analysis.* Clin Cancer Res, 2020. **26**(16): p. 4280-4288.
51. Papaemmanuil, E., et al., *Genomic Classification and Prognosis in Acute Myeloid Leukemia.* N Engl J Med, 2016. **374**(23): p. 2209-2221.
52. Montesinos, P., et al., *Long-term results from the AGILE study of azacitidine plus ivosidenib vs placebo in newly diagnosed IDH1-mutated AML.* Blood Adv, 2025. **9**(20): p. 5177-5189.
53. Gruber, E., et al., *Inhibition of mutant IDH1 promotes cycling of acute myeloid leukemia stem cells.* Cell Rep, 2022. **40**(7): p. 111182.

54. Paul, F., et al., *Transcriptional Heterogeneity and Lineage Commitment in Myeloid Progenitors.* Cell, 2015. **163**(7): p. 1663-77.
55. Lekstrom-Himes, J.A., *The role of C/EBP(epsilon) in the terminal stages of granulocyte differentiation.* Stem Cells, 2001. **19**(2): p. 125-33.
56. Yamanaka, R., et al., *Impaired granulopoiesis, myelodysplasia, and early lethality in CCAAT/enhancer binding protein epsilon-deficient mice.* Proc Natl Acad Sci U S A, 1997. **94**(24): p. 13187-92.
57. Friedman, A.D., *Transcriptional control of granulocyte and monocyte development.* Oncogene, 2007. **26**(47): p. 6816-28.
58. Kwok, I., et al., *Combinatorial Single-Cell Analyses of Granulocyte-Monocyte Progenitor Heterogeneity Reveals an Early Uni-potent Neutrophil Progenitor.* Immunity, 2020. **53**(2): p. 303-318 e5.
59. Evrard, M., et al., *Developmental Analysis of Bone Marrow Neutrophils Reveals Populations Specialized in Expansion, Trafficking, and Effector Functions.* Immunity, 2018. **48**(2): p. 364-379 e8.
60. Saha, S.K., et al., *Mutant IDH inhibits HNF-4alpha to block hepatocyte differentiation and promote biliary cancer.* Nature, 2014. **513**(7516): p. 110-4.
61. Sasaki, M., et al., *IDH1(R132H) mutation increases murine haematopoietic progenitors and alters epigenetics.* Nature, 2012. **488**(7413): p. 656-9.
62. Landberg, N., et al., *IDH1-mutant preleukemic hematopoietic stem cells can be eliminated by inhibition of oxidative phosphorylation.* Blood Cancer Discov, 2024. **5**(2): p. 114-131.

Figure Legends

Figure 1: *IDH1*-mutant myeloid neoplasms show a defect in neutropoiesis. (A, B) PCA with visualization of PC1 and PC2 of HSPCs, myeloid progenitors and mature myeloid cells together with 16 *IDH1*-mutant **(A)** and 29 *IDH2*-mutant AML **(B)** based on the 5000 mvCpGs identified across all healthy hematopoietic cell types shown. HSPCs: hematopoietic stem and progenitor cells, CMPs: common myeloid progenitor cells, GMPs: granulocyte-monocyte progenitors, MEPs: megakaryocyte-erythrocyte progenitors, monocytes, neutrophils, eosinophils, natural killer cells, B cells, T cells. **(C, D)** Visualization of PC2 and PC3 from the PCA based on the 5000 mvCpGs found across all healthy hematopoietic cell types shown in C) and D). *IDH1*-mutant **(C)** and *IDH2*-mutant **(D)** AML samples are shown. **(E,F)** Percentage of bone marrow granulopoiesis (BM granulopoiesis) as determined by cytomorphology from diagnostic BM smears of AML patients from the Munich Leukemia Laboratory (MLL, n = 3670) **(E)** and Düsseldorf (n = 255) **(F)** cohorts with *IDH1*-mutant (IDH1), *IDH2*-mutant (IDH2), or *IDH1/2*-wildtype (WT) AML. (**G, H**) Absolute neutrophil counts (ANC) in peripheral blood of AML patients. **(G)** Data from n = 857 AML patients analyzed in MLL are depicted. **(H)** Data from n = 255 AML patients analyzed and documented in the Düsseldorf registry (Düsseldorf). Pairwise comparisons between groups were performed using the Mann-Whitney U test with Benjamini–Hochberg correction for multiple testing. Only p-values < 0.05 are displayed.

