TFEB Links MYC Signaling to Epigenetic Control of Myeloid Differentiation and Acute Myeloid Leukemia

Seongseok Yun1, Nicole D. Vincelette1, Xiaoqing Yu2, Gregory W. Watson1, Mario R. Fernandez3, Chunying Yang3, Tafts Hitesugi4, Chia-Ho Cheng2, Audrey R. Freischel5, Ling Zhang5, Weimin Li3, Hsinan Hou6, Franz X. Schaub5, Alexis R. Vedder1, Ling Cen2, Kathy L. McGraw1, Jungwon Moon1, Daniel J. Murphy7, Andrea Ballabio8,9,10,11,12, Scott H. Kaufmann4, Anders E. Berglund2, and John L. Cleveland3
**ABSTRACT**

MYC oncoproteins regulate transcription of genes directing cell proliferation, metabolism, and tumorigenesis. A variety of alterations drive MYC expression in acute myeloid leukemia (AML), and enforced MYC expression in hematopoietic progenitors is sufficient to induce AML. Here we report that AML and myeloid progenitor cell growth and survival rely on MYC-directed suppression of Transcription Factor EB (TFEB), a master regulator of the autophagy–lysosome pathway. Notably, although originally identified as an oncogene, TFEB functions as a tumor suppressor in AML, where it provokes AML cell differentiation and death. These responses reflect TFEB control of myeloid epigenetic programs by inducing expression of isocitrate dehydrogenase-1 (IDH1) and IDH2, resulting in global hydroxylation of 5-methylcytosine. Finally, activating the TFEB-IDH1/IDH2–TET2 axis is revealed as a targetable vulnerability in AML. Thus, epigenetic control by an MYC–TFEB circuit dictates myeloid cell fate and is essential for maintenance of AML.

**SIGNIFICANCE:** Alterations in epigenetic control are a hallmark of AML. This study establishes that a MYC–TFEB circuit controls AML differentiation and epigenetic programs by inducing IDH1/IDH2 and hydroxylation of 5-methylcytosine, that TFEB functions as a tumor suppressor in AML, and that this circuit is a targetable vulnerability in AML. See related commentary by Wu and Eisenman, p. 116.

**INTRODUCTION**

Acute myeloid leukemia (AML) is an aggressive malignant hematologic disorder characterized by the accumulation of proliferating self-renewing myeloid progenitors arrested at various stages of differentiation, leading to impaired hematopoiesis and bone marrow (BM) failure (1). Despite significant advances in our understanding of the molecular pathogenesis of AML, there remains a pressing need for new therapeutic strategies to improve survival outcomes, which are disappointing and largely unchanged for the past three decades (2). Recent studies have identified a variety of chromosomal abnormalities and somatic mutations in epigenetic regulators, transcription factors, splicing components, and prosurvival proteins that contribute to malignant transformation and leukemic clonal expansion (1). Among these, MYC gene amplification, copy-number variation, and somatic mutations are frequent in both adult and pediatric AML (1, 3, 4).

Several observations suggest that these MYC alterations contribute to leukemogenesis. MYC oncoproteins [c-MYC (MYC), N-MYC, and L-MYC] are basic helix-loop-helix leucine zipper (bHLH-Zip) transcription factors that coordinate the expression of genes involved in glycolysis, glutaminolysis, nutrient transport, cell proliferation, and survival (5), and enforced MYC expression is sufficient to induce AML in mouse models (6). Moreover, human AMLs with increased levels of MYC protein have a poor prognosis independent of other prognostic variables (7). Finally, in mouse AML models, MYC is necessary for leukemias provoked by FLT3–ITD, PML–RARα, BCR–ABL1, and AML1–ETO fusion genes (8–11). Despite these findings, how MYC drives or contributes to the development and maintenance of AML has not been resolved. Further, it is unclear if MYC control of AML involves alterations in myeloid epigenetic programs.

Transcription factor EB (TFEB), an MYC-related bHLH-Zip transcription factor, activates genes that harbor CLEAR sites (TACCATGA) in their promoters (12). TFEB is a master regulator of genes that control autophagy and lysosome biogenesis, a central catabolic recycling pathway that regulates cell survival (13). Although TFEB is activated by chromosomal translocations and functions as an oncogene to drive renal cell cancer (14, 15), surprisingly, several common chromosomal deletions found in AML delete key autophagy genes (16). Further and paradoxically, autophagy is known to impair AML cell growth and trigger cell death and differentiation (16).
Given the oncogenic effects of MYC in AML (6) and that the induction of autophagy compromises AML cell growth and survival (16), we tested if MYC controls TFEB transcription programs during myelopoiesis and in AML. Here we report that MYC directly suppresses TFEB expression and thus its functions in myeloid progenitors and AML ex vivo and in vivo, that TFEB expression is dynamically regulated during myelopoiesis, and that TFEB functions as a tumor suppressor that provokes normal and malignant myeloid progenitor differentiation and cell death. Strikingly, these responses are therapeutically targetable and rely on epigenetic control, where TFEB expression is directly induced by a variety of transcription factors in AML and TCGA AML cohorts (Fig. S1A). Further, inducible MYC expression promoted colony formation in both BM (Supplementary Fig. S1F) and in vivo (Supplementary Fig. S1G). Conversely, inducible knockout of MYC in primary myeloid BM cells from Rosa26-CreERT2,Myeloid cells (Supplementary Fig. S1R) compromised myeloid cell proliferation and colony formation while triggering increases in CD11b and Gr-1 expression (Supplementary Fig. S1S–S1U). Thus, MYC is necessary and sufficient to suppress myeloid progenitor and AML cell differentiation.

Given the inverse relationship between MYC and myeloid differentiation, and the fact that loss of autophagy in hematopoietic stem cells (HSC) is sufficient to provoke a myeloproliferative, preleukemic phenotype (16), we reasoned that MYC’s ability to promote AML and suppress myeloid differentiation might rely on its suppression of TFEB expression and/or function. In accord with this notion, we observed that TFEB expression is the lowest in AML cell lines where MYC expression is high (Supplementary Figs. S1A and S2A). Further, the expression of TFEB and its downstream target genes (13) inversely correlates with that of MYC in three independent AML cohorts: the TCGA, National Taiwan University Hospital, and normal karyotype (NK) AML patient (GSE15434) cohorts (Fig. 1A; Supplementary Fig. S2B–S2D). In addition, in comparison with AML with NKS or BM from healthy donors, MYC expression is high and TFEB expression is low in AML subtypes where leukemogenesis is MYC dependent (Fig. 1B–E; Supplementary Table S3), that is, inv(16), t(15;17), t(8;21), and t(9;22) (8, 10, 11, 19). MYC and TFEB mRNA and protein levels are also inversely correlated across AML cell lines (Fig. 1F–H) and in BM blasts from patients with AML (Fig. 1I and J). Finally, the inverse relationship of MYC and TFEB expression manifest in AML appears context dependent, as this was not evident in other human B-cell leukemia (B-cell acute lymphoblastic leukemia, chronic lymphocytic leukemia), multiple myeloma, and B-cell lymphoma (mantle cell lymphoma, diffuse large B-cell lymphoma) cell lines (Supplementary Figs. S1A and S2A).

Subsequent gain- and loss-of-function studies established that MYC indeed suppresses TFEB expression and function in normal myeloid progenitors and AML. Inducible MYC expression in 32D.3 myeloid cells, as well as in K562 and THP-1 leukemia cells, was sufficient to suppress expression of TFEB and its target genes (e.g., SQSTM1, CTSD, CTSD, and PSAP), and to impair lysosome biogenesis, as evidenced by significant reductions inLysoTracker Red or Green staining (Fig. 1I–K; Supplementary Fig. S2E and S2F). Conversely, MYC knockout or knockdown in NB4 AML and 32D.3 myeloid progenitors, respectively, provoked increased expression of TFEB mRNA and protein, as well as increased expression of TFEB target genes (Fig. 1O; Supplementary Fig. S2G).

To test if MYC control of the TFEB axis was also manifest in vivo, FVB/N mice were transplanted with congenic fetal liver stem cells from wild-type, Rosa26DM-LSL-MYC/+ or Rosa26DM-LSL-MYC/LSL-MYC (20) donors that were transduced with MSCV-Cre-ER<sup>T2</sup>-GFP or control MSCV-GFP retrovirus prior to transplant (Supplementary Fig. S2H and S2I). Following engraftment, MYC was induced by 4-hydroxytamoxifen (4OHT)–mediated Cre-ERT2 deletion of theloxP-stop-loxP (LSL) cassette, as evidenced by increased levels of nuclear MYC in BM (Supplementary Fig. S2J). As expected, increased MYC expression promoted colony formation in both BM and spleen cells harvested from Rosa26DM-LSL-MYC/+ or Rosa26DM-LSL-MYC/LSL-MYC recipient mice (Supplementary Fig. S2K–S2M), and this was accompanied by the repression of<sup>Tf</sup>eb and its downstream target genes (Supplementary Fig. S2N and S2O). Conversely, inducible Myc deletion in primary myeloid progenitors cultured from BM of

### RESULTS

**MYC Represses TFEB Expression and Function in AML and Myeloid Progenitor Cells**

Among 27 different human cancer cell types in the Cancer Cell Line Encyclopedia (CCLE), AML cells rank fourth highest in MYC expression (Supplementary Fig. S1A). Further, elevated MYC expression is associated with an increased number of immature myeloblasts in the peripheral blood (PB) at the time of AML diagnosis in The Cancer Genome Atlas (TCGA) AML cohort (Supplementary Fig. S1B; Supplementary Table S1). Similarly, patients with AML expressing high levels of MYC protein have higher blast percentages (46.4% vs. 28.8%, P = 0.047) in their BM at their time of diagnosis in the Total Cancer Care (TCC) cohort from the H. Lee Moffitt Cancer Center (Supplementary Fig. S1C; Supplementary Table S2). As AML is characterized by developmental arrest at distinct stages of myelopoiesis, we also examined the relationship between MYC expression and myeloid differentiation. Our analysis of TCGA and GSE15434 data set demonstrates a significant positive correlation between the expression of MYC and c-KIT, a marker of immature stem cells, and a significant negative correlation of MYC with the myeloid differentiation markers CD11b and CD14 (refs. 17, 18; Supplementary Fig. S1D–S1G).

