Targeting Mitochondrial Structure Sensitizes Acute Myeloid Leukemia to Venetoclax Treatment

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ABSTRACT

The BCL2 family plays important roles in acute myeloid leukemia (AML). Venetoclax, a selective BCL2 inhibitor, has received FDA approval for the treatment of AML. However, drug resistance ensues after prolonged treatment, highlighting the need for a greater understanding of the underlying mechanisms. Using a genome-wide CRISPR/Cas9 screen in human AML, we identified genes whose inactivation sensitizes AML blasts to venetoclax. Genes involved in mitochondrial organization and function were significantly depleted throughout our screen, including the mitochondrial chaperonin CLPB. We demonstrated that CLPB is upregulated in human AML, it is further induced upon acquisition of venetoclax resistance, and its ablation sensitizes AML to venetoclax. Mechanistically, CLPB maintains the mitochondrial cristae structure via its interaction with the cristae-shaping protein OPA1, whereas its loss promotes apoptosis by inducing cristae remodeling and mitochondrial stress responses. Overall, our data suggest that targeting mitochondrial architecture may provide a promising approach to circumvent venetoclax resistance.

SIGNIFICANCE: A genome-wide CRISPR/Cas9 screen reveals genes involved in mitochondrial biological processes participate in the acquisition of venetoclax resistance. Loss of the mitochondrial protein CLPB leads to structural and functional defects of mitochondria, hence sensitizing AML cells to apoptosis. Targeting CLPB synergizes with venetoclax and the venetoclax/azacitidine combination in AML in a p53-independent manner.

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INTRODUCTION

Acute myeloid leukemia (AML) is a hematopoietic neoplasm characterized by the proliferation and accumulation of aberrant immature myeloid progenitor cells. AML is associated with poor clinical outcome and high mortality, with an overall five-year survival rate of 15% to 30%. For patients with AML, standard therapies often fail to achieve complete remission, disease relapse is often fatal, and salvage and bone marrow transplantation are applicable to only a few select patients with AML, highlighting the need for novel targeted treatments. A number of such emerging treatments have targeted essential “hallmarks” of cancer, including the regulation of cancer cell survival by members of the BCL2 protein family. Among these members, B-cell lymphoma 2 (BCL2) is found upregulated in AML cells (1) and, specifically in leukemic stem cells (LSC; ref. 2). BCL2 overexpression is a poor-risk factor in AML and is associated with poor response to standard cytotoxic therapy (1, 3).

Mechanistically, BCL2 is the founding member of the BCL2 protein family, and prevents programmed cell death by binding to proapoptotic members of the same family (4). Following a death signal, the proapoptotic factors are released from BCL2 and induce the oligomerization of BAX and BAK on the outer mitochondrial membrane (OMM; ref. 5). This is a crucial step for the release of cytochrome c from mitochondria to the cytosol where, together with dATP, APAF1, and caspase 9, it forms the apoptosome, a complex that subsequently activates the executioner caspases and thus apoptosis (6, 7).

In cancer cells, enhanced BCL2 expression supports cell survival, as it leads to the suppression of mitochondrial-mediated apoptosis. Inhibiting BCL2 via a selective BH3 mimic such as venetoclax has proven to be an efficient strategy to promote caspase-dependent cell death in AML (8). Venetoclax is an orally bioavailable drug that is approved for chronic lymphocytic leukemia and other hematologic malignancies. Venetoclax recently received FDA approval for the treatment of newly diagnosed elderly patients with AML in combination with hypomethylating agents (azacitidine or decitabine; ref. 9), as proposed by our group (10, 11), or with low-dose cytarabine. However, approximately 30% of patients do not respond up front, and many patients with AML still develop resistance while on treatment (12).
highlights the need for a greater mechanistic understanding of venetoclax resistance, both in combination and as monotherapy. To that end, multiple combinational therapies for venetoclax have been proposed, including CDK (CDK9) and MCL1 inhibition, as we and others have suggested (13–15), with several of these combinations having entered clinical trials (16).

To shed light on the mechanisms of resistance to venetoclax, and to propose novel venetoclax treatment combinations, we performed a genome-wide CRISPR/Cas9 loss-of-function screen in human AML cells in the presence or absence of venetoclax and identified CLPB as one of the top candidates whose ablation sensitizes AML cells to the drug treatment. We show that the mitochondrial protein CLPB is upregulated in patients with AML and protects AML cells against caspase-dependent apoptosis and mitochondrial dysfunction. In particular, we demonstrate that CLPB is essential for sustaining the correct mitochondrial cristae morphology by its direct interaction with OPAL, the master regulator of mitochondrial dynamics. Moreover, CLPB deficiency leads to mitochondrial dysfunction, which causes ATF4-mediated mitochondrial stress responses and alterations at the cell transcriptome and metabolome. Therefore, depleting CLPB sensitizes AML cells to venetoclax-induced programmed cell death in vitro and in vivo. Our work suggests that targeting mitochondrial structure could be a promising strategy to overcome venetoclax resistance in patients with AML.

RESULTS
CRISPR Screen Identifies Synthetic Lethal Vulnerabilities for Venetoclax in AML

To systematically identify key factors that regulate venetoclax resistance in human AML, we performed a genome-wide CRISPR/Cas9 loss-of-function screen (17). We transduced MOLM-13 AML cells with the Brunello single-guide RNA (sgRNA) library (18), cultured them in the presence of venetoclax or DMSO for 16 days, and sequenced the distribution of sgRNAs at days 8 and 16 after drug treatment (Fig. IA; Supplementary Fig. S1A). Our strategy ensured that genes confer resistance to or synergize with venetoclax would be positively or negatively selected, respectively, following 8 and 16 days of drug treatment compared with DMSO treatment (Fig. 1B–D; Supplementary Fig. S1B and Supplementary Table S1). BAX and PMAIP1 (NOXA) were among the positively selected genes (genes whose loss confers resistance), a finding consistent with the mechanism of action of the drug (14, 19, 20). Interestingly, the tumor suppressor p53 (TP53) was also positively selected in the screen, in agreement with recent studies (15) as well as the accompanying CRISPR screen performed by NIchiporuk and colleagues in this issue. MDM2, encoding an E3 ubiquitin ligase that targets p53 for degradation (15, 21) and MCL1, a primary mode of resistance to venetoclax (13, 22), were among the negatively selected genes (genes whose loss synergizes with venetoclax) at both days 8 and 16 of drug treatment (Fig. 1B–D; Supplementary Fig. S1B). We further validated a number of positively and negatively selected genes (GID8, BAX, TP53, PMAIP1, SLC25A1, RTN4IP1, and CLPB) throughout the screen by selecting the top sgRNAs for each gene and monitoring their ability to confer resistance to or synergize with venetoclax in AML cells performing a competition-based assay (Supplementary Fig. S1C). Our results confirmed that all the sgRNAs targeting the positively selected genes demonstrated significant resistance to venetoclax, whereas the sgRNAs targeting the negatively selected genes strongly sensitized MOLM-13 cells to the drug treatment (Fig. 1E; Supplementary Fig. S1D). Likewise, a significant increase of IC50 in KASUMI-1 and MOLM-13 cells upon deletion of BAX and PMAIP1 validated the gain of resistance of venetoclax in BAX- and PMAIP1-deficient AML cells. Notably, TP53 sgRNAs increased the venetoclax IC50 in p53 wild-type MOLM-13 but not in TP53-mutant KASUMI-1, confirming the specificity of the guides used in this study (Supplementary Fig. S1E and S1F). Together, our genome-wide CRISPR/Cas9 loss-of-function screen successfully revealed potential modes of resistance to venetoclax as well as synthetic lethal partners in AML.