Figure 2: *IDH1* is transcriptionally upregulated specifically in the myeloid lineage. UMAP representation and annotation of healthy adult human **(A,B)** and murine **(C,D)** bone marrow scRNA-sequencing data. Cell type annotation was done based on marker gene expression **(A,C)**. Normalized *IDH1/Idh1* transcript levels in hematopoietic cells from adult human **(B)** and mouse **(D)** bone marrow. **E)** DNA methylation tracks surrounding the TSSs of *Idh1* transcript variants 1 (TV-1) and 2 (TV-2) in normal murine hematopoietic cells [35, 36]. HSC - hematopoietic stem cell, MPP - multipotent progenitor, CMP - common myeloid progenitor, MEP – megakaryocyte-erythroid progenitor, preMegE – pre-megakaryocyte-erythroid progenitor, MkP – megakaryocyte progenitor, CFU-E – colony forming unit-erythroid, MDP – monocyte-dendritic cell progenitor, GMP – granulocyte-monocyte progenitor, cMoP – common monocyte progenitor, Mono – Monocytes, Neutro – Neutrophil granulocytes, Eosino – Eosiophil granulocytes, CDP – common dendritic progenitor, DC – dendritic cells, PDC – plasmacytoid dendritic cells, CLP – common lymphoid progenitor. **(F, G)** TV-specific qRT-PCR from myeloid progenitors and mature myeloid cells normalized to the expression of erythrocyte/megakaryocyte-primed primed $CD55^{neg}$ CMPs (n = 3). Raw expression data from *Idh1* transcript variant 1 **(F)** and *Idh1* transcript variant 2 **(G)** were normalized to *Gapdh* and expression of each transcript variant is plotted relative to the expression found in $CD55^{pos}$ CMPs.

Figure 3: An inducible mouse model of *Idh1*-mutant hematopoiesis reveals a neutrophil differentiation block in myeloid progenitor cells. A) Experimental overview for generating bone marrow chimeric *Idh1*-WT and *Idh1*-R132H mice by transplanting 10^5 $Lin^{neg}EYFP^{pos}$ cells from primary induced donor mice into lethally irradiated recipients. **(B – E)** Frequency of **(B)** $CD55^{neg}$ and **(C)** $CD55^{pos}$ CMPs, **(D)** $Ly6C^{neg}$ and **(E)** $Ly6C^{pos}$ GMPs in the bone marrow of *Idh1*-WT (n = 5) and *Idh1*-R132H (n = 5) mice. **(F – H)** Frequency of **(F)** $CD11b^{pos}$ mature myeloid cells (n = 13 for *Idh1*-WT and n = 12 for *Idh1*-R132H), **(G)** neutrophils (n = 42 for *Idh1*-WT and n = 43 for *Idh1*-R132H) and **(H))** Ly6C+ monocytes (n = 26 for *Idh1*-WT and n = 24 for *Idh1*-R132H). All comparisons between groups were performed using the Mann–Whitney U test. Only p-values < 0.05 are displayed.

Figure 4: *Idh1*-R132H myeloid progenitors exhibit a cell-intrinsic neutrophil differentiation defect. A) Experimental workflow for *in vivo* differentiation. Bone marrow of *Idh1*-WT and *Idh1*-R132H mice was isolated 16 weeks after transplantation. Bone marrow was subjected to lineage depletion and FACS to isolate $EYFP^{pos}Lin^{neg}cKit^{pos}Sca1^{neg}CD34^{pos}CD16/32^{neg}CD55^{neg}$ CMPs. $EYFP^{pos}CD55^{neg}$ CMPs were transplanted into non-irradiated secondary recipients. Secondary recipient bone marrow was subjected to flow cytometric analysis 7 days after adoptive transfer. (**B – D)** Flow cytometry analysis of bone marrow isolated on day 7 after adoptive transfer (n = 6). All cell fractions are relative to all $EYFP^{pos}$ cells detected. **B)** Frequency of $CD11b^{pos}$ myeloid cells. **C)** Frequency of mature neutrophils. **D)** Frequency of monocytes. **(E – H)** Analysis of an *in vitro* model of *Idh1*-mutant granulopoiesis. **E)** Experimental workflow for generating *Idh1*-mutant 32D cells and inducing neutrophil differentiation via G-CSF treatment in the absence of IL-3. **F)** Number of living cells at day 6 of differentiation. **G)** Frequency of $CD11b^{high}$ cells and **H)** mature neutrophils at day 6 of differentiation. All comparisons between groups were performed using the Mann-Whitney U test.