Given these clinical observations, experiments were performed to assess whether MYC directly blocks myeloid differentiation and is necessary to sustain AML cell growth and survival. CRISPR-mediated knockout of MYC in K562 leukemia cells that express high levels of MYC provoked marked increases in CD11b<sup>+</sup> cells (Supplementary Fig. S1H–S1J). Conversely, inducible MYC overexpression reduced Gr-1<sup>+</sup> populations in mouse 32D.3 myeloid progenitor cells cultured in IL3 medium (Supplementary Fig. S1K–S1M) and abolished GM-CSF<sup>+</sup>, PMA<sup>+</sup>, and ATRA-induced myeloid differentiation of 32D.3, THP-1, and NB4 cells, respectively (Supplementary Fig. S1K–S1Q). Further, inducible knockout of Myc in primary myeloid BM cells from Rosa26-CreERT2,Myeloid mice (Supplementary Fig. S1R) compromised myeloid cell proliferation and colony formation while triggering increases in Cd11b and Gr-1 expression (Supplementary Fig. S1S–S1U). Thus, MYC is necessary and sufficient to suppress myeloid progenitor and AML cell differentiation.

Given the inverse relationship between MYC and myeloid differentiation, and the fact that loss of autophagy in hematopoietic stem cells (HSC) is sufficient to provoke a myeloproliferative, preleukemic phenotype (16), we reasoned that MYC’s ability to promote AML and suppress myeloid differentiation might rely on its suppression of TFEB expression and/or function. In accord with this notion, we observed that TFEB expression is the lowest in AML cell lines where MYC expression is high (Supplementary Figs. S1A and S2A). Further, the expression of TFEB and its downstream target genes (13) inversely correlates with that of MYC in three independent AML cohorts: the TCGA, National Taiwan University Hospital, and normal karyotype (NK) AML patient (GSE15434) cohorts (Fig. 1A; Supplementary Fig. S2B–S2D). In addition, in comparison with AML with NKS or BM from healthy donors, MYC expression is high and TFEB expression is low in AML subtypes where leukemogenesis is MYC dependent (Fig. 1B–E; Supplementary Table S3), that is, inv(16), t(15;17), t(8;21), and t(9;22) (8, 10, 11, 19). MYC and TFEB mRNA and protein levels are also inversely correlated across AML cell lines (Fig. 1F–H) and in BM blasts from patients with AML (Fig. 1I and J). Finally, the inverse relationship of MYC and TFEB expression manifest in AML appears context dependent, as this was not evident in other human B-cell leukemia (B-cell acute lymphoblastic leukemia, chronic lymphocytic leukemia), multiple myeloma, and B-cell lymphoma (mantle cell lymphoma, diffuse large B-cell lymphoma) cell lines (Supplementary Figs. S1A and S2A).

Subsequent gain- and loss-of-function studies established that MYC indeed suppresses TFEB expression and function in normal myeloid progenitors and AML. Inducible MYC expression in 32D.3 myeloid cells, as well as in K562 and THP-1 leukemia cells, was sufficient to suppress expression of TFEB and its target genes (e.g., SQSTM1, CTSD, CTSD, and PSAP), and to impair lysosome biogenesis, as evidenced by significant reductions in LysoTracker Red or Green staining (Fig. 1I–K; Supplementary Fig. S2E and S2F). Conversely, MYC knockout or knockdown in NB4 AML and 32D.3 myeloid progenitors, respectively, provoked increased expression of TFEB mRNA and protein, as well as increased expression of TFEB target genes (Fig. 1O; Supplementary Fig. S2G).

To test if MYC control of the TFEB axis was also manifest in vivo, FVB/N mice were transplanted with congenic fetal liver stem cells from wild-type, Rosa26DM-LSL-MYC/+ or Rosa26DM-LSL-MYC/LSL-MYC (20) donors that were transduced with MSCV-Cre-ER<sup>T2</sup>-GFP or control MSCV-GFP retrovirus prior to transplant (Supplementary Fig. S2H and S2I). Following engraftment, MYC was induced by 4-hydroxytamoxifen (4OHT)–mediated Cre-ERT2 deletion of theloxP-stop-loxP (LSL) cassette, as evidenced by increased levels of nuclear MYC in BM (Supplementary Fig. S2J). As expected, increased MYC expression promoted colony formation in both BM and spleen cells harvested from Rosa26DM-LSL-MYC/+ or Rosa26DM-LSL-MYC/LSL-MYC recipient mice (Supplementary Fig. S2K–S2M), and this was accompanied by the repression of<sup>Tf</sup>eb and its downstream target genes (Supplementary Fig. S2N and S2O). Conversely, inducible Myc deletion in primary myeloid progenitors cultured from BM of
A TFEB–IDH Epigenetic Circuit Controls AML Cell Fate

MYC suppresses TFEB and the autophagy–lysosome pathway in AML and myeloid progenitor cells. A, Heat map of MYC, TFEB, and TFEB target gene expression in patients with AML from the TCGA. B–E, MYC and TFEB mRNA expression levels in BCR–ABL1 and core binding factor (CBF)–positive patients with AML from the TCGA and Taiwan AML data sets. Demographic profiles, clinical parameters, and laboratory values of these patients are provided in Supplementary Tables S1 and S3. F and G, MYC and TFEB protein levels (F) and their correlation (G) in human AML cell lines. MYC and TFEB protein levels in G were first normalized to Actin levels in each cell line and then normalized by MYC and TFEB levels in the Molm13 and Molm16 cell lines, respectively. H, Negative correlation of TFEB and MYC expression in human AML cell lines (CCLE data set). I and J, MYC and TFEB IHC staining in patients with AML with myelodysplasia-related changes from the TCC data set. MYC protein levels were calculated as MYC staining–positive cells out of total counted blasts in the selected area with sheets of blasts. Higher than 5% staining was counted as high MYC.

Rosa26-Cre-ERT2,MycΔ18 mice (Fig. 1P) was sufficient to trigger upregulation of Tfeb and its target genes (Fig. 1Q), as well as increased lysosome biogenesis (Fig. 1R). Likewise, inhibition of MYC function using several different strategies—including treatment with the MYC-MAX dimerization inhibitors 10058-F4, NSC11656, or NSC49689 (21, 22), inhibition of MYC expression with the BET inhibitor JQ1(+)(23), or treatment of NB4 AML cells with ATRA—induced expression of TFEB and its target genes (Supplementary Fig. S2P–S2S). Finally, inhibition of mouse Myc function or expression in 32D.3 myeloid cells also augmented autophagolysosome formation and autophagic flux (Fig. 1S and T; Supplementary Fig. S2T and S2U). In short, MYC is both sufficient and necessary to repress TFEB expression and TFEB functions in normal myeloid progenitor cells and in AML.

**MYC-Directed Repression of TFEB in AML Leads to the Suppression of TFEB Target Genes**

In some cancer cell lines, MYC has been shown to bind to TFEB target gene promoters and to suppress their expression by antagonizing TFEB binding to its target genes (24). These results and our findings regarding MYC-directed suppression of TFEB and its target genes in AML and normal...
myeloid cells (Fig. 1K, M, O, and Q; Supplementary Fig. S2F, S2N, and S2O) suggested models where MYC-directed suppression of TFEB transcription leads to reductions in TFEB target gene expression and/or that MYC directly and effectively competes with TFEB for binding to TFEB target genes. To test this notion, we first assessed the effects of CRISPR-directed knockout of MYC in HL60 AML cells on the activity of a TFEB promoter-luciferase reporter. Notably, loss of MYC was associated with the induction of TFEB promoter activity, which was also induced by the activation of a TFEBS211A transgene (Fig. 2A), a constitutively active nuclear form of TFEB (13), consistent with a TFEB autoregulatory circuit (13).

To test if MYC transcriptionally represses TFEB, both HeLa and OCI-AML3 cells were engineered to (i) inducibly express TFEBS211A-FLAG; (ii) constitutively express MYC-HA; or (iii) inducibly express TFEBS211A-FLAG along with constitutively expressed MYC-HA (Fig. 2B; Supplementary Fig. S2V). In HeLa cells, chromatin immunoprecipitation (ChIP) experiments demonstrated, as expected, robust binding
TFEB Provokes Myeloid and AML Cell Differentiation and Apoptosis

Though TFEB can function as an oncogene (14, 15), collectively our studies supported the hypothesis that TFEB might function as a tumor suppressor in myeloid cells and AML. To test this hypothesis, we established a variety of mouse myeloid progenitor and AML cell line models that inducibly express TFEBS211A via doxycycline (Dox)-regulated expression systems. Notably, dose-response studies demonstrated that even relatively low levels of TFEBS211A expression impair the proliferation of 32D.3 myeloid cells, as well as the proliferation of HL60, OCI-AML2, and OCI-AML3 AML cells (Fig. 3A–D; Supplementary Fig. S3A–S3E). In addition, induction of TFEBS211A provoked rampant apoptosis in all models, as judged by marked increases in Annexin V binding as well as by caspase-3 and Parp cleavage (Fig. 3E–H; Supplementary Fig. S3F).