Next, we aimed to identify key pathways and biological processes that are enriched in our screen. We performed Gene Ontology (GO) analysis focusing first on positively selected genes that were at least 8-fold [log fold change (LFC) > 3] enriched. The majority of these genes significantly clustered into key biological processes, categories that regulate the intrinsic apoptotic signaling pathway, including cytochrome c release and mitochondrial outer membrane permeabilization (Supplementary Fig. S1G). STRING protein–protein interaction network analysis was then performed using the positively selected genes at both days 8 and 16 of venetoclax treatment (Supplementary Fig. S1H). One of the major clusters from this network was the p53-mediated apoptotic signaling pathway in mitochondria (Supplementary Fig. S1I). We were particularly interested in the negatively selected genes, as they are potential therapeutic targets for circumventing venetoclax resistance. GO analysis of the “sensitizer” genes revealed strong enrichment for mitochondrial processes, such as mitochondrial transport, organization, and oxidative phosphorylation (Supplementary Fig. S1J). Moreover, when we combined the two time points of our screen, we successfully identified 353 common negatively selected genes (Fig. 1F). Among these, 55 were genes that encode proteins functioning in mitochondria, highlighting the relevance of mitochondrial processes in the sensitization of AML cells to venetoclax. Consistently, mapping this 353-gene set on the STRING database generated a highly connected network consisting mainly of genes that regulate mitochondrial functions, as well as chromatin remodelers, ubiquitin ligases, signal transducers, and components of mRNA processing (Fig. 1G). Therefore, our genome-wide CRISPR/Cas9 loss-of-function screen identified mitochondrial processes as key potential synthetic lethal vulnerabilities for venetoclax in AML.

Venetoclax Treatment Leads to Aberrant Mitochondrial Structure and Depolarization in AML

Given that venetoclax action converges on mitochondria which actively participate in programmed cell death, our screen results intrigued us to further investigate how BCL2 inhibition affects mitochondrial structure and function.
Targeting Mitochondria Sensitizes AML to Venetoclax

Figure 1. Genome-wide CRISPR screen identifies genes controlling mitochondrial physiology as synthetic lethal with venetoclax treatment in AML.

A, Schematic outline of the viability-based, genome-wide CRISPR/Cas9 loss-of-function screen. B, Volcano plot showing both positively and negatively selected genes in the CRISPR screen at day 8 after drug treatment. A number of positively and negatively selected genes are shown in red and green, respectively. Known regulators of venetoclax resistance are shown in orange (positively selected) and blue (negatively selected), respectively.

C and D, Frequency histograms of the delta CRISPR score of the negative control guides (top), and selected genes at days 8 (C) and 16 (D) after drug treatment.

E, Validation of selected genes in the CRISPR screen using a competition-based survival assay in MOLM-13 cells. The normalized enrichment scores were calculated as shown in Supplementary Fig. S1C. Data, mean ± SEM (n = 4 for each sgRNA).

F, Venn diagram of the negatively selected genes (“sensitizers”) in the CRISPR screen at day 8 (log fold change < −1) and day 16 (log fold change < −3) after drug treatment.

G, STRING protein–protein interaction network of the 353 common negatively selected genes as defined in F. The minimum required interaction score was set to 0.5, and the disconnected dots were removed. k-means clustering was applied with the number of clusters set to 6. Data with statistical significance are as indicated: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
in AML. Mitochondria change their shape early during the process of apoptosis; the individual inner membrane lamellae, named cristae, fuse, and the opposing faces of the cristae membrane (cristae junctions) open, allowing the redistribution of cytochrome c from the cristae lumen to the intermembrane space (23, 24). To determine the mitochondrial structure alterations in AML, we performed electron microscopy–based morphometric analysis of AML cells (THP-1 and MOLM-13) treated with venetoclax. Indeed, mitochondria exhibit abnormal ultrastructure after venetoclax treatment, characterized by a lower number of cristae and increase of the cristae lumen width (Fig. 2A–C; Supplementary Fig. S2A–S2C). The normal mitochondrial structure is crucial for the maintenance of the mitochondrial membrane potential, which is the driving force behind ATP production and mitochondrial homeostasis. Early during apoptosis, the mitochondrial membrane potential collapses, leading to the complete cytochrome c release from mitochondria to the cytosol (25). We therefore predicted that venetoclax, as an inducer of apoptosis, causes loss of mitochondrial membrane potential in leukemic cells. Indeed, staining of AML cells with the cell-permeant, cationic fluorescent dye tetramethylrhodamine methyl-ester (TMRM) revealed depolarization of mitochondria after treatment with the BCL2 inhibitor (Fig. 2D; Supplementary Fig. S2D).

To further investigate how venetoclax treatment may be causing widening of the cristae, we turned to the mitochondrial protein Optic Atrophy 1 (OPA1), which functions as a molecular staple at cristae junctions to prevent cytochrome c mobilization and release (26). In physiologic conditions, membrane-associated (long) and soluble, cleaved (short) forms of OPA1 coexist to form oligomers and complexes that contribute to cristae maintenance. Upon an intrinsic death stimulus, OPA1 proteolytic cleavage is enhanced, allowing the “opening” of the cristae junctions and the cytochrome c redistribution (25). We observed a decrease in the long (L-OPA1) forms and accumulation of short (S-OPA1) forms of OPA1 in response to venetoclax treatment, suggesting its proteolysis following BCL2 inhibition (Fig. 2E and F). Venetoclax treatment also led to caspase activation, as indicated by caspase-3 cleavage (Supplementary Fig. S2E) and subsequently cell death, as demonstrated using Annexin V staining (Supplementary Fig. S2F).