Figure 5: *Idh1*-R132H neutrophil progenitors present impaired *Cebpe* expression A) Experimental workflow. Bone marrow of *Idh1*-WT (n = 2) and *Idh1*-R132H (n = 2) mice was collected and subjected to FACS isolation. To enrich each sample with hematopoietic stem and progenitor cells, equal number of cells (4000) per layer ($CD45^+$ TBM, Lin^-cKit^+ and $Lin^-cKit^+Sca1^+$) were sorted into the same tube which was then subjected to library preparation according to the Chromium 10X protocol. **B)** After dimension reduction and clustering analyses, clusters were annotated to hematopoietic cell states based on the expression of state-specific marker genes (Giladi et al., 2018; Paul et al., 2015). All 46 clusters were annotated separately and summarized in lineage groups for visualization. **C)** Projection of the GMP expression signature (GMP-score) into the UMAP. **D)** Quantification of GMP-score in *Idh1*-WT and *Idh1*-R132H LNPs. Statistical testing was performed with Mann–Whitney U test. **E)** Projection of the NeutroP expression signature (NeutroP-score) into the UMAP. **F)** Quantification of NeutroP score in *Idh1*-WT and *Idh1*-R132H LNPs. Statistical testing

was performed with Mann–Whitney U test. (**G-H)** Expression of key markers for neutrophil identity and differentiation towards neutrophilic granulocytes in cells derived from **G)** *Idh1*-WT and **H)** *Idh1*-R132H bone marrow. Overall, per genotype, the same number of cells was subjected to analysis. Columns represent single cells which were ordered according to their diffusion map pseudotime with the trajectory spanning from early myeloid progenitors (EMPs; on the left) to LNPs (on the right). Each row represents a specific gene. Row clustering was performed based on z-score transformed gene expression of WT cells using Euclidean distance. Gene expression heatmap of *Idh1*-R132H cells was row-matched. **(I – L**) Expression of key differentiation genes in LNPs. Statistical testing was performed with Mann–Whitney U test**. M)** Slingshot trajectory analysis of myeloid clusters reproducing two lineages corresponding to the monocytic and neutrophilic lineage. **N)** Density curves showing the distribution of bone marrow cells from the neutrophil lineage of *Idh1*-WT and *Idh1*-R132H mice ordered by their differentiation pseudotime calculated with slingshot. Annotated cell types are shown as bar plots below the density curves. Statistical testing was performed with Mann–Whitney U test. **O)** *Cebpe* expression along the myeloid progenitor trajectory starting from annotated early myeloid progenitors (EMPs) to LNPs. **P)** Expression of *Cebpe* in 32D-EV (n = 8) and 32D-R132H (n = 8) cells in steady-state cell culture conditions measured with qRT-PCR. Expression was normalized to the expression of a randomly chosen 32D-EV sample. Statistical testing was performed with Mann–Whitney U test.

Figure 6: *Idh1*-R132H myeloid progenitors are responsive to G-CSF treatment. **A)** Experimental workflow for sorting and differentiating *Idh1*-WT (n = 3) and *Idh1*-R132H (n = 3) EYFP^{pos}CD55^{neg} CMPs in *ex vivo* conditions with or without instructive cytokines. **B)** Frequency of CD11b^{pos} myeloid cells, monocytes and neutrophils after 96 h of differentiation in the absence of instructive cytokines. **C)** Frequency of CD11b^{pos} myeloid cells, monocytes and neutrophils after 96 h culture in the presence of M-CSF. **D)** Frequency of CD11b^{pos} myeloid cells, monocytes and neutrophils after 96 h culture in the presence of G-CSF. All comparisons between groups were performed using the Mann-Whitney U test. Only p-values < 0.05 are displayed.

Figure 7: Re-activation of *CEBPE* expression restores neutrophil differentiation in *Idh1*-R132H cells.
A) D2HG concentration in DMSO or AGI-5198 treated 32D-EV (n = 3) and 32D-R132H (n = 3) cells 5 days after start of treatment. **B)** Number of cells on the last day of differentiation with or without AGI-5198 treatment. **C)** Frequency of CD11b^{pos} cells and **D)** of mature neutrophils on the last day of differentiation with or without AGI-5198 treatment. **E)** Expression of *Cebpe* after five days of AGI-5198 treatment in steady-state 32D-EV and 32D-R132H cells. **F)** Experimental workflow for introducing inducible CEBPA into 32D-EV and 32D-R132H cells and for inducing neutrophil differentiation. Puromycin-selected cells were subjected to G-CSF-mediated differentiation with or without the activation of CEBPA-ER construct. Differentiation

was stopped after four days. **G)** Expression of *Cebpe* without CEBPA activation in steady-state culture. **H)** Expression of *Cebpe* after differentiation with or without activation of CEBPA. *Cebpe* expression was normalized to steady-state expression of a randomly chosen 32D-EV sample. **I)** Frequency of $CD11b^{pos}$ and **J)** of mature neutrophils 96 h after the start of differentiation with or without activation of CEBPA. **K)** Genome browser tracks presenting chromatin accessibility and DNA methylation signal in 32D-EV and 32D-R132H cells at the *Cebpe* locus. Chromatin accessibility was assessed using ATAC-seq; DNA methylation was analyzed with long-read Oxford Nanopore sequencing. **L)** Expression of *Cebpe* in steady-state 32D-EV and 32D-R132H cells after 5 days of treatment with AGI-5198, 5-azacitidine (Aza) or combined treatment. **M)** Neutrophil differentiation of 32D-WT and 32D-R132H cells after 6 days of G-CSF following 5 day treatment with AGI-5198, Aza or AGI-5198 + AZA. For all statistical analyses, analysis of variance with Bonferroni correction was performed. Only adjusted p-values < 0.05 are shown. In M) repetitive comparisons of the 32D-EV control conditions to untreated 32D-R132H were omitted for clarity.