To verify these findings using an independent system, we generated an in-frame TFEBS211A–ERT2 fusion protein whose nuclear localization is dependent upon treatment with 4OHT, which we verified when expressed in 32D.3
TFEB compromises AML and myeloid cell growth and survival. A-D, Puromycin-resistant 32D.3 myeloid cells and HL60 leukemia cells that were selected after transduction with either pRRL-EV-puro or pRRL-TFEBS211A-puro lentivirus were treated for 5 days with the indicated doses of Dox and assessed by MTS assays (A and B); values for vehicle-treated cells were set at 100%. Supplementary Fig. S3A and S3B show TFEB protein levels after 72-hour incubation with Dox at 0, 0.0625, 0.125, 0.25, 0.5, and 1 μg/mL. C and D, Representative images of cells treated with 1 μg/mL of Dox for 120 hours are shown. E-H, The indicated cells were treated for 48 hours with Dox (250 ng/mL [immunoblotting] or 1 μg/mL [apoptosis assays]) for 32D.3 and 1 μg/mL for HL60 and assessed for levels of Annexin V+ cells (E and F), and for TFEB, cleaved caspase-3, and cleaved Parp levels by immunoblotting (G and H). I, Schematic of HL60 and OCI-AML3 leukemia cell xenograft studies. One day after sublethal irradiation (250 Rads), recipient NSG mice were injected (via tail vein) with HL60 cells or OCI-AML3 cells transduced with either pRRL-EV-puro (EV) or pRRL-TFEBS211A-puro (TFEBS211A) and were placed on regular or Dox chow. CBC, complete blood count. J, Doc induction of TFEB expression was confirmed by qRT-PCR analyses of mononuclear cells from PB from the indicated cohorts of mice (n = 3 for each cohort). (continued on following page)

myeloid cells (Supplementary Fig. S3G). Treatment of these cells with 4OHT resulted in dose-dependent suppression of cell proliferation and the induction of apoptosis (Supplementary Fig. S3H–S3J). Further, 4OHT activation of the TFEBS211A::ERT2 transgene was sufficient to induce TFEB functions, including increased lysosome biogenesis and the induction of TFEB target genes (Supplementary Fig. S3K and S3L).

To assess potential tumor suppressor roles of TFEB, mouse xenograft experiments were performed using HL60 and OCI-AML3 cells transduced with Dox-inducible TFEBS211A lentivirus (Fig. 3I). Following transplant, recipient mice were placed on normal chow or Dox chow and followed for disease (Fig. 3I). Selective, Dox-induced expression of the TFEBS211A transgene (~3-fold in HL60 transplants) was evident in PB mononuclear cells (Fig. 3J). Notably, this rather modest induction of TFEBS211A was sufficient to markedly delay the development of leukemia, as manifested by reductions in AML cell infiltrates in BM and peripheral tissues such as spleen and liver (Fig. 3K; Supplementary Fig. S3M), reduced numbers of human AML (CD33+) cells in the PB (Fig. 3L; Supplementary Fig. S3N; Supplementary Tables S4 and S5), reduced leukocytosis and splenomegaly, and improved platelet counts and hemoglobin levels compared with control cohorts (Fig. 3M–P; Supplementary Fig. S3O–S3R; Supplementary Tables S4 and S5). Finally, forced expression of TFEBS211A significantly improved overall survival (OS) of recipient mice.
A TFEB–IDH Epigenetic Circuit Controls AML Cell Fate

Figure 3. (Continued) K, Hematoxylin and eosin (H&E; 20×)-stained images of BM, spleen, and liver tissues harvested from EV- or TFEB$^{S211A}$-expressing HL60 leukemia cell transplanted mice at week 3 following transplant on normal versus Dox chow. The inset shows lower magnification of the same H&E-stained slides. L, Leukemic burden was determined from analyses of PB samples collected pretransplantation and at weeks 1 and 3 after transplant by flow cytometry for viable human CD33$^+$ myeloid cells that are negative for mouse CD45 and Ter119. M–O, CBC PB analyses of white blood cells (WBC; M), platelets (N), and hemoglobin (O) were performed at the indicated times posttransplant. P, Spleen weight (at week 3) was normalized to total body weight (BW; baseline body weights were measured 1 day before transplant). Q, Kaplan-Meier curves of OS. Demographic profiles and laboratory parameters are described in Supplementary Table S4. Error bars in A, B, E, and F indicate mean ± SEM of three independent assays. Error bars in J indicate mean ± SEM of three samples. Box plots in L–P represent data from 10 to 16 mice in each group. * in A, B, E, F and J, $P < 0.05$ compared with the control group. * in L, M, N, O, and P, $P < 0.05$ compared with the Dox chow group transplanted with HL60 EV cells.
bearing either HL60- or OCI-AML3-derived leukemia (Fig. 3Q; Supplementary Fig. S3S). Thus, TFEB functions as a tumor suppressor in AML.

**TFEB Controls Monocytic/Granulocytic Lineage Determination and Promotes Myeloid Differentiation**

These findings suggested diametrically opposed roles for MYC and TFEB in controlling myeloid cell fate. Consistent with this notion, MYC and TFEB expression is inversely regulated during mouse and human monopoiesis and granulopoiesis, where expression of TFEB and its targets increase during terminal differentiation when MYC levels fall (Fig. 4A and B; Supplementary Fig. S4A and S4B). Interestingly, this inverse regulation of MYC and TFEB expression was selective, as this relationship is not evident in progenitors undergoing megakaryocytic or erythrocytic differentiation (Supplementary Fig. S4C and S4D). There were also clear negative or positive correlations between the expression of TFEB and immature (c-KIT and MPO) or mature (CD11b and CD14) myeloid markers, respectively, in both adult and pediatric patients with AML from the TCGA, patients with NK AML, and TARGET cohorts (refs. 17, 25; Fig. 4C–G; Supplementary Fig. S4E–S4L).

In accord with the notion that TFEB plays roles in monocytic and granulocytic differentiation, Dox-inducible expression of TFEB211A in normal 32D.3 myeloid cells (cultured in IL3), and in HL60 and OCI-AML3 cells, was sufficient to promote monocytic and granulocytic differentiation, as judged by morphologic changes, staining of adherent cells with crystal violet, and the acquisition of mature monocytic and granulocytic markers (Fig. 4H–L; Supplementary Fig. S4M–S4V). Furthermore, high TFEB expression in the myelomonocytic (M4) and monocytic (M5) AML positively

---

**Figure 4.** TFEB provokes differentiation of AML and myeloid progenitor cells. A, Myc expression is the inverse of Tfeb and myeloid differentiation-specific genes during mouse monopoiesis (left and right), and of Tfeb target genes (middle). C. Mono, classical monocyte; CMP, common myeloid progenitor; GMP, granulocyte-monocyte progenitor; GRA, granulocyte; LT-HSC, long-term HSC; MPP, multipotent progenitor cell; N.C. Mono, nonclassical monocyte; ST-HSC, short-term HSC. B, MYC expression is the inverse of TFEB and myeloid differentiation-specific genes during human monopoiesis (left and right), and of TFEB target genes (middle). C. Schematic of markers of HSCs (c-KIT, MPO) and monocytic cells (CD11b, CD14).
correlates with the expression of groups of genes (s-mpp, s-myly, r-myly, and d-my, boxed in Fig. 4M) that are HSC-to-monocytic and HSC-to-granulocytic lineage specific (ref. 26; Fig. 4M and N; Supplementary Fig. S4W). In contrast, TFEB expression did not positively correlate with groups of genes associated with HSCs (stem), B lymphopoiesis (d-ly), or megakaryopoiesis and erythropoiesis (s-ery) in any AML subtype (Supplementary Fig. S4W and S4X).

To identify TFEB targets that might contribute to myeloid/granulocytic differentiation, we performed RNA-sequencing (RNA-seq) analysis of HL60 leukemia cells engineered to inducibly express the TFEBS211A transgene. Using a cutoff of fold change >4 with q < 0.01, a total of 1,152 genes were differentially regulated (1,149 upregulated and only 3 downregulated) following the induction of TFEBS211A in HL60 cells (Fig. 4O and P). As expected, this included the robust induction of nearly all TFEB target genes associated with the autophagy–lysosome pathway (ref. 12; Fig. 4O) but also of genes such as STAT1, KLF4, KLF6, CEBPB, CSF1, and GATA2 (Fig. 4Q; Supplementary Fig. S4Y) that are necessary and/or sufficient to provoke monocytic and granulocytic differentiation, including that of AML cells (27–29). Thus, TFEB induces a cast of myeloid lineage determination genes that are upregulated in myelomonocytic AML.
TFEB Induces 5-methylcytosine Hydroxylation in AML and Myeloid Progenitors by Upregulating the IDH1/IDH2-TET2 Axis

Surprisingly, several observations indicated that TFEB controls myeloid lineage genes, at least in part, by regulating DNA methylation. IDH1 and IDH2 expression positively correlates with TFEB in s-myly and d-my lineage AML (Fig. 4M), and among genes induced by TFEB in HL60 cells is IDH1 (Fig. 4P and Q), which catalyzes the production of α-ketoglutarate (α-KG), a required substrate of the TET family of dioxygenases that convert 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), the initial step in DNA demethylation (30). Notably, TFEB-induced genes are similar to those induced by TET2 in HL60 cells (Fig. 4R; ref. 31), suggesting a connection between TFEB upregulation and gene demethylation. In support of this notion, the genes induced by TFEB are downregulated in HL60 cells by mutant IDH1 (Fig. 4S; ref. 32), which produces the TET2 enzyme inhibitor 2-hydroxylutarate (2-HG) rather than α-KG. Finally, although TET2 and IDH2 expression is generally high in all AML subtypes, analyses of TCGA AML and TARGET AML subtypes reveal that the expression of TFEB and IDH1 is selectively upregulated in the M4 and M5 subtypes, where MYC expression is lowest (Supplementary Fig. S5A and S5B).
The ability of TFEB to control expression and function of the IDH1/IDH2–TET axis was confirmed by molecular and gain- and loss-of-function studies in both AML models and normal myeloid progenitors. First, TFEB5211A expression induces the expression of IDH1 and/or IDH2 and TET family members in HL60 and OCI-AML3 leukemia cells both \textit{ex vivo} and \textit{in vivo} (Fig. S5A–D; Supplementary Fig. S5C and S5D). TFEB also upregulated IDH1/IDH2 mRNA and/or protein levels in HeLa cells (Fig. S5E and F), although the specific IDH family member induced, and its fold induction, was cell context dependent. Second, in HL60 and OCI-AML3 leukemia cells, and in HeLa cells, activated TFEB induced \textit{IDH1} promoter activity (Fig. S5G and H), and in ChIP assays of OCI-AML3 cells, activated TFEB bound to the \textit{IDH1} promoter (Supplementary Fig. S5E). Furthermore, knockout of \textit{MYC} in HeLa cells induced TFEB as well as IDH1 and IDH2, and these changes were abrogated by shRNA-directed knockdown of \textit{TFEB} (Fig. S5I). Moreover, MYC-HA did not bind to the \textit{IDH1} promoter in OCI-AML3 cells and did not significantly affect binding of TFEB-FLAG to the \textit{IDH1} promoter (Supplementary Fig. S5F). Thus, consistent with findings from MYC-HA and TFEB-FLAG coexpression