**Venetoclax Resistance Is Coupled to OPA1 Overexpression and Tighter Mitochondrial Cristae**

To further investigate the mechanisms of venetoclax resistance, we generated four human AML clones highly resistant to the drug, by growing the parental MOLM-13 and MV4-11 cells in increasing doses of the drug for over 8 weeks. IC_{50} analysis verified more than 100-fold increase of venetoclax concentration required for the 50% cell death in the venetoclax-resistant (VR) cell lines in respect to the original clones (Par; Fig. 2G). Driven by the role of BCL2 inhibition in mitochondrial architecture, we aimed to closely examine the organelle’s structure in the venetoclax-resistant AML cells. We discovered that venetoclax-resistant AML cells exhibit tighter cristae (approximately 14–15 nm in diameter) and a higher number of cristae per mitochondrion than the parental sensitive clones (Fig. 2H and I; Supplementary Fig. S2G–S2I). This phenotype could be explained as the decrease in the ratio of OPA1 protein expression in the AML venetoclax-resistant cell lines (Fig. 2J; Supplementary Fig. S2J). Notably, this is not simply the result of a general increase of mitochondrial biogenesis, as the mitochondrial protein TOM20 does not display similar expression changes (Supplementary Fig. S2K).

These data suggested that mitochondria change their shape in venetoclax-resistant AML cells, most likely to antagonize the cell-intrinsic apoptotic signaling cascade initiated by BCL2 inhibition.

Because MCL1 upregulation has been previously implicated in the development of venetoclax resistance (12), we aimed to examine the protein levels of the major BCL2 family members (MCL1, BCL2, and BCL-XL) in the generated venetoclax-resistant cell lines. We observed a slight increase of MCL1 and BCL2 protein levels in MOLM-13-VR cells. Also, we found no increase of either BCL-XL protein or any of the examined BH3 antiapoptotic proteins in the MV4-11-VR cell lines. These results suggest that BCL2 and MCL1 upregulation may serve as one of the potential mechanisms of cellular resistance to venetoclax; however, at least in AML, there appear to exist alternative modes of resistance acquisition (Supplementary Fig. S2L). In agreement with that notion, and based on the generated resistant AML lines, OPA1 upregulation and mitochondrial structural adaptations appear to represent a more universal mechanism of venetoclax resistance.
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**A**  
DMSO  
Venetoclax

**B**  
Maximal cristae width (nm)

**C**  
Mitochondria with abnormal ultrastructure (%)

**D**  
% TMRM-low cells

**E**  
Ratio

**F**  
Long form/postformal OPA1

**G**  
IC₅₀ of venetoclax

**H**  
MOLM-13 Par  
MOLM-13 VR

**I**  
Maximal cristae width (nm)

**J**  
Cell viability (% Annexin V−, DAPI−)

**K**  
Genes encoding mitochondrial proteins (LFC > 1 or LFC < −1, FDR < 0.05)

**L**  
CRISPR screen vs. RNA-seq in VR cells

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**GO biological process complete**

- Response to type I interferon
- Type I interferon signaling pathway
- Mitochondrial depolarization
- Defense response to virus
- Cellular amino acid metabolic process
- Regulation of mitochondrial membrane potential
- Coenzyme metabolic process
- Negative regulation of ATP metabolic process
- Negative regulation of purine nucleotide metabolic process
- Negative regulation of nucleotide metabolic process
- Membrane depolarization
- Neurotransmitter catabolic process
- Alpha-amino acid metabolic process
- Negative regulation of ATP metabolic process
- Negative regulation of nucleotide metabolic process

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**Abbreviations**

- LFC: Log fold change
- FDR: False discovery rate
- CRISPR: Clustered regularly interspaced short palindromic repeats
- RNA-seq: RNA sequencing
To further explore the molecular mechanisms underpinning venetoclax resistance, RNA sequencing (RNA-seq) was performed. Indeed, the transcriptional landscape of the AML cells changed significantly upon acquisition of resistance to venetoclax (Supplementary Fig. S3A–S3B). Interestingly, among the differentially expressed genes, there were many involved in various mitochondrial processes. We focused on such genes (“Genes encoding mitochondrial proteins,” using the Mitominer data set; Supplementary Fig. S3A–S3B) and performed GO analysis to dissect the functional changes in mitochondria upon acquisition of venetoclax resistance. Strikingly, pathways that regulate mitochondrial membrane organization, potential, and depolarization were strongly enriched in both MOLM-13-VR and MV4-11-VR, though strong variances were observed at the global transcriptomic level in these cell lines (Fig. 2K; Supplementary Fig. S3C–S3D), which is consistent with our findings that mitochondrial structure adaptation is universally required for gain of venetoclax resistance in AML cells. Besides, metabolic processes of key cellular components, such as amino acids, coenzyme, ATP, and nucleotides, were also enriched within the top 20 enriched pathways, suggesting the metabolic adaptation is likewise important for venetoclax resistance. Finally, we observed enhanced interferon responses in MOLM-13-VR cells, which might be a result of mitochondrial DNA release to the cytosol as a consequence of incomplete apoptosis and partial mitochondrial dysfunction during the persistent venetoclax treatment (Fig. 2K; Supplementary Fig. S3D; ref. 27). Overall, these data suggested that AML blasts require transcriptional and post-translational mitochondrial adaptation to acquire resistance to venetoclax.

Targeting the Mitochondrial Protein CLPB Overcomes Venetoclax Resistance in AML

We next attempted to determine whether there is a correlation between genes differentially expressed in the resistant cell lines and gene hits in our CRISPR screen. We initially focused on the downregulated genes in MOLM-13-VR cells compared with MOLM-13-Par cells, whose ablation confers resistance to venetoclax (positively selected genes). We identified the validated genes TP53, BAX, and PMAIP1, as well as the proapoptotic genes BAD and BCL2L11 (also known as BIM), and SLC27A2, a gene involved in fatty-acid metabolism. Moreover, we also focused on the upregulated genes in MOLM-13-VR cells compared with MOLM-13-Par cells, whose ablation sensitizes cells to the drug (negatively selected genes). We identified genes encoding the mitochondrial proteins CLPB and SLC25A1, as well as antiapoptotic proteins such as MCL1 and HAX1. We also found ATPAF1 and MSTO1, whose products are involved in the maintenance of mitochondrial structure and functions (Fig. 2L).