Figure 1

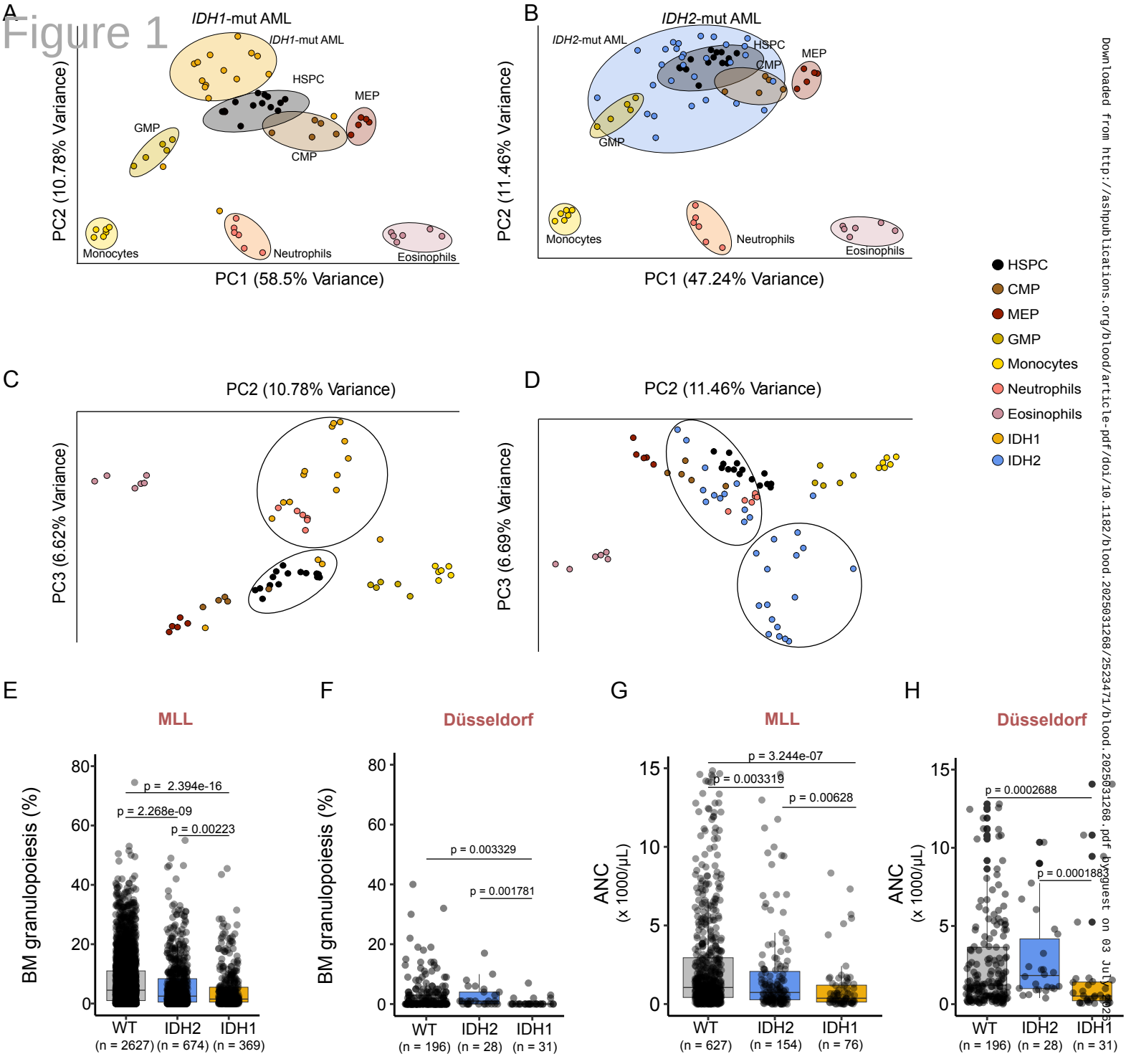

Figure 2