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5}
\caption{Figure 5. (Continued) N. Forty-eight hours after vehicle or Dox (1 \(\mu\)g/mL) treatment, EV- and TFEB5211A-expressing HL60 leukemia cells were harvested for gas chromatography–mass spectroscopy determination of \(\alpha\)-KG levels. O–R, EV- and TFEB5211A-expressing HL60 leukemia cells, TFEB5211A, MYC-, or Myc shRNA-expressing 32D.3 myeloid cells, and primary myeloid cells from Rosa26-CreERT2; CD15 cytometry \((U\) and \(V\)), 5hmC dot blot assays \((V\) and \(W\)), and assessed by immunoblotting \((X\) and \(W\)), and crystal violet staining \((X\) and \(W\)). TFEB also upregulated IDH1/IDH2 and TET family members in HL60 and OCI-AML3 leukemia cells both \textit{ex vivo} and \textit{in vivo} (Fig. S5A–D; Supplementary Fig. S5C and S5D). TFEB also upregulated IDH1/IDH2 mRNA and/or protein levels in HeLa cells (Fig. S5E and F), although the specific IDH family member induced, and its fold induction, was cell context dependent. Second, in HL60 and OCI-AML3 leukemia cells, and in HeLa cells, activated TFEB induced \textit{IDH1} promoter activity (Fig. S5G and H), and in ChIP assays of OCI-AML3 cells, activated TFEB bound to the \textit{IDH1} promoter (Supplementary Fig. S5E). Furthermore, knockout of \textit{MYC} in HeLa cells induced TFEB as well as IDH1 and IDH2, and these changes were abrogated by shRNA-directed knockdown of \textit{TFEB} (Fig. S5I). Moreover, MYC-HA did not bind to the \textit{IDH1} promoter in OCI-AML3 cells and did not significantly affect binding of TFEB-FLAG to the \textit{IDH1} promoter (Supplementary Fig. S5F). Thus, consistent with findings from MYC-HA and TFEB-FLAG coexpression...}
\end{figure}
studies in OCI-AML3 cells (Fig. 2). MYC regulation of IDH1/2 is indirect and is mediated via MYC-directed suppression of TFEB. Third, inducible Tfeb loss in ex vivo–cultured primary mouse BM-derived Rosa26-Cre-ER
t

expression resulted in elevated levels of MYC-directed transcriptional repression of Tet1-3, Idh1, and Idh2 (Fig. 5J and K). Consistent with these results, knockout of Myc in Rosa26-Cre-ER

primary myeloid progenitors following treatment with 4OH-T led to significant increases in the expression of Tet1-3 and Idh1 and Idh2 (Fig. 5L and M), whereas MYC overexpression in vivo in BM progenitors repressed the expression of Tet1-3, Idh1, and Idh2 (Supplementary Fig. S5G). Similarly, MYC overexpression in K562 leukemia cells repressed IDH2 expression (Supplementary Fig. S5H), while CRISPR-directed knockout or shRNA-mediated knockdown of MYC in K562, HL60, and NB4 leukemia cells led to increased levels of TFEB, IDH1 (in HL60 and NB4 cells), and IDH2 proteins (Supplementary Fig. S51–S5L). Finally and importantly, IDH1 or IDH2 levels were increased by pharmacologic inhibition of MYC protein function using a validated inhibitor of MYC:MAX dimerization (22) in HL60 cells, but these changes were abrogated by concomitant TFEB knockdown (Supplementary Fig. S5M), collectively indicating that MYC suppression of IDH1/2 depends on MYC-directed transcriptional repression of TFEB.

Notably, these changes in IDH1 and IDH2 expression were associated with changes in DNA methylation. In particular, Tfeb

expression resulted in elevated levels of α-KG, the product of IDH1 and IDH2, but not of the oncometabolite 2-HG (Fig. 5N; Supplementary Fig. S5N). Strikingly, the induction of Tfeb

provoked significant increases in global levels of ShmC in genomic DNA of HL60 leukemia cells both ex vivo and in vivo (Fig. 5O; Supplementary Fig. S5O), as well as in normal 32D.3 myeloid progenitors (Fig. 5P). Consistent with these results, inducible MYC expression in 32D.3 myeloid progenitors repressed ShmC levels (Fig. 5Q, left), whereas knockdown or knockout of Myc in 32D.3 myeloid cells and primary myeloid progenitors increased ShmC levels (Fig. 5Q, right, and R). Finally, the effects of Myc knockdown on the expression of Tet1-3, Idh1, and Idh2 were phenocopied in 32D.3 myeloid progenitors by pharmacologic inhibition of MYC protein function (Supplementary Fig. S5P). Similarly, treatment of OCI-AML3 leukemia cells with these MYC:MAX inhibitors led to marked increases in global levels of ShmC (Supplementary Fig. S5Q).

The presence of a MYC–TFEB–IDH1/IDH2–TET axis is also supported by observations in acute promyelocytic leukemia, a leukemia subtype where ATRA treatment provokes granulocytic differentiation (33) that is associated with MYC repression (34). In our studies, ATRA treatment of NB4 AML cells suppressed MYC (Supplementary Figs. S2S and S2R) and induced TFEB and IDH1 expression and ShmC levels (Supplementary Figs. S2S, S3R, and S3S).

Important roles of the TFEB–IDH1/IDH2–TET axis are also manifest in other AML subtypes and myeloid progenitors. First, the antiproliferative effects of inducible Tfeb

expression were impaired by 2-HG treatment (Supplementary Fig. S5T). Second, coexpression of FLAG-tagged mutant IDH1

(Fig. 5S), which effectively suppressed increases in ShmC levels that are induced by TFEB (Fig. 5T), impaired TFEB-induced myeloid differentiation of HL60 and OCI-AML3 leukemia cells, as evidenced by reductions in CD15 expression and crystal violet staining (Fig. 5U–X). Collectively, these results support a model whereby a MYC–TFEB–IDH1/IDH2–TET circuit dictates myeloid cell fate via epigenetic control of genome methylation.

**TFEB Epigenetically Controls Genes that Contribute to Monocytic and Granulocytic Differentiation**

To assess the global effects of TFEB on 5mC and 5hmC landscapes, we performed paired reduced representation bisulfite (BS) and oxidation bisulfite (oxBS) sequencing (BS-seq and oxBS-seq) as described (35–37). Generation of paired BS and oxBS converted genomic DNA libraries from the same sample allowed a direct comparison of the levels of 5mC and 5hmC in individual SC loci in each sample, and these were determined in HL60 cells with or without Tfeb

overexpression. As predicted, Tfeb

induced both loss and gains of 5mC, but there were more losses (n = 722) than gains (n = 459; Fig. 6A and B). 5mC loss occurred most commonly in distal intergenic regions (40.34%), followed by loss in other introns (25.85%) and promoter regions (20.6%; Fig. 6A). Similarly, 5mC gains occurred most commonly in the distal intergenic regions (33.86%), followed by promoters (31.83%) and other introns (22.12%; Fig. 6A). About 16% and 28% of loss and gain of 5mC, respectively, occurred in CpG islands (Fig. 6B). Quite remarkably, and consistent with TFEB-provoked increases of ShmC signals in dot blot assays (Fig. 5O; Supplementary Fig. S5O), Tfeb

exclusively induced ShmC gains (in a total of 863 genes), and 37% and 36% of these ShmC gains occurred in promoter regions and CpG islands (Fig. 6C). Both 5mC and ShmC changes occurred across all 22 chromosomes (Fig. 6D and E).

Underscoring roles for TFEB in controlling cell differentiation programs, gene set enrichment analyses (GSEA) revealed that a significant number of TFEB-induced differentially methylated and hydroxymethylated genes are involved in cell differentiation, development, and neuron differentiation (Fig. 6F). Comparison of BS- and oxBS-seq versus RNA-seq analyses of HL60 cells expressing TFEB revealed significant changes in mRNA levels (log2 fold change >1 with adjusted P < 0.01) for a total of 137 and 111 genes, respectively (Fig. 6G), that also showed concomitant differential changes in 5mC and ShmC marks (Fig. 6G and H; Supplementary Table S6). Notably, these genes include KLFL, KLF6, STAT3, TP73, and FOXO1, which have pivotal roles in controlling myeloid differentiation and cell death (refs. 27, 28; Fig. 6H), indicating that TFEB is a master regulator of these cell-fate determination genes. However, a subset of genes (i.e., CSF1, GATA2, RUNX1, RUNX2, MPO, FLT3, PUMA, BAX, and BCAT1) are significantly induced or repressed by TFEB without significant changes in their 5mC and/or ShmC marks (Fig. 6H). Thus, TFEB may also regulate cell death and differentiation through direct activation or repression of selection transcription targets, and/or these changes are secondary to the effects of TFEB on epigenetic programs.