Driven by these findings, we hypothesized that targeting proteins regulating mitochondrial cristae maintenance and function might open up potential synthetic lethal vulnerabilities for venetoclax treatment. To further refine the candidate list from the screen, we excluded 97 core cellular fitness genes (17), genes that are generally required for all cell types and therefore may not serve as ideal future therapeutic targets. From the remaining 256 genes, we focused on genes that displayed higher mRNA expression levels in AML patient samples compared with healthy primary CD34+ hematopoietic stem and progenitor cells (HSPC) according to The Cancer Genome Atlas (TCGA). After applying these criteria, we identified 18 candidates (Fig. 3A). One of the top-scoring candidates was CLPB, which encodes a mitochondrial AAA+ ATPase chaperonin. Our expression studies revealed that CLPB gene expression is significantly higher in AML patient samples when compared with normal CD34+ HSPCs (Fig. 3B). Moreover, CLPB median expression in AML was the second highest among all other cancer subtypes in the TCGA data set and the third highest among all cancer cell lines in the Cancer Cell Line Encyclopedia database (Fig. 3C; Supplementary Fig. S4A).

As a further suggestion of a role in this process, we observed higher levels of CLPB protein in venetoclax-resistant human AML cell lines when compared with the parental sensitive clones (Fig. 3D and E). Based on our RNA-seq data (Supplementary Fig. S4C), CLPB transcripts are only slightly upregulated in venetoclax-resistant cells, indicating that this overexpression is post-translational. Importantly, the protein levels of the mitochondrial transcription factor A (TFAM) were unchanged, indicating that the total mitochondrial mass was not altered. Furthermore, when we treated AML cells with venetoclax, we observed a significant increase in the CLPB protein, indicating that cells that survive venetoclax treatment have higher CLPB levels (Supplementary Fig. S4D–S4E).
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A. Upregulated in AML patients vs. CD34+ HSPCs. 2,220 genes. Common negatively selected genes in the screen. 353 genes. Core essential genes. (numTKO hits > 1)

B. FPKM of CLPB mRNA. CD34+ HSPCs. Patients with AML.


D. MOLM-13, MV4-11.

E. CLPB band intensity (relative to Actin).

F. MOLM-13, MV4-11. % Viable cells vs. log (conc.) (nmol/L).

G. MOLM-13-VR1, MV4-11-VR1. % Viable cells vs. log (conc.) (nmol/L).

H. sgRosa, Vehicle, Ven. sgCLPB #1, Ven.

I. % GFP+ cells in PB days after injection.

J. Bioluminescence intensity (photons/sec/mouse) days after injection.

K. % Cell survival days after injection.

L. Vehicle, Ven. CLPB, OPA1, GAPDH.
In addition, we quantified the CLPB protein levels in 4 different AML cell lines (MOLM-13, MV4-11, OCI-AML3, and THP-1) and found that cell lines with high CLPB protein levels were more resistant to venetoclax than those with low CLPB levels (Supplementary Fig. S4F–S4H); however, the small statistical sample of cell lines analyzed precludes us from suggesting a definite predictive role of CLPB protein levels in venetoclax sensitivity.

To further evaluate the importance of CLPB function across different human AML cells, we depleted CLPB in multiple AML cell lines using CLPB-targeting sgRNAs (Supplementary Fig. S6A), and we subsequently measured the venetoclax IC_{50}. CLPB depletion significantly reduced the IC_{50} of venetoclax in all the cell lines tested (Fig. 3F; Supplementary Fig. SSA). Additionally, CLPB-deficient AML cells were negatively selected upon venetoclax addition at a much faster pace than the DMSO control groups in a competition-based viability assay (Supplementary Fig. SSB), confirming that CLPB ablation can sensitize AML cells to venetoclax treatment. Considering that CLPB is stabilized in venetoclax-resistant AML cells (Fig. 3D and E), we sought to validate the sensitization effect of CLPB depletion in our venetoclax-resistant cells. Similar to the results observed in the parental clones (Fig. 3F), ablation of CLPB significantly sensitized VR cells to the drug treatment (Fig. 3G; Supplementary Fig. SSC–SSD). The synthetic lethal relationship between CLPB ablation and venetoclax is specific to venetoclax mechanisms of action, because no synergistic effect was detected in AML cells treated with cytarabine, idarubicin, or JQ-1 treatment (Supplementary Fig. S4K). Moreover, in contrast to venetoclax, treatment of MOLM-13 cells with idarubicin did not lead to increase of CLPB protein levels in the surviving cells (Supplementary Fig. S4K). Finally, to avoid potential side effects of CRISPR-mediated gene deletion, silencing of CLPB using four distinct shRNAs with different efficiencies further validated the dose-dependent competitive disadvantage of CLPB loss in MOLM-13 cells treated with venetoclax (Supplementary Fig. S6B–S6D). Altogether, our findings revealed a functional role of mitochondrial CLPB in the response of AML cells to venetoclax and suggested that targeting CLPB can overcome venetoclax resistance in AML.

Finally, we sought to examine the synergistic effects of CLPB deletion with venetoclax in vivo by using preclinical animal models. Specifically, NOD/SCID gamma (NSG) mice were transplanted with MOLM-13-Par cells or MOLM-13-VR cells transduced with sgRosa or sgCLPB. After transplantation, we treated the mice with venetoclax or vehicle from days 6 to 20. Bioluminescence imaging and examination of peripheral blood circulating leukemic cells (GFP) indicated that CLPB deletion synergizes efficiently with venetoclax in both venetoclax-sensitive and venetoclax-resistant xenografts (Fig. 3H–K; Supplementary Fig. SSE–SSJ). In the course of these in vivo experiments, we determined that mice bearing wild-type tumors and being treated with venetoclax exhibit significantly elevated CLPB and OPA1 protein levels at late stages of tumor progression compared with the vehicle-treated mice (Fig. 3L), suggesting once more that mitochondrial structure adaptation also occurs in vivo as a mechanism of resistance to the drug.

**CLPB Ablation Impairs Mitochondrial Structure in AML Cells, Rendering Them More Susceptible to Apoptosis**

Next, we aimed to investigate how CLPB loss promotes venetoclax-induced programmed cell death in AML cells. First, to confirm the subcellular localization of CLPB, we performed confocal imaging, which indicated that endogenous CLPB colocalizes with the mitochondrial marker TOM20 in THP-1 and HeLa cells (Supplementary Fig. S7A). Notably, CLPB expression in patients with AML correlates with the transcription of genes whose products participate in the control of mitochondrial organization, such as OPA1 and ATAD3A (Supplementary Fig. S7B and S7C), suggesting a similar function of CLPB in leukemic cells. To test the hypothesis that CLPB participates in the organization of mitochondrial ultrastructure, we performed electron microscopy in CLPB-knockout AML cells. Mitochondria lacking CLPB exhibited an aberrant structure, characterized by wider cristae lumen (maximal cristae width—from a mean of 18 nm in the controls to 27 nm in the knockout). As expected, the mitochondrial structure abnormalities were exacerbated when the cells were treated overnight with venetoclax. It is worth noting that CLPB depletion was associated with a significant increase in mitochondrial cristae width in both venetoclax-sensitive and venetoclax-resistant xenografts (Fig. 3H–K; Supplementary Fig. SSE–SSJ). Furthermore, we examined the effects of CLPB expression on mitochondrial ultrastructure in multiple AML cell lines using CLPB-targeting sgRNAs (Supplementary Fig. S1A). Collectively, these findings indicate that CLPB ablation significantly impairs mitochondrial structure in AML cells, sensitizing them to mitochondria-mediated cell death.