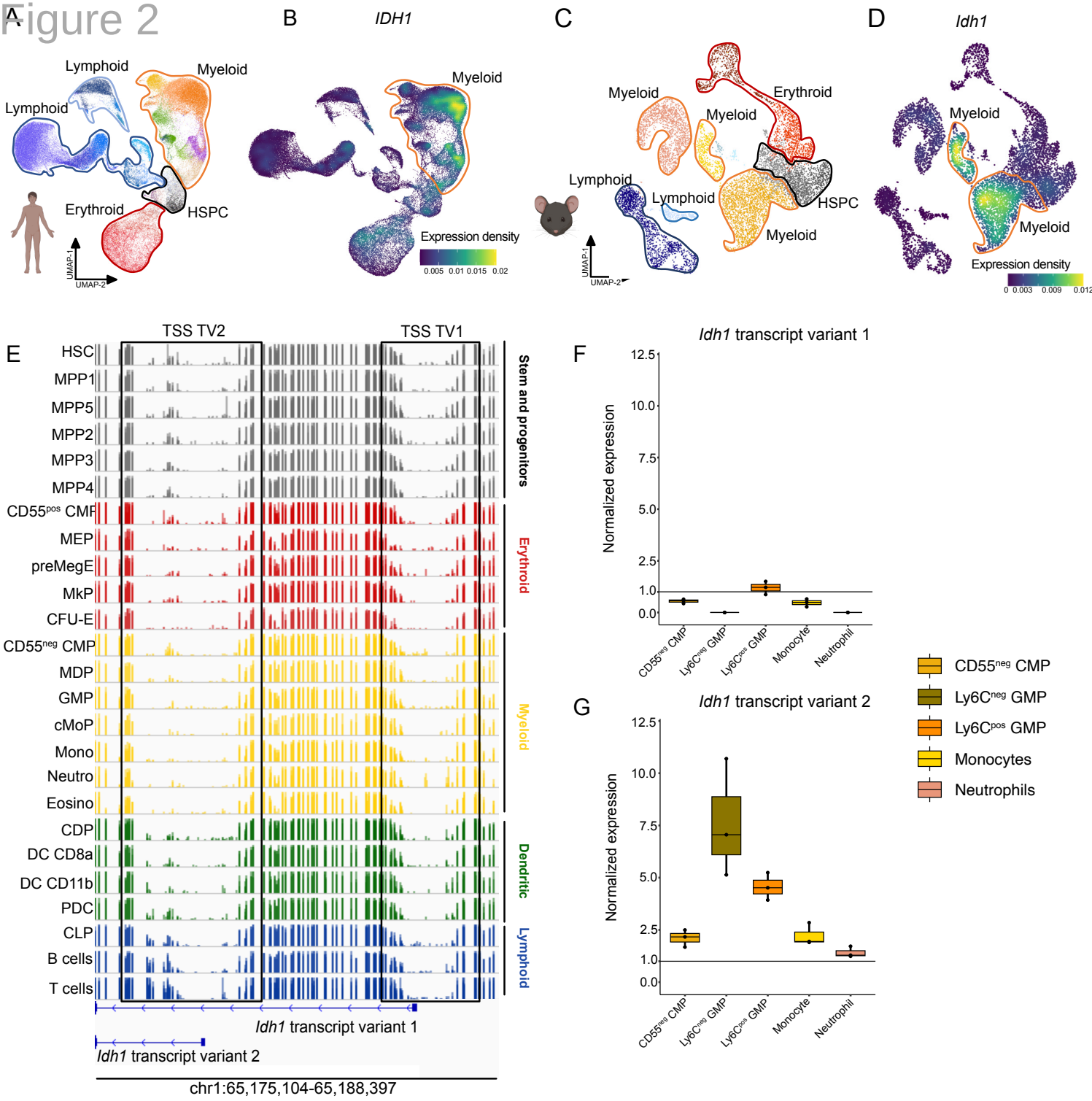

Figure 3