To assess if TFEB control of 5mC and ShmC marks in HL60 leukemia cells was associated with similar expression changes in patients with AML, the results from the BS- and oxBS-seq were compared with gene-expression profiles in the
Figure 6. TFEB induces global changes in 5mC and ShmC in HL60 leukemia cells. A-C, Reduced representation BS- and oxBS-seq analyses were performed as described in Methods. HL60-EV-puro and HL60-TFEB S211A-puro cells were treated with vehicle or Dox (1 μg/mL) for 48 hours, and genomic DNA was extracted. Libraries were generated after MspI digestion and analyzed on Illumina NextSeq 500. Hyper- and hypomethylated 5C (5mC) were determined using criteria credible methylation difference (CDIF) >0.06 for both groups, and mean CDIF (TFEB group) − mean CDIF (non-TFEB groups) >0 for both groups, and mean CDIF (TFEB group) − mean CDIF (non-TFEB groups) >0.06 for hyper-hydroxymethylation and <−0.06 for hypo-hydroxymethylation were used. The positions of changes in 5mC and ShmC within genes that are differentially methylated following the induction of TFEB are indicated.

D and E, TFEB-induced genes in HL60 leukemia cells with significant gains (red) or losses (blue) in their levels of 5mC (D) or ShmC (E) were mapped across all 22 human chromosomes. F, GSEA of genes that are differentially methylated or hydroxymethylated following the induction of TFEB in HL60 leukemia cells. CNS, central nervous system; GO, Gene Ontology. (continued on next page)
that activation of TFEB may synergize with DNA hypomethylating agents that are commonly used in the AML clinic, to further suppress 5mC and provoke AML differentiation and cell death (Fig. 7A; ref. 38). As predicted, the combination of azacitidine with TFEBS211A overexpression significantly augmented cell death compared with azacitidine treatment or TFEBS211A overexpression alone in HL60 and OCI-AML3 cells, and also in 32D.3 myeloid progenitors (Fig. 7B–G), and this response was associated with significant increases in the levels of 5hmC (Fig. 7H–J). Further, in Dox-inducible TFEBS211A-expressing HL60 and OCI-AML3 xenographs, cohorts treated with both azacitidine and Dox chow (to induce TFEBS211A) had reduced numbers of circulating AML cells and reduced PB white blood cell (WBC) counts, and significantly improved OS versus the other cohorts (Fig. 7K–N; Supplementary Fig. S7A and S7B; Supplementary Tables S7 and S8). Thus, agents activating the TFEB–IDH1/2–TET2 axis can be effectively combined with DNA hypomethylating agents.
TFEB function is held in check by phosphorylation of residue S211 via mTORC1, which leads to 14-3-3 binding and sequestration of phospho-TFEB in the cytoplasm (13). As an alternative means to induce TFEB signaling, we used GSK-621, an AMPK activator that inhibits mTORC1 (39), to indirectly activate TFEB signaling. As expected, GSK-621 treatment of HL60 and OCI-AML3 reduced mTORC1 activity, as evidenced by reduced levels of phospho-4EBP1 (Fig. 7O), treatment of HL60 and OCI-AML3 reduced mTORC1 activity, as evidenced by reduced levels of phospho-4EBP1 (Fig. 7O), indicating GSK-621 can also provoke differentiation phenotypes in primary AML (Fig. 7U–W; Supplementary Fig. S7M–S7P; Supplementary Table S9). Collectively, these findings suggest the MYC–TFEB–IDH1/2–TET2 axis can be therapeutically targeted with small molecules activating TFEB signaling, especially when combined with DNA hypomethylating agents, to improve treatment responses and survival outcomes in patients with AML lacking IDH1/2 or TET2 somatic mutations (Fig. 7X).

**DISCUSSION**

Recent genetic studies in AML have shown that up to 90% of cases have a variety of somatic mutations and copy-number variations that dysregulate RNA splicing, epigenetic control, metabolism, and signaling cascades. Among these, MYC gene copy-number variation, rearrangement, or somatic mutations are frequently manifested in both pediatric and adult patients with AML (1, 3, 4). Although previous studies have shown that MYC is sufficient to induce AML (6), how MYC drives AML is poorly understood. In accord with recent cell line studies (24), here we have shown that MYC directly represses the expression and thus the functions of TFEB, a transcription factor that has oncogenic roles in other contexts (14, 15). Strikingly, we show TFEB functions as a potent tumor suppressor in AML that provokes myeloid cell differentiation and cell death via epigenetic control by inducing
Figure 7. (Continued) K–N, After conditioning irradiation (250 Rad), NSG mice (10 mice for each cohort) were injected (via tail vein) with control or TFEBCR11A-expressing HL60 or OCI-AML3 cells, and were placed on normal or Dox chow ± AZA treatment. Numbers of circulating human CD33+ leukemia cells were determined (K and L), and OS (M and N) was estimated by the Cox regression model. Detailed parameters of these xenograft studies are provided in Supplementary Tables S6 and S7. O and P, HL60, K562, and OCI-AML3 cells were treated with GSK-621 (25 μmol/L), and effects on the phosphorylation status of the mTORC1 target 4EBP1 (O) and TFEB nuclear localization (P) were determined by immunoblotting total cell lysates (O) or nuclear (Nuc) and cytoplasmic (Cyt) fractions (P). WCL, whole cell lysate. Q and R, HL60 or OCI-AML3 cells were treated with vehicle or GSK-621 (25 μmol/L), effects on the expression of TFEB and its targets were determined by qRT-PCR (Q), and effects on leukemia cell survival were monitored by staining with Annexin V (R). S and T, The indicated leukemia cells were treated with vehicle or GSK-621 (25 μmol/L) for 48 hours and assessed for IDH1 and IDH2 protein levels by immunoblotting (S) and 5hmC levels by DNA dot blot assays (T). U–W, Myeloblasts isolated from an AML patient were treated with vehicle or GSK-621 (25 μmol/L) for 48 hours, and effects on nuclear and cytoplasmic levels of TFEB were determined by immunoblotting (U). Effects of GSK-621 on expression of TFEB and its targets were determined by qRT-PCR (V), and effects on 5hmC levels were determined by DNA dot blot assays (W). Patient demographic and clinical parameters are provided in Supplementary Table S9. X, Graphical summary of the epigenetic MYC–TFEB-IDH1/2–TET circuit in AML. Error bars in B–D, Q, and R indicate mean ± SEM of three independent experiments. Box plots in K and L represent data from 6 to 10 mice in each group. Error bars in V indicate mean ± SEM of three technical triplicates. * in B–D, Q, R, and V, P < 0.05 compared with the control group.

the IDH1/2–TET2 circuit, a frequent target of mutation in AML (1).

Clonal expansion of leukemic stem cells (LSC) is manifested when AMLs progress and/or relapse, and residual leukemic clones are associated with inferior clinical outcomes (40). However, given their quiescent nature, LSCs are chemoresistant (41). Accordingly, the field has focused on identifying new therapeutically actionable targets that can provoke AML differentiation and cell death. Among the many somatic mutations and genetic alterations identified in patients with AML, mutually exclusive mutations in IDH1, IDH2, and TET2 impair hematopoiesis, augment self-renewal capacity, and facilitate malignant transformation of HSCs (42). Further, the newly developed inhibitors ivosidenib and enasidenib have been shown to induce myeloid differentiation and improve survival outcomes in patients with AML harboring IDH1 and IDH2 somatic mutations, respectively (43, 44). However, only 15% to 25% of patients with AML have IDH1 or IDH2 mutations (1), and the majority of patients have limited options when AML relapses. Our findings support the concept that provoking TFEB signaling may be a generalizable approach for inducing AML cell differentiation and death in IDH1 and IDH2 wild-type patients with AML, where TFEB induces the IDH1/IDH2–TET circuit to provoke global changes of 5hmC and 5mC that are associated with the induction of established monocytic and granulocytic lineage fate genes, differentiation, and cell death. Excitingly, this circuit is therapeutically tractable, as AMPK...
agonists such as GSK-621 can be effectively combined with DNA hypomethylating agents as a differentiation-based therapeutic strategy.

As documented herein, TFEB also appears to play important physiologic roles in myeloid differentiation programs. In particular, TFEB also activates the IDH1/IDH2–TET circuit in normal 32D.3 myeloid precursors and in primary mouse BM cells, in both cases provoking myeloid differentiation. Further, MYC and TFEB are inversely regulated during human and mouse monopoiesis and granulopoiesis as HSCs undergo differentiation. Similarly, in accord with cell line studies (24), we have observed inverse relationships in the expression of MYC and the TFEB orthologs TF3 and MiTF during monopoiesis and granulopoiesis in and AML patient data (17). Collectively, these observations suggest that TFEB, and perhaps its orthologs, play important roles in sustaining monocytic and granulocytic differentiation, which is an important area for future investigation. Similarly, as MYC plays essential roles in leukemogenesis provoked by numerous oncoproteins (FLT3–ITD, BCR–ABL, etc.; refs. 8–11, 19), it will be important to test whether TFEB or its orthologs have tumor suppressor roles in other AML subtypes.

Of note, expression of wild-type IDH1 protein alone failed to induce AML cell differentiation or death, indicating that IDH1 is necessary but not sufficient for the TFEB tumor suppressor phenotype. This observation suggests that TFEB might regulate distinct parallel pathways that cooperate with 5hmC-dependent epigenetic changes (Fig. 7X). We posit this occurs via direct transcriptional induction and/or repression of target genes by TFEB, as TFEB regulates the expression of several differentiation- and apoptosis-regulating genes, including CSF1, GATA2, RUNX1, RUNX2, TP73, FLT3, PUMA, and BAX, without significant changes in their levels of 5hmC or 5mC. Thus, future studies to uncover the underlying mechanisms of activation and/or repression of these genes, and their interaction with IDH1/2–TET2–dependent epigenetic changes, are certainly warranted (Fig. 7X).