**Figure 4.** CLPB loss induces mitochondrial ultrastructure defects sensitizing AML cells to mitochondria-mediated cell death. A, Representative electron micrographs of THP-1 transduced with sgRNAs targeting CLPB or control (sgRosa) and treated with 4 μmol/L venetoclax or DMSO for 16 hours. Scale bars, 2 μm (top) and 500 nm (bottom). B, Quantification of the percentage of mitochondria with abnormal ultrastructure in experiments as in A (n = 100 mitochondria per condition; top). Quantification of the maximal cristae width in 60 randomly selected mitochondria from 15 cells in experiments as in A (n = 200 cristae per condition; bottom). C, Equal amounts (30 μg) of protein from THP-1 infected with sgRNAs targeting CLPB or control (sgRosa) were separated by SDS-PAGE and immunoblotted using the indicated antibodies. D, Quantitative densitometric analysis of the ratio of OPA1 long (L-OPA1) versus short (S-OPA1) forms in experiments as in C. Data represent mean ± SEM of 8 independent experiments. E, Representative electron micrographs and morphometric analysis of BAX/BAK double-knockout MOLM-13 cells transduced with sgRNAs targeting CLPB or control (sgRosa). Scale bars, 500 nm. Morphometric analysis was performed in 60 randomly selected mitochondria (n = 200 cristae per condition) in two independent experiments. F, Quantification of the membrane potential loss after staining with TMRM in THP-1 infected with sgRNAs targeting CLPB or control (sgRosa) and treated with 4 μmol/L venetoclax or DMSO for 16 hours. Data, mean ± SD (n = 3 for each group). G, BH3 profiling mitochondrial depolarization measured by JC-1 in permeabilized THP-1 transduced with sgRosa or sgCLPB upon stimulation with BIM and BID peptides. Repolarization (%) was calculated based on the area under the curve for each condition and normalized to CCCP-positive control and 1% DMSO as negative control as previously described (48). H, Isolated mitochondria from THP-1 transduced as indicated were treated with recombinant cBID for 30 minutes and centrifuged at 12,000 x g for 10 minutes. Pellet and supernatant (SN) of each sample were separated with SDS-PAGE and immunoblotted for cytochrome c (cyt c). % Cyt c release is calculated as the percentage of the supernatant to the total (pellet and supernatant) cytochrome c band intensity. I, AML cells were transduced with sgRNAs targeting CLPB or control (Rosa), treated with venetoclax or DMSO for 16 hours, and cell death was determined by flow cytometry using Annexin V. Data are mean ± SD (n = 3). J, Liver mitochondrial lysates were immunoprecipitated with anti-CLPB coupled to magnetic protein G beads. Coprecipitated proteins were separated by SDS-PAGE and immunoblotted against CLPB and OPA1. Input was diluted 1:10. Data with statistical significance are indicated: *P < 0.05; **P < 0.01; ***P < 0.001; N.S., not significant.
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**Figure A**
- sgRosa
- sgCLPB
- DMSO
- Venetoclax

**Figure B**
- Mitochondria with abnormal ultrastructure (%)
- Max cristae width (nm)

**Figure C**
- L-OPA1/S-OPA1
- TFAM
- Actin

**Figure D**
- Ratio L-OPA1/S-OPA1 forms

**Figure E**
- BAX/BAK DKO

**Figure F**
- TMRM low cells (%)
- % depolarization

**Figure G**
- Cyt c release

**Figure H**
- Untr
- cBID

**Figure I**
- Cell death (Annexin V+ cells, % of GFP)

**Figure J**
- IP CLPB
- Neg control CLPB
- OPA1
venetoclax, and they were more significant in mitochondria lacking CLPB compared with wild-type (Fig. 4A and B; Supplementary Fig. S7D). The structural perturbations of CLPB-ablated mitochondria were accompanied by a prominent accumulation of short OPA1 forms, indicating excessive OPA1 processing, as demonstrated by Western blotting (Fig. 4C and D). These phenomena were also observed in cells deficient for BAX and BAK, which do not respond to cell death stimuli, suggesting that the mitochondrial morphologic defects are not a simple result of undergoing apoptosis (Fig. 4E). In addition, TMRM staining after venetoclax treatment indicated an elevated percentage of cells with depolarized mitochondria in AML cells lacking CLPB, suggesting that CLPB-knockout cells are more prone to venetoclax-induced mitochondrial depolarization (Fig. 4F; Supplementary Fig. S7E). To expand on these results, we performed a “BH3 profiling” in which permeabilized sgCLPB- and sgRosa-expressing AML cells were treated with increasing doses of BH3 peptides, and mitochondrial membrane potential was monitored over time using the JC-1 dye. As expected, CLPB-deficient AML cells undergo faster depolarization and are more primed for cell death than wild-type counterparts upon treatment with the BH3-only “activators” BIM and BID (Fig. 4G; Supplementary Fig. S7F–S7G). Given the wider cristae and the higher sensitivity to mitochondrial membrane potential loss upon apoptosis stimulation, we speculated that CLPB-deficient mitochondria would readily release cytochrome c following a death stimulus. To address this hypothesis, we isolated functional mitochondria from wild-type and CLPB-knockout THP-1 cells, stimulated them with the recombinant proapoptotic caspase-8 cleaved BID (cBID), and centrifuged them to retrieve both the pellet (mitochondria) and supernatant (released factors) fractions. Western blot analysis indicated that CLPB-deficient mitochondria are more sensitive to cytochrome c release upon cBID stimulation compared with the wild-type organelles (Fig. 4H). All of the above suggest an increased responsiveness of CLPB-ablated AML cells to programmed cell death. Indeed, immunoblotting against pro- and cleaved caspase-3 verified a prevalent caspase-3 activation upon BCL2 inhibition in AML cells lacking CLPB relative to the wild-type (Supplementary Fig. S7H). Moreover, Annexin V staining in puromycin-selected AML cells transduced with sgRNAs targeting CLPB confirmed hypersensitivity of these cells to venetoclax compared with the negative controls (Fig. 4I). Taken together, these data suggest that CLPB is an important protein for the maintenance of physiologic mitochondrial cristae, as well as the control of cytochrome c release and ultimately the execution of cell death.