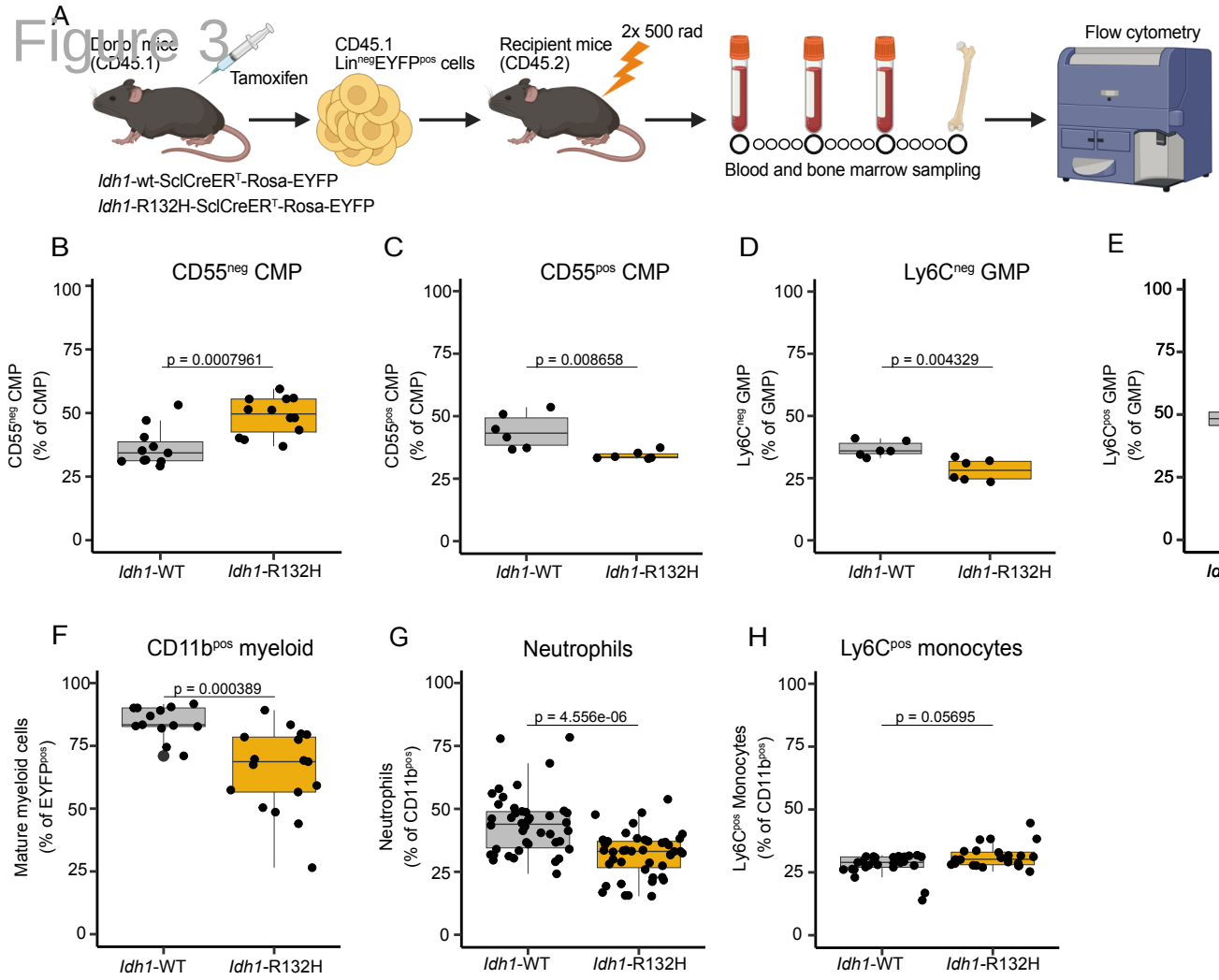

Figure 4

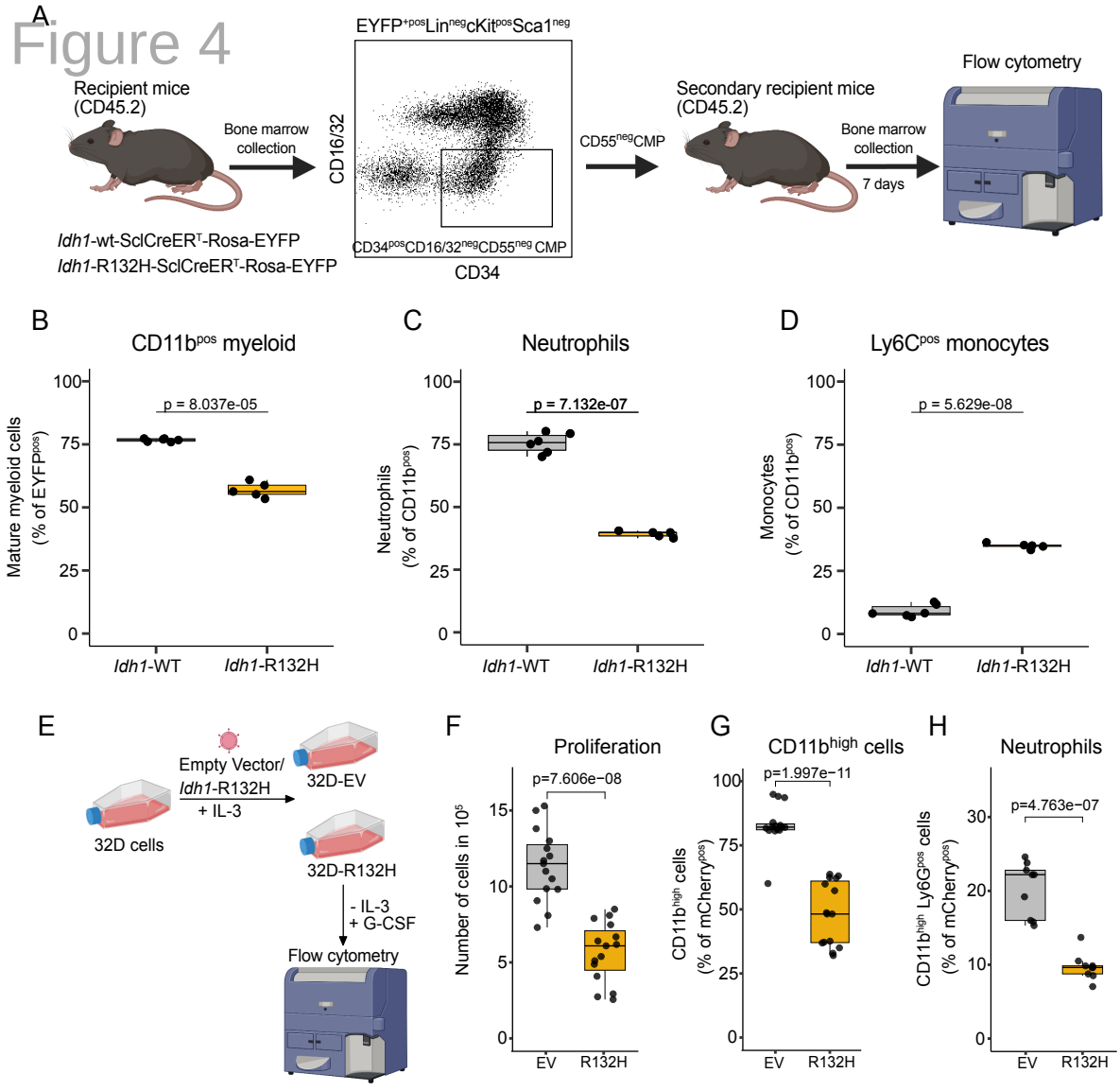