Our findings establish epigenetic control by MYC relies on its direct suppression of TFEB, where reduced representation B5- and oxBS-seq analyses show that, remarkably, the induction of TFEB exclusively leads to 5hmC gains in a large cast (>800) of genes across the entire HL60 leukemia cell genome. Notably, generation of 5hmC is the first step in DNA demethylation, where 5hmC can be directly converted into cytosine, as DNMT1 activity is significantly reduced for DNA substrates harboring 5hmC (45). Further, TET enzymes can convert 5hmC to 5-formylcytosine and then to 5-carboxylcytosine, which is demethylated by thymine–DNA glycosylase to produce cytosine (46, 47). Interestingly, TFEB also provokes both loss and gain of 5mC in the leukemia genomes. Reduced levels of 5mC may reflect increases in 5hmC that is converted to cytosine and/or reduced methylation of cytosine by DNMT1 (45). In contrast, our studies indicate that TFEB may provoke 5mC gains in genes involved in cell differentiation via direct upregulation of the DNMT3L enzyme that stimulates DNA methylation by DNMT3A (48).

Finally, it is notable that our GSEA revealed that TFEB induces 5hmC and 5mC changes in a number of genes involved in neurogenesis and neuronal differentiation, and that IDH1 mutations are frequently observed in low-grade gliomas and transformed glioblastoma (49). Further, it has recently been shown that TFEB triggers cell death in a subset of premyelinating oligodendrocytes via PUMA upregulation and activation of BAX (50), and these proapoptotic genes are also induced by TFEB in AML cells. Thus, TFEB may function as a tumor suppressor across a broad spectrum of malignancies and particularly in the large group of tumors driven by MYC family oncoproteins. If so, agents that target TFEB signaling in combination with DNA hypomethylating agents may be effective strategies to treat not only AMLs but these aggressive cancers as well.

**METHODS**

See Supplementary Materials for additional Methods and Supplementary Tables S10 and S11 for key resource and sequence information.

**Human Studies**

Using the TCC Moffitt Cancer Center (MCC) data set, we retrospectively identified cases diagnosed with AML with myelodysplasia-related changes (AML-MRC) from 2011 to 2018. The patients had provided written informed consent to be included in the data set, and our study received Institutional Review Board (IRB) approval. MYC and TFEB protein expression was assessed by IHC staining as described below. Somatic mutations were tested by Sanger sequence targeted next-generation sequencing (developed at MCC) as described (40, 51). Clinical variables and disease-related prognostic factors, including age, gender, cytogenetics, and treatment regimens, were characterize at the time of AML-MRC diagnosis and were annotated using descriptive statistics. The OS was estimated with the Kaplan–Meier method and compared using the log-rank test. All statistical analyses were performed using SPSS v24.0 and GraphPad Prism 7.

**Animal Studies**

All animal studies were performed in compliance with the NIH Guidelines under a protocol approved by the H. Lee Moffitt Cancer Center and Research Institute and the University of South Florida Institutional Animal Care and Use Committee. NSG and FVB/N mice (6–8 weeks of age) were used for xenograft transplant studies as described (52).

For human AML cell line xenograft experiments, mice were randomized into the indicated cohorts, conditioned with 250 cGy irradiation, and then transplanted with log phase HL60 or OCI-AML3 cells via tail-vein injection 24 hours after irradiation. Mice were placed on regular or Dox chow diet 1 day after transplantation. Azacitidine was administered intraperitoneally on days 7, 9, and 11 (2.5 mg/kg in 100 μl normal saline, total three doses), and mice in control groups received 100 μl of normal saline as described (53). Baseline complete blood counts (CBC) and leukemic burden were assessed 1 week prior to transplant, and serial analyses were performed at weeks 1 and 3 after transplantation using PB samples. For red blood cell lysis, PB samples were incubated in ACK buffer twice for 5 minutes. Cells were incubated with Zombie Near IR (viability dye) for 10 minutes and then washed with FACS buffer [2% fetal bovine serum (FBS) in phosphate-buffered saline (PBS)]. After incubation with mouse and human FC blocking antibody for 10 minutes, cells were stained with mouse CD45.1, mouse Ter119, and human CD33 primary antibodies, fixed with 4% paraformaldehyde for 10 minutes, washed with FACS buffer three times, and then subject to flow cytometry as described (54). Femur, liver, and spleen tissues were harvested at week 3 after transplant or at the endpoint per protocol. Tissues were incubated in neutral buffered formalin for 24 hours and then stored in 70% EtOH until used. Hematoxylin
and eosin staining was performed as described (55). Spleen weight was normalized to baseline body weight measured before transplant.

For the congenic transplant experiments, day 15 fetal liver stem cells from FVB/N Rosa26Gm1::LSL-MYC/LSL-MYC, Rosa26::Gm1::LSL-MYC, and Rosa26::/ mice were transduced with MSCV-Cre-ER\(^{+}\) and expanded ex vivo. After confirming genotypes and Cre-ER\(^{+}\) expression by qRT-PCR, 1 x 10^6 cells (per mouse) were injected via tail vein into FVB/N wild-type recipient mice (8 weeks of age) 24 hours following lethal irradiation (1,100 cGy). Mice were placed on Baytril water (0.25 mg/mL) for 72 hours prior to irradiation to prevent opportunistic bacterial infections. Engraftment was monitored biseekly by determining CBC. Tamoxifen was prepared in corn oil (final concentration 20 mg/mL) and administered via oral gavage (180 \(\mu\)L daily for 5 days) at 7 weeks when counts recovered. Mice were humanely sacrificed at week 10 to harvest primary BM and spleen cells under sterile conditions for the colony-forming assays (as described below) and qRT-PCR analyses.

**Tissue Culture**

Cell lines were propagated at densities of \(<1\times10^6 \text{ cells/mL} in RPMI-1640\) medium containing 10% heat-inactivated FBS, 100 U/mL penicillin G, and 2 \(\mu\)mol/L glutamine (medium A) except for 32D.3 myeloid cells, which received medium A with IL3 (2 ng/mL) and penicillin G. Primary BM cells were harvested from 7-week-old C57BL/6J mice (8 weeks of age) 24 hours following lethal irradiation (1,100 cGy). They were then cultured in EMEM medium containing 10% FBS and 100 U/mL penicillin G, and 2 \(\mu\)mol/L glutamine (medium A) except for 32D.3 myeloid cells, which received medium A with IL3 (10 ng/mL), mouse IL6 (10 ng/mL), mouse stem cell factor (SCF; 10 ng/mL), and penicillin G (100 U/mL).

**Immunoblotting**

Following treatment with indicated concentrations of drugs and/or gene manipulation, cells (5 \times 10^6/aliquot) were washed with cold Dulbecco’s PBS, lysed with RIPA [10 mmol/L Tris (pH 7.4), 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 10% heat-inactivated FBS, 100 U/mL HEPES (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 2.5 mmol/L EGTA, 1% Tween-20] buffer containing complete mini tablet (1 tablet/10 mL), PMSF (1 mmol/L), beta-glycerophosphate (10 mmol/L), sodium fluoride (1 mmol/L), and sodium orthovanadate (1 mmol/L). Lysates were sonicated, centrifuged at 15,000 rpm for 30 seconds or 2 minutes, and then supernatant was carefully collected. Protein concentration was determined using a BCA Assay. Protein was separated on SDS-PAGE, transferred to nitrocellulose membranes, and blotted with specific primary antibodies as listed in the Key Resources table (Supplementary Table S10). Images were captured using Odyssey Fc Imaging System (LI-COR).

**RNA Preparation and qRT-PCR Assays**

RNA from primary BM cells, human AML cells, HeLa cells, and mouse myeloid progenitor cells was isolated using the NucleoSpin RNA II Kit. cDNA was prepared using an iScript cDNA synthesis kit, and qRT-PCR was performed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Data were analyzed using the following equations: \(\Delta\Delta Ch = C(\text{sample}) - C(\text{endogenous control})\); \(\Delta Ch = C(\text{sample}) - C(\text{untreated or control})\); fold change = 2 \(^{-\Delta\Delta Ch}\). Ubiquitin and Gapdh served as the endogenous controls. Primers used, and their sequences, are provided in Supplementary Table S11.

**Assessment of Apoptosis and Proliferation**

After cells were treated with Dox, GSK-621, 4-OHT, and/or azacitidine, they were stained with Annexin V and propidium iodide (PI) to assess cell death using flow cytometry as described (56). For cell proliferation, aliquots containing 2 \times 10^4 cells in 100 \mu L medium were incubated at 37°C with the indicated concentrations of Dox for 5 days. MTS plates were then treated with phenazine methosulfate and were incubated for 2 to 8 hours, and absorbance at 490 nm was determined relative to vehicle or media controls.

**Myeloid Cell Differentiation**

After treatment with the indicated drugs, cells were washed with PBS, stained with Zombie Near IR (viability dye) for 10 minutes, and then washed with flow cytometry analysis buffer (2% FBS in PBS). Cells were then incubated with mouse or human FC block for 10 minutes, followed by staining with primary antibodies for 15 minutes at room temperature. Cells were analyzed using LSR II (BD Biosciences) or FACSCanto (BD Biosciences).

**Crystal Violet Staining and Quantification**

Log phase cells (0.5 \times 10^6/mL) were plated into 12-well plates and incubated for 72 hours. After removing media, cells were stained with crystal violet staining solution (0.05% w/v crystal violet, 1% formaldehyde, and 1% methanol in 1x PBS) for 20 minutes at room temperature. After removing the staining solution, plates were gently washed by dipping into water several times and were air-dried overnight. For quantification, 1 mL of 10% of acetic acid was added into each well and incubated for 20 minutes on shaker. Then 100 \mu L of each sample was transferred to 96-well plate, and optical density was measured at a wavelength of 590 nm.