**Figure 5.** CLPB ablation leads to cell growth suppression in AML in vitro and in vivo. **A**, Oxygen consumption rate (OCR), respiration (bar plot), and extracellular acidification rate (ECAR) of wild-type and CLPB-knockout MOLM-13 determined by Seahorse Extracellular Flux Analysis. Data, mean ± SEM (n = 5). **B**, Representative flow cytometry plots showing EdU cell-cycle analysis of wild-type and CLPB-knockout MV4-11 (left). Bar charts, mean percentage of cell populations ± SD (n = 3) in MV4-11 or THP-1 AML cells (right). **C**, Cell growth analysis of AML cells transduced with sgRNAs targeting CLPB or control (sgRosa; mean ± SD, n = 3). **D**, Schematic outline of the mouse model of CLPB dependency in AML. **E**, Bioluminescent images of mice transplanted with MOLM-13 cells transduced with sgRosa (n = 3) or sgCLPB #1 (n = 6). Representative images of two mice per sgRNA construct are shown. The same mice are depicted at each time point. **F**, Quantification of bioluminescence emitted from the whole body of each mouse transduced with sgRosa or sgCLPB #1 construct at the indicated time points. **G**, Flow cytometry analysis of GFP-sgRNA-expressing leukemia cells in the peripheral blood of MOLM-13 leukemia recipient mice at indicated time points. **H**, Kaplan-Meier survival curves of recipient mice transduced with sgRosa and sgCLPB #1 are plotted. The P values were determined using the log-rank Mantel-Cox test. Data with statistical significance are as indicated: *P* < 0.05, **P** < 0.01, ***P*** < 0.001; N.S., not significant.

**CLPB Interacts with the Antiapoptotic Mitochondrial Proteins HAX1 and OPA1**

To investigate how CLPB participates in the preservation of the proper mitochondrial ultrastructure and the regulation of apoptosis, we sought to identify the interacting partners of this chaperonin in human AML cells. We performed immunoprecipitation (IP) of the endogenous human CLPB followed by mass spectrometry (MS) in whole THP-1 cell lysates (Supplementary Fig. S7I). Our analysis uncovered 64 mitochondrial proteins found exclusively in the CLPB pulldown and not in the IgG sample (negative control), indicating their potential association with CLPB. Among the top enriched CLPB interactors, we identified two proteins involved in the regulation of mitochondrial morphology and apoptosis, HAX1 and OPA1 (26, 28), ranked No. 1 and 3, respectively (Supplementary Table S2). Western blotting of the CLPB immunoprecipitation in THP-1 lysates and mouse liver mitochondria confirmed the physical interaction of CLPB with HAX1 and OPA1 (Fig. 4J; Supplementary Fig. S7J–S7K). Altogether, these data suggest that CLPB regulates mitochondrial cristae morphology and apoptosis in AML cells, via its specific interactions with OPA1 and HAX1.
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in leukemia progression in mice receiving CLPB-deficient MOLM-13 cells, as determined by bioluminescent imaging (Fig. 5E and F). FACS analysis of peripheral blood from recipient mice detected a significantly lower percentage of circulating leukemia cells harboring CLPB sgRNAs compared with the negative control (Fig. 5G), which correlated with the significant prolonged survival seen in mice harboring CLPB-deficient MOLM-13 cells (Fig. 5H). These data suggested that CLPB is required for AML maintenance, which is consistent with the physiologic function of CLPB in regulating key mitochondrial biological processes, including cristae structure, membrane potential, and oxidative phosphorylation.

**Loss of CLPB Induces Mitochondrial Stress Response**

To further focus on the underlying mechanisms of CLPB function, we performed RNA-seq in AML cells upon CLPB deletion. We used two CLPB-targeting sgRNAs and profiled cells at days 6 and 8 after CLPB depletion (Fig. 6A). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the differentially expressed genes in both CLPB-deficient MOLM-13 cells (sgCLPB #1 and #2) showed strong enrichment on metabolic pathways, such as biosynthesis of amino acids and one carbon metabolism (Fig. 6B), all consistent with the functions of CLPB in regulating mitochondrial processes (Fig. 5). Ingenuity pathway analysis (IPA) was performed to reveal the upstream transcriptional regulators that are responsible for the transcriptional landscape changes upon CLPB ablation. Interestingly, ATF4, a key transcription factor induced by the integrative stress response (29), ranked first in MOLM-13 cells transduced with any of the CLPB-targeting sgRNAs (Fig. 6C). Indeed, qPCR analysis verified the upregulation of ATF4 and its downstream effector CHOP in AML cells lacking CLPB (Fig. 6D; Supplementary Fig. SBD–S8F). Considering our previous results and given that ATF4 has been identified as a key regulator of the mitochondrial stress response in mammals (30), we speculated that CLPB-deficient AML cells are under mitochondrial stress. To this end, we performed gene-set enrichment analysis (GSEA) using a combined mitochondrial stress expression gene signature extracted from the RNA-seq studies described by Quirós and colleagues (30). Such analysis revealed that genes associated with mitochondrial stress response were indeed strongly enriched in CLPB-deficient MOLM-13 cells at both time points (Fig. 6E; Supplementary Fig. S8A). To functionally confirm the mitochondrial stress in CLPB-deficient AML cells, we measured mitochondrial reactive oxygen species (ROS) upon treatment with the complex III inhibitor antimycin A using MitoSOX Red. CLPB deficiency in MOLM-13 cells led to a significant elevation of the mean MitoSOX fluorescent intensity after antimycin A addition relative to the steady-state levels, indicating greater ROS accumulation inside CLPB-ablated mitochondria upon challenge (Supplementary Fig. S8B), thus demonstrating that AML cells are under mitochondrial stress upon CLPB depletion. Given that proapoptotic BH3-only proteins can be induced by ATF4 (31), we wondered if these pro-death sensitizers were upregulated in response to CLPB depletion. Indeed, PMAIP1, BBC3, and HRK gene expression was significantly upregulated in CLPB-deficient AML cells (Fig. 6F; Supplementary Fig. S8G). In line with this finding, BH3 profiling revealed increased sensitivity of CLPB-ablated cells to the peptide “sensitizers” PUMA and BMFγ, as well as to the MCL1 inhibitor (MS1; Supplementary Fig. S8H). Such findings were also consistent with our previous results showing that CLPB-ablated AML cells are more primed to apoptosis (Fig. 4G and H).