Figure 5

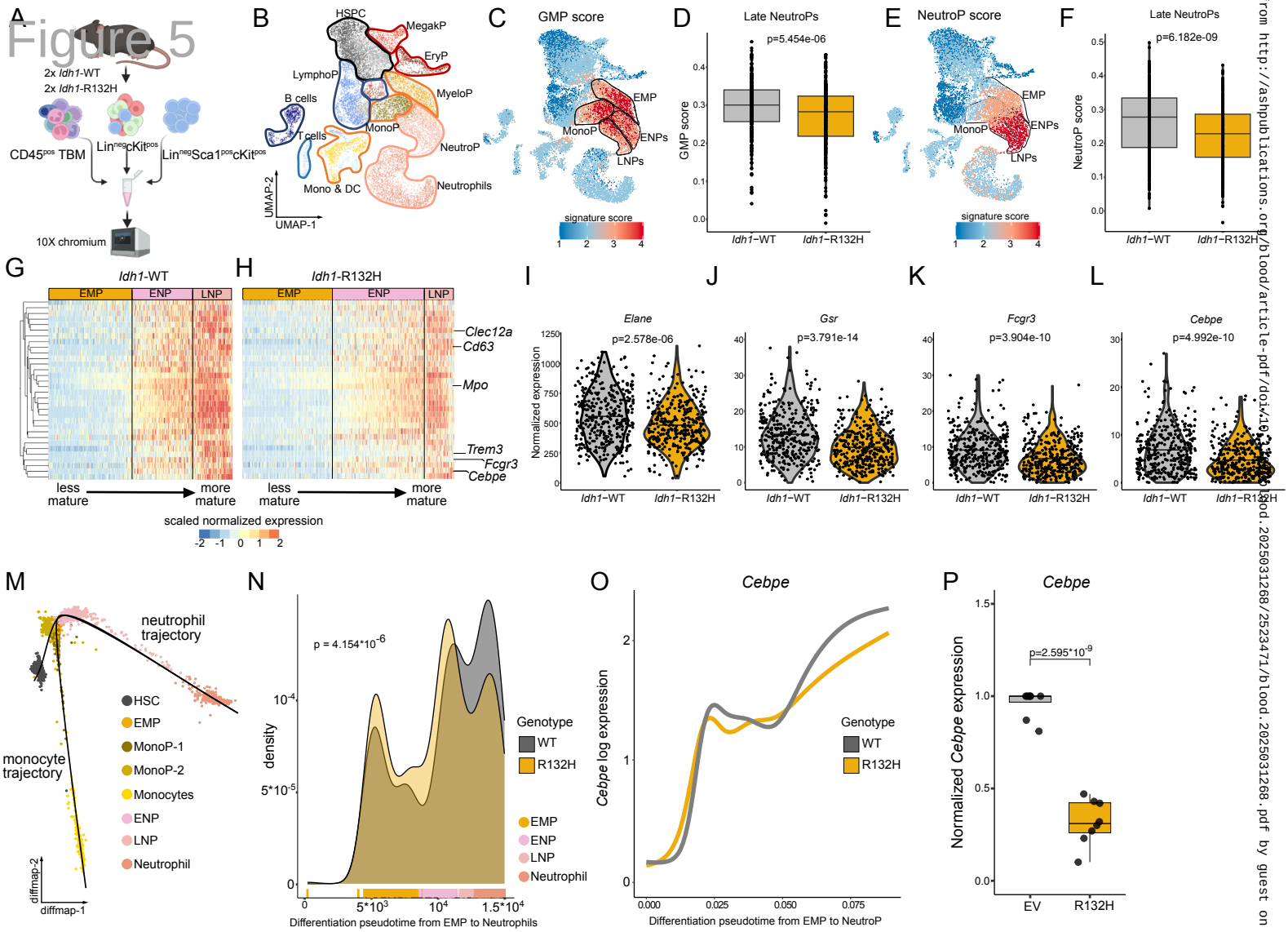