**Assessment of Autophagic Flux, Autophagosomes, and Lysosomes**

32D.3 myeloid cells engineered to express the mCherry-GFP-LC3 autophagic flux reporter were cultured in IL3 medium and treated with the indicated drugs, incubated for 24 to 48 hours at 37°C, and subjected to flow cytometry analyses. The ratio of mCherry to GFP was calculated, and the percentage of high flux (ratio >1) was plotted as histograms.

To assess autophagosome formation by quantifying cytoplasmic fluorescent LC3 punctae, cells were cytotoxicentrified, fixed in 2% (wt/vol) formaldehyde in PBS, stained with DAPI in PBS, and imaged on the Leica SP8 Laser Scanning confocal microscope to assess cytoplasmic fluorescent punctae as described (57). Lysosomal mass was determined by staining cells with 25 \mu M LysoTracker Red or LysoTracker Green for 20 minutes in growth media at 37°C and analyses by flow cytometry.

**TFEB and IDH1 Promoter-Luciferase Reporter Assays**

For TFEB promoter assays, 2 \times 10^4 HL60 cells were transfected with plasmid containing TFEB promoter firefly luciferase reporter (70 ng) and pRL-TK Renilla (30 ng) with or without pL-CRISPR-gMYC-puro (100 ng) or MSCV-TFEB\(^{S211A}\)-ERT2-puro plasmid (100 ng) using FuGENE (Promega) as instructed by the supplier. Following overnight incubation, TFEB\(^{S211A}\)-ERT2-expressing cells were treated for 24 hours with vehicle or 4OHT (1 \mu M/L), lysed in passive lysis buffer, and assayed sequentially for firefly and Renilla luciferase using a Synergy HTX plate luminometer (BioTek).

To assess IDH1 promoter activity, a total of 2 \times 10^4 HeLa-WT-pRRL-EV (empty vector), HeLa-WT-pRRL-MYC, or HeLa-TFEB-GFP-pRRL-EV cells were transfected with the IDH1 promoter firefly luciferase reporter (70 ng) and the pRL-TK Renilla reporter (30 ng), treated with Dox (0.5 \mu g/mL) for 24 hours, and then assessed for firefly and Renilla luciferase as above. In addition, HL60 pRRL-EV or HL60 pRRL-TFEB\(^{S211A}\) and OCI-AML3 pRRL-EV or pRRL-TFEB\(^{S211A}\) cells were transfected with pCAG-IDH1 promoter-Hygro, and hygromycin-resistant cells were selected. A total of 4 \times 10^4 cells were treated with Dox (0.5 \mu g/mL) for 48 hours, and then firefly luciferase activity was measured as noted above.

Downloaded from https://bloodcancerdiscov.aacrjournals.org by guest on August 27, 2021. Copyright 2020 American Association for Cancer Research.
ChIP Assays

OCI-AML3 cells were cultured in media with Dox for 48 hours, fixed, and harvested. Chromatin was processed using the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology) according to the manufacturer’s protocol and immunoprecipitated with normal rabbit IgG (2729, Cell Signaling Technology) or antibodies specific for TFEB (37785, Cell Signaling Technology) and H3 (4620, Cell Signaling Technology). For the engineered HeLa and OCI-AML3 cells that express TFEB<sup>211A</sup>-MYC-FLAG or/and MYC-HA, anti-FLAG and anti-HA agarose beads were used for the ChIP. qPCR was run on the chromatin, and the percentage of chromatin bound by TFEB<sup>211A</sup>-MYC-FLAG or MYC-HA was normalized by 2% input. The sequence of primers used for the qPCR is provided in Supplementary Table S11.

Retroviral Transduction

EcoPack cells were transfected with the indicated retroviral plasmids using Profection system (Promega). Retrovirus was collected four times (at days 2, 3, 4, and 5 following transfection), filtered through a 0.45-μm pore size filter, and then concentrated using a 50,000 MW cutoff filter (VIVASPIN 20, Sartorius). Concentrated virus was applied to 32D.3 myeloid cells expressing rtTA2 and RIEP generated previously (58), and cells were then spin-infected (2,500 rpm, 90 minutes) at 30°C in the presence of polybrene. After expansion, GFP- or dTomato-positive cells were sorted by FACS or were selected by growth in medium containing 1 to 2 μg/mL puromycin.

Lentiviral Transduction

HEK293T cells were transfected with the indicated lentiviral plasmids using FuGENE (Promega) or Lipofectamine 2000 (Thermo Fisher) as instructed by the manufacturer. Lentivirus was collected, filtered, and concentrated as described above. Concentrated virus was applied to human AML cells or 32D.3 myeloid cells, and transduced cells were sorted for the presence of GFP or were selected by growth in medium containing 1 to 2 μg/mL puromycin (1 μg/mL for NB4 cells, 2 μg/mL for the remaining cells) and/or 150 μg/mL hygromycin.

IHC Analyses

Paraffin-embedded BM trephine biopsies were used for IHC analyses as described (7). Blocks were sectioned 4 μm in thickness. Unstained slides were deparaffinized using an automated system with EZ Prep solution (Ventana Medical System) and stained with MYC and TFEB antibodies using a Ventana Discovery XT automated system (Ventana) as per the manufacturer’s protocols. Slides were reviewed by an independent hematopathologist. Protein expression levels were calculated as MYC- or TFEB-positive cells out of total counted blasts in the selected area with sheets of blasts as described (59).

Genomic DNA Isolation and 5hmC Dot Blot Assay

Genomic DNAs were isolated from 2 to 10<sup>5</sup> cells using the QIAamp DNA mini kit. DNAs were sonicated to generate 200 to 500 bp fragments and denatured at 95°C for 10 minutes, followed by incubation on ice for an additional 10 minutes. DNA samples were then mixed with 1 volume of 2M NH₄SO₄ (pH 7.0) and diluted to a final concentration of 50 to 200 ng/μL. DNA was applied as indicated on the nylon membrane (Amersham Hybond N<sup>+</sup>). After drying the membrane, DNAs were cross-linked to nylon membrane at 1,200 J/m² using UV Crosslinker (UVP/Analytik Jena). The membrane was incubated in blocking buffer for 30 minutes at room temperature. Blots were then washed three times with 1x TBST, incubated with HRP-conjugated secondary antibody at 1:2,000 in blocking buffer for 30 minutes, and then washed three times with 1x TBST. Images were captured using Odyssey Fc Imaging System (LI-COR). PI staining of genomic DNA served as a loading control.

RNA-seq Analysis

HL60-pRRL-EV-puro or HL60-pRRL-TFEB<sup>211A</sup>-puro cells were treated with vehicle or 1 μg/mL Dox for 48 hours. Total RNA was extracted using the NucleoSpin RNA II Kit. After RNA sample quality was assessed by RNA Integration Number using Agilent 2100 Bioanalyzer and the RNA 6000 Nano LabChip, an Ovation Human FFPE RNA-Seq Library Systems (NuGEN) was used to generate cDNA for the next-generation sequencing. RNAs were subjected to reverse transcription with random primers and second-stand synthesis to generate double-stranded cDNA. Ends were filled and then ligated with nucleotide analogue-marked adapters. The cDNAs were then denatured and PCR-enriched to generate the final genomic library, which was analyzed on an Illumina NextSeq 500. Read adapters were detected using BBMerge (60) and subsequently removed with cutadapt (61). Processed raw reads were then aligned to human genome hs37d5 using STAR (v2.5.3a; ref. 62). Gene expression was evaluated as read count at gene level with HTSeq (v0.6.1; ref. 63) and Gencode gene model v19. Gene-expression data were then normalized, and differential expression of TFEF versus non-TFEB-expressing groups was evaluated using DESeq2 (64).

Reduced Representation Bisulfite and Oxidation Bisulfite Sequencing Analysis

HL60-pRRL-EV-puro or HL60-pRRL-TFEB<sup>211A</sup>-puro cells were treated with vehicle or 1 μg/mL Dox for 48 hours. Total genomic DNAs were extracted using the QIAamp DNA mini kit. After digestion with MspI restriction enzyme at 37°C for 1 hour, DNAs were ligated with adaptors and ends repaired. Each DNA sample was split into two aliquots and then subjected to bisulfite conversion or to an oxidation reaction followed by bisulfite conversion. After desulfonation and purification, DNAs were denatured and PCR-enriched to generate genomic libraries, which were analyzed on Illumina NextSeq 500. Each sample had at least 10 million counts with paired-end 150 base reading. Raw sequence reads were first assessed for their quality using the program FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and they were then trimmed with a paired-end mode to remove low-quality ends and adapters using the program Trim Galore (v0.4.5, www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Additional sequences added by the diversity adapters were further removed using a custom python script provided by NuGEN (https://github.com/nugenotechnologies/NuMetRRBS/). Trimmed reads were aligned to human genome hs37d5 using bismark v0.20.0 (65) in paired-end mode. CpG methylation calls were then extracted using bismark methylation extractor (v0.20.0) with the default settings. TFEF-induced changes in 5mC were detected by comparing methylation calls from oxBS-seq data using MOABS v1.3.4 (66). Hypermethylated and hypomethylated 5C were determined using criteria credible methylation difference (CDIF) >0.2 and <−0.2, respectively, as suggested by MOABS. 5hmC levels were first inferred by the differences between BS-seq and oxBS-seq of the paired samples. TFEF-induced 5hmC changes were further measured by comparing the resulting CDIF values using a t test. Criteria for differentially methylated 5hmC were chosen as suggested by MOABS (66): FDR-controlled P value <0.05, mean CDIF >0 for both groups, and mean CDIF (TFEB group) - mean CDIF (non-TFEB group) >0.06 for hyper-hydroxymethylation and <−0.06 for hyp-hydroxymethylation. Identified differentially methylated and hydroxymethylated 5C were annotated to genomic regions and CpG islands using the annotation functions implemented in R package ChIPseeker (67) and methylKit (68).

Isolation of Primary AML Patient Samples

Primary BM aspirates from patients with AML were obtained with IRB approval. Cells were isolated on Histopaque-1077 step gradients, washed with RPMI-1640 media, and then cultured for 48 to 72 hours in 10% FBS RPMI-1640 media with vehicle or GSK-621 as indicated.
Cells were harvested for immunoblotting, qRT-PCR analysis, and 5hmC DNA dot blot assays as described above.

**Cell Fractionation Studies**

After treatment with vehicle, 4OH-T, or GSK-621 for 48 hours, cells were sedimented at 200 x g for 5 minutes and then washed twice with ice-cold PBS. Cells were then incubated for 15 minutes at 4°C in hypotonic buffer (25 mmol/L HEPES (pH 7.5), 5 mmol/L MgCl2, and 1 mmol/L EDTA) supplemented immediately before use with 1 mmol/L α-phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL pepstatin), and were then Dounce homogenized. After confirming cells were ruptured using trypan blue staining, samples were sedimented at 8000 x g to separate nuclei. The supernatant was saved as the cytoplasmic fraction. Fractionated samples were applied to SDS-PAGE.

**Gas Chromatography–Mass Spectroscopy Quantification of α-KG and 2-HG**

α-KG and 2-HG extracted from HL60 cells were measured by gas chromatography–mass spectroscopy (GC-MS) system as described (69). In brief, 25 x 10^6 cells were quickly rinsed in ice-cold PBS and lysed with 50% methanol. The crude lysates were spiked with 4 μg stable isotope standards of 13C4-α-KG (Cambridge Isotope Laboratories) and 2-HG-d3 (Toronto Research Chemicals), freeze–thawed three times, and centrifuged to remove debris. The resulting supernatants were evaporated to dryness under nitrogen gas, derivatized in DMF and MTBSTFA + 1% TBDMS, and then injected (3 μL) onto Agilent 7890B gas chromatograph networked to an Agilent 5977A mass selective detector with following chromatographic conditions: Initial temperature 70°C for 1 minute, increasing to 215°C at 15°C/minute over 10 minutes, further increasing to 280°C at 15°C/minute over additional 10 minutes, and finally increasing to 325°C at 25°C/minute for 5 minutes. The front inlet temperature was 230°C operating with a splitless mode. MSD ion source and interface temperature were 230°C. The MSD operated in EI mode at 70 eV. Scan mode of 50 to 700 m/z was used for the analyses. The carrier gas was helium (1.0 mL/minute). GC-MS data were acquired and processed using Agilent MassHunter WorkStation software. The relative ion intensities of α-KG (m/z 431) and 2-HG (m/z 433) with respect to stable isotope standards of 13C4-α-KG (m/z 435) and 2-HG-d3 (m/z 436) were calculated by IsoPat2 software to quantify α-KG and 2-HG levels with taking account of natural abundances of isotopomers.

**TCGA AML Data Set**

The expression data for the TCGA AML RNA-seq samples were extracted from the “EBIPlusPlusAdjustPANCAN_HumaHiSeq_ RNASeqv2_geneExp.txt” file that is available from the following link: https://gdac.cancer.gov/about-data/publications/pancanatlas and was log2 transformed, log2(α + 1). Sample annotation was retrieved from the TCGA AML data set using Supplementary Table S1 at https://gdac.cancer.gov/node/876. TCGA methylation data were downloaded as raw IDAT files and were processed using normalization via internal controls followed by background subtraction using the methylumi R package from the Bioconductor Open Source Software for Bioinformatics that is available at https://bioconductor.org/packages/release/bioc/html/methylumi.html.

**GSE15434 Data Set**

Raw CEL files were downloaded and normalized using IRON (70). Only BM samples were analyzed.

**GSE42519 Data Set**

The GSE42519 CEL files were downloaded from Gene Expression Omnibus (GEO) along with monocyte samples from GSE11864 (GSM299556 and GSM299561) and from E-MEXP-1242 (Immcon2cd14poscd16neg241104 and Immcontcl14poscd16neg241104) as described (71). The files were normalized using IRON (70).

**TARGET AML Data Set**

TARGET AML lvl3 data (TARGET_AML_GE_level3.txt) was downloaded from the TARGET Data Matrix, https://ocg.cancer.gov/programs/target/data-matrix (17). Only samples labeled as “Primary Blood Cancer BM” were used in the analysis. Use of this data set was approved by a TARGET AML study investigator (NIH/NCI).

**GSE15907 ImmGen Data**

The GSE15907 ImmGen data were downloaded from GEO as a Series Matrix File and log2 transformed, log2(x + 1) (72).

**Taiwan AML Data Set**

Taiwan AML data were provided by Dr. Hsin-An Hou at the Taiwan National University Hospital.

**CCLE Data**

The CCLE data were downloaded from the following website: www.cbioportal.org.

**Lineage-Affiliated Signatures**

The individual Excel files for each of the nine lineage-affiliated signatures containing the probe sets were downloaded from the following website: http://www.massgeneral.org/cbrc/research/researchlab.aspx?id=1070 (26). The expression of all the genes within a signature was summarized using principal component analysis (73).

**Quantification and Statistical Analyses**

Under the assumption of independent variables, normal distribution, and equal variance of samples, statistical significance was assessed using an unpaired two-tailed Student t test for in vitro experiments. Error bars presented in the figures indicate the mean ± SEM. The statistical parameters are described in the individual figure legends. For the survival analyses, disease-free survival and OS were estimated with the Kaplan–Meier method and compared using a log-rank test. Statistical analyses were performed using SPSS v24.0, GraphPad Prism 7, and MATLAB. A P value less than 0.05 was considered statistically significant.

**Data and Software Availability**

RNA-seq, BS-seq, and oxBS-seq data have been deposited in the GEO under accession files GSE132584, GSE132585, and GSE132586, respectively.

**Authors’ Disclosures**

F.X. Schaub reports grants from Swiss National Science Foundation during the conduct of the study. D.J. Murphy reports grants from Puma Biotech and Merck Group outside the submitted work. A. Ballabio reports personal fees from Casma Therapeutics, Inc outside the submitted work. F. X. Schaub reports grants from Swiss National Science Foundation during the conduct of the study. D.J. Murphy reports grants from Puma Biotech and Merck Group outside the submitted work. A. Ballabio reports personal fees from Casma Therapeutics, Inc outside the submitted work. F.X. Schaub reports grants from Swiss National Science Foundation during the conduct of the study. D.J. Murphy reports grants from Puma Biotech and Merck Group outside the submitted work. A. Ballabio reports personal fees from Casma Therapeutics, Inc outside the submitted work. S.H. Kaufmann reports grants from Takeda Pharmaceuticals outside the submitted work. J.L. Cleveland reports grants from Leukemia & Lymphoma Society during the conduct of the study. No disclosures were reported by the other authors.

**Authors’ Contributions**

S. Yun: Conceptualization, resources, data curation, formal analysis, funding acquisition, validation, investigation, visualization, methodology, writing–original draft, writing–review and editing. N.D. Vincellete: Conceptualization, data curation, formal analysis, validation, investigation, visualization, methodology, writing–original draft, writing–review and editing. J. L. Cleveland: Conceptualization, resources, data curation, formal analysis, funding acquisition, validation, investigation, visualization, methodology, writing–original draft, writing–review and editing.
draft, writing-review and editing. X. Yu: Software, formal analysis, methodology, writing-review and editing. G.W. Watson: Data curation, formal analysis, investigation. M.R. Fernandez: Data curation, formal analysis. C. Yang: Data curation. T. Hirotsugi: Data curation, formal analysis, funding acquisition, methodology. C.-H. Cheng: Software, formal analysis, methodology. A.R. Freischel: Data curation, formal analysis. L. Zhang: Data curation, formal analysis, methodology. W. Li: Data curation. H. Hou: Resources, data curation. F.X. Schaub: Resources. A.R. Vedder: Data curation. L. Cen: Software, methodology. K.L. McGraw: Resources. J. Moon: Data curation. D.J. Murphy: Resources, writing-original draft, writing-review and editing. A. Ballabio: Conceptualization, resources, writing-review and editing. S.H. Kaufmann: Resources, data curation, writing-original draft, writing-review and editing. A.E. Berglund: Data curation, software, formal analysis, visualization, methodology, writing-original draft, writing-review and editing. J.L. Cleveland: Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing-original draft, project administration, writing-review and editing.

Acknowledgments

The authors thank the Flow Cytometry, Molecular Genomics, Tissue, Biostatistics and Bioinformatics, and Analytical Microscopy Cores of the H. Lee Moffitt Cancer Center and Research Institute, and the Comparative Medicine Department of the University of South Florida (USF). The pRRL-puro plasmid was a kind gift from Dr. Johannes Zuber (IMP, Vienna, Austria). This work benefited from data assembled by the ImmGen Consortium, and the authors also thank AML team members of TARGET for providing expression data. This work was supported in part by an LLS-SCOR grant (J.L. Cleveland), by NIH grant CA225680 (T. Hitosugi), by Deutsche Krebsfalle grant 109220 (D.J. Murphy), by F32 grant CA203217 (M.R. Fernandez), by the Cortner-Couch Endowed Chair for Cancer Research from the USF School of Medicine (J.L. Cleveland), by NIH grant K08 CA237627 (S. Yun), by a Research Training Award for Fellows (RTAF) from the American Society of Hematology (ASH; S. Yun), by a Scholar Award from ASH (S. Yun), by a research grant from the Graduate Medical Education at USF (S. Yun), and by NCI Comprehensive Cancer Grant P30-CA076292 to the H. Lee Moffitt Cancer Center and Research Institute.

Received March 2, 2020; revised October 30, 2020; accepted December 15, 2020; published first December 21, 2020.

REFERENCES