Considering that CLPB-deficient AML cells are under mitochondrial stress and harbor dysfunction in the biosynthesis of several metabolites (Fig. 6B), a more detailed metabolic profiling was performed using mass spectroscopy. Among the 150 metabolites tested, the intensity of 16 substances was significantly changed in CLPB-deficient MOLM-13 cells (Fig. 6G; Supplementary Table S3). Pathway analysis of the metabolomics data revealed significant enrichment in amino acid–related pathways (glycine, serine, and threonine metabolism, arginine and proline metabolism, aminoacyl-tRNA biosynthesis; Fig. 6H). Indeed, the glycine, serine, and threonine biosynthesis is one of the most altered metabolic pathways in cells under mitochondrial stress (30). Notably, the majority of the altered metabolites upon CLPB depletion were strongly correlated with the differentially expressed genes shown in Fig. 6A, highlighting the consistency of our RNA-seq and metabolomics data (Supplementary Fig. S8C). These findings suggest that CLPB deficiency amplifies proapoptotic signals through the induction of the ATF4-mediated mitochondrial stress response.

**CLPB Targeting Overcomes p53-Mediated Venetoclax Resistance and Sensitizes AML Cells to Combined Venetoclax and Azacitidine Treatment**

Stabilization of the p53 protein synergizes with venetoclax in AML (15). Based on data included here and in the
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KEGG pathway analysis

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Activation Z-score

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mRNA expression

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Arginine and proline metabolism

- Aminoacyl-tRNA biosynthesis
- Amino sugar and nucleotide sugar metabolism
- Pyrimidine metabolism
- Cysteine and methionine metabolism
related article by Nechiporuk and colleagues in this issue, p53 deletion can confer resistance to the drug in AML cells (Fig. 1B–E; Supplementary Fig. S1B and S1D–S1F). Therefore, the key question of obvious clinical significance is whether CLPB deletion can sensitize p53-deficient AML cells to venetoclax treatment. To this end, we deleted p53 in MOLM-13 cells using two independent sg RNAs targeting TP53 (Supplementary Fig. S9A). Our studies showed that CLPB-deficient AML cells were more sensitive to venetoclax treatment, even in a TP53-knockout (p53-KO) background (Fig. 7A). More importantly, CLPB deletion was able to fully sensitize p53-deficient MOLM-13 cells to venetoclax treatment by inducing the ATF4-mediated upregulation of proapoptotic sensitizers (Fig. 7B; Supplementary Fig. S9D–S9E). Likewise, CLPB ablation led to a synergistic effect with venetoclax also in an AML cell line with naturally occurring TP53 mutation (KASUMI-1; Supplementary Fig. S9B–S9C). Collectively, CLPB-mediated resensitization of venetoclax-resistant AML cells is p53-independent.

Because resistance to venetoclax monotherapy rapidly ensues in AML (14, 32), multiple combinational therapies for venetoclax have been proposed and tested, some of which are in clinical trials (https://www.cancer.gov/about-cancer/treatment/clinical-trials/intervention/Venetoclax). Among these, the combination of venetoclax with hypomethylating agents (HMA), such as azacitidine, is currently in clinics due to its favorable responses in the clinical trials (33). To investigate if CLPB inhibition can enhance the efficacy of venetoclax and azacitidine combined treatment, we tested the possibility that CLPB depletion can further sensitize AML cells to the combined treatment. Indeed, CLPB-deficient AML cells were negatively selected upon combined treatment at a much faster pace than the DMSO control groups (Fig. 7C), confirming that CLPB ablation can sensitize AML cells to combined venetoclax/azacitidine treatment. Altogether, our findings highlight that loss of the mitochondrial protein CLPB leads to structural and functional defects of mitochondria, hence sensitizing AML cells to apoptosis. Therefore, targeting CLPB synergizes with venetoclax and venetoclax/azacitidine combination in AML in a p53-independent manner (Fig. 7D).

**DISCUSSION**

The recent FDA approval for venetoclax introduces an exciting and novel strategy to target AML. In the initial single-agent venetoclax studies in AML, response rates were modest and rather short-lived (12). Our genome-wide CRISPR/Cas9 screen identified both possible mechanisms of resistance and synthetic lethal combinations with venetoclax in AML, including the targeting of a number of proteins regulating mitochondrial structure. These proteins can control the architecture of both the OMM and the mitochondrial cristae. Interestingly, both these structures are essential for BCL2 family function (5), cytochrome c release, and cell death (34). For the complete release of the cytochrome c stores, the inner mitochondrial membrane needs to be remodeled and the cristae widened by the proteolytic cleavage of OPA1 and the disruption of its complexes, which normally keep two opposing cristae membranes tight (26, 35). Both OMM rupture and cristae remodeling is what we observed by electron microscopy upon venetoclax treatment in AML cells. The changes in mitochondrial architecture could, at least partially, explain the reported impairment in oxidative phosphorylation following BCL2 inhibition in the LSC derived from patients with AML (2). On the opposite side, venetoclax-resistant AML cells display mitochondria with narrow cristae lumen and increased protein levels of the cristae “staple” OPA1, possibly rendering them more resistant to the cytochrome c release upon stimulation.

One of the top-ranked genes revealed by our screen to act synergistically with venetoclax is the mitochondrial chaperonin CLPB. Although a plethora of studies in bacteria and yeast have extensively characterized the homolog of CLPB as a protein chaperone that disaggregates misfolded proteins (36–38), the exact function of the mammalian CLPB has not been investigated thoroughly. Of note, the amino acid sequence of the human CLPB is only about 20% identical to the ortholog in *Escherichia coli* (38). CLPB belongs to the AAA+ ATPase superfamily, whose members act both as general protein chaperones and as targeted proteases that degrade specific substrates. Other mitochondrial AAA+ ATPases, such as YME1L, AFG3L2, ATAD3, and m-AAA protease, participate in the organelle’s protein quality control, mitochondrial protein synthesis, and maintenance of mitochondrial architecture (39–43). Worthington and colleagues, who studied the mutations in CLPB in patients with an autosomal recessive metabolic syndrome, hypothesized a possible role for CLPB in apoptosis, due to its in situ–predicted interaction with HAX1 (44). Our study provides the biochemical evidence of the association of CLPB with HAX1 and uncovers its function in the cell protection from intrinsic mitochondria-mediated apoptosis. Furthermore, our work reveals that CLPB physically interacts with OPA1—possibly as a means to protect it from its proteolytic

**Figure 7.** CLPB targeting overcomes p53-mediated venetoclax resistance and sensitizes AML cells to combined venetoclax and azacitidine treatment. **A,** Synergistic effect of CLPB depletion and BCL2 inhibition in TP53-knockout (KO) MOLM-13 cell lines (two clones, B10 and B11) generated as shown in Supplementary Fig. S9. Plotted are GFP+ percentages measured during 4 days in culture and normalized to day 0 of drug treatment. Negative control (sgRosa) and two independent sgRNAs targeting TP53 (Supplementary Fig. S9A). **B,** IC50 curves of venetoclax in p53 wild-type (WT) and p53-deficient (KO; two clones, B10 and B11) MOLM-13 cells transduced with sgCLPB #1 or sgRosa. Transduced AML cells were selected with puromycin and then treated with venetoclax for 48 hours. Viable cells were measured by CellTiter-Glo. Data, mean ± SD (n = 6 for each group). **C,** Validation of the synergistic effect of CLPB depletion and venetoclax + azacitidine combined treatment using a competition-based survival assay in MOLM-13 (left) and MV4-11 (right) cells. Plotted are GFP+ percentages measured during 6 days (for MOLM-13) or 4 days (for MV4-11) in culture and normalized to day 0 of drug treatment. Negative control (sgRosa) and two independent sgRNAs targeting CLPB are shown in the graphs. Data, mean ± SD (n = 6 for MOLM-13 and n = 4 for MV4-11). **D,** Schematic outline of this study. Targeting the mitochondrial CLPB sensitizes AML cells to venetoclax treatment by (i) promoting apoptotic cristae remodeling and (ii) inducing mitochondrial stress response which will amplify the programmed cell death pathway. Data with statistical significance are as indicated. ***P < 0.001.
Targeting Mitochondria Sensitizes AML to Venetoclax

A

\[ \text{p53-KO-MOLM-13 (B10)} \]

\[ \text{p53-KO-MOLM-13 (B11)} \]

\[ \text{p53-WT-MOLM-13} \]

\[ \text{% GFP + cells} \]

\[ \text{% Viable cells} \]

\[ \text{Days after drug treatment} \]

\[ \text{IC_{50} of venetoclax} \]

\[ \text{Drug resistance CLPB high} \]

\[ \text{Drug sensitization CLPB low} \]

\[ \text{Cristae remodeling Mitochondrial stress} \]

\[ \text{Proapoptotic sensitizers} \]

\[ \text{Activation of executioner caspases} \]

\[ \text{Resisting cell death} \]

\[ \text{Cytochrome c} \]

\[ \text{Activation} \]

\[ \text{Cytochrome c} \]

\[ \text{Activation of executioner caspases} \]

\[ \text{Cell death} \]

\[ \text{Nucleus} \]

\[ \text{BAX BAX} \]

\[ \text{BCL2} \]

\[ \text{BAX BAX} \]

\[ \text{ATF4} \]

\[ \text{BAX BAX} \]
cleavage—and thus demonstrates a role for CLPB in the maintenance of the physiologic mitochondrial structure. Loss of CLPB results in abnormal mitochondria with wider cristae than the normal; hence, such organelles are more prone to release their cytochrome c following a death signal, such as venetoclax treatment. Our work proposes a specific CLPB dependency of AML cells. Its depletion leads to cell-cycle suppression, possibly due to the defects in both oxidative phosphorylation and glycolysis. As a consequence of the mitochondrial structural and functional impairment, cells lacking CLPB are more sensitive to oxidative stress. As a response, the CLPB-deficient AML cells alter their transcriptional program and reprogram their metabolism in an ATF4-dependent manner, thus leading to cell-cycle arrest and enhancement of apoptotic pathways. Overall, we show that CLPB is most likely required for AML progression and, importantly, can synergize with venetoclax to induce rapid cell death both in vitro and in vivo.

Recently, Pollyea and colleagues demonstrated that the FDA-approved combined treatment of venetoclax with azacitidine disrupts the tricarboxylic acid cycle in LSCs of patients with AML and that relapsed patients with AML remodel their metabolism (45, 46). These studies support the notion that targeting mitochondrial functions concomitantly with venetoclax and azacitidine could have a clinical relevance for patients with AML. In line with this idea, we show that depleting CLPB, which affects the cellular metabolic status of the AML cells, sensitizes them to the combined treatment in a p53-independent way. Interestingly, a bacterial CLPB inhibitor has been developed and proposed to be utilized as an antimalarial agent (47). Because this inhibitor targets the conserved domain of the bacteria and the human orthologs (44) and displays moderate toxicity in human cell lines (47), it could synergize with venetoclax treatment in AML cells. In general, targeting mitochondrial proteins that would promote apoptotic cristae remodeling should be investigated as a potential way to overcome venetoclax resistance in AML, in addition to ongoing trials targeting other BCL2 family members, such as MCL1 (R. Tibes, personal communication), either directly with novel MCL1 inhibitors, or indirectly via cyclin-dependent kinase inhibition affecting MCL1 transcript expression, as we and others have proposed (11, 16). In conclusion, targeting CLPB and possibly its interactome in combination with venetoclax treatment directly promotes the apoptotic cristae remodeling and indirectly induces mitochondrial stress responses, which will both amplify the programmed cell death pathway and suppress the growth of AML cells. Our data open a potential avenue for novel venetoclax drug combinations in AML.

**METHODS**

**Cell Lines and Cell Culture**

All human leukemia cells (MOLM-13, THP-1, MV4-11, CuTLL-1, Nalm-6, and K562) were cultured in RPMI medium supplemented with 10% FBS and 1% penicillin and streptomycin. The adherent cell lines HeLa and HEK293T were grown in DMEM with 10% FBS and 1% penicillin and streptomycin. SK-MEL-239 cells were cultured in RPMI, 10% FBS, and 1% penicillin and streptomycin. Cas9-expressing cell lines transduced with retroviral Cas9-2A-blast (Addgene, plasmid no. 73310) were selected with blasticidin (InvivoGen) 48 hours after transduction. All transfections were performed in HEK293T cells using polyethyleneimine reagent at 4:2:3 ratios of sgRNA or shRNA construct (Supplementary Table S4: pSVG: pPAX2 in OPTI-MEM solution. Viral supernatant was collected 36 and 48 hours after transfection. Spin infections were performed at room temperature at 1,500 × g for 30 minutes with polybrene reagent (1:2,000; Fisher Scientific).

For the generation of the venetoclax-resistant cell lines, MOLM-13 and MV4-11 cells were cultured in media containing increasing doses of venetoclax (from 5 to 1,000 nmol/L) for more than 8 weeks to achieve complete resistance to the drug.

Cell line information is as follows: MOLM-13, DSMZ, ACC554; THP-1, ATCC, TIB-202; MV4-11, ATCC, CRL-9591; CuTLL-1, Adolfo Ferrando Lab; Nalm-6, ATCC, CRL-3273; K562, ATCC, CCL-243; HEK293T, ATCC, CRL-1573; SK-MEL-239, Eva Hernando Lab; and HeLa, ATCC, CCL-2.

All cell lines were determined negative for Mycoplasma, as indicated by the latest Mycoplasma test (December 2018) using the LookOut Mycoplasma PCR Detection Kit