Figure 6

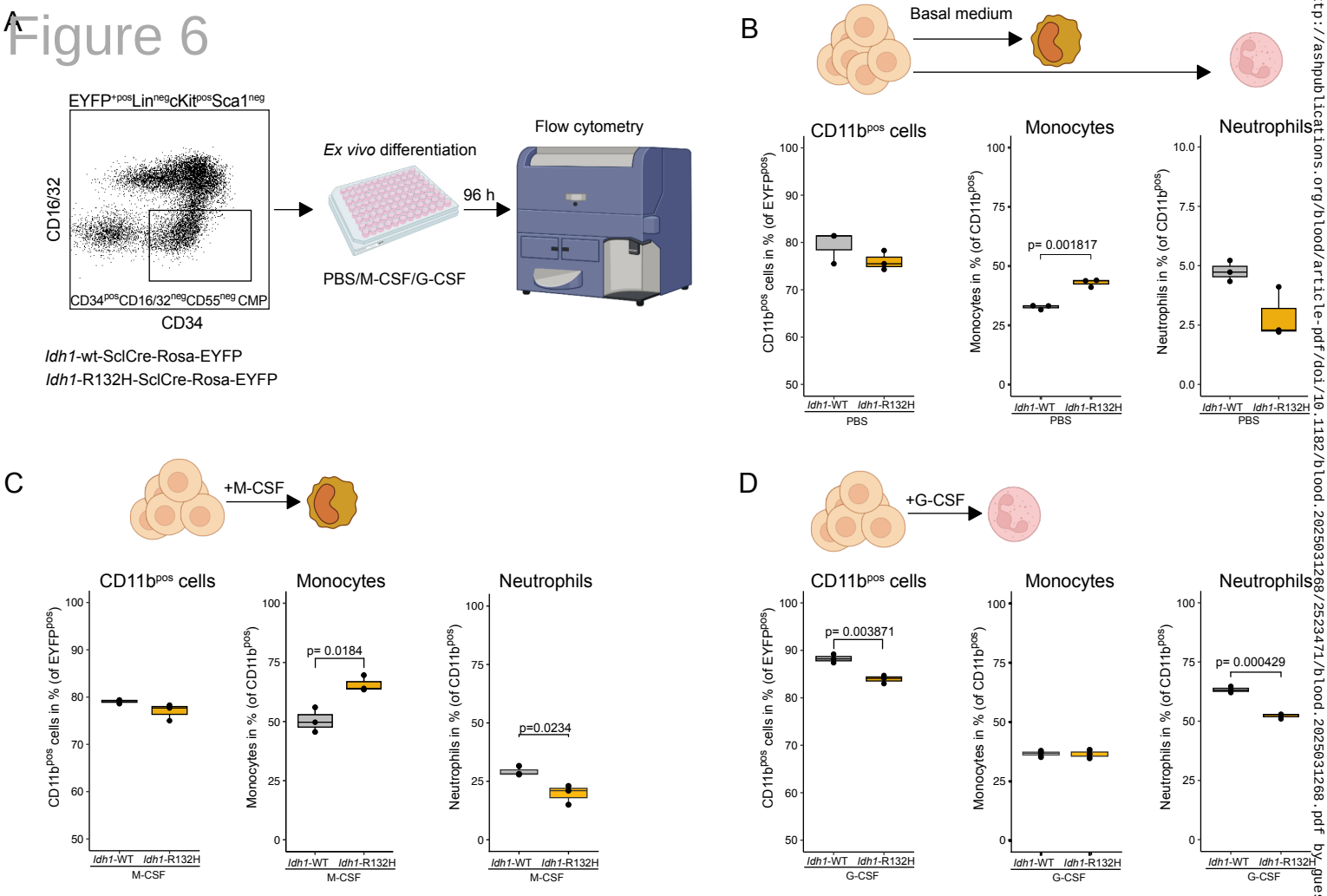

Figure 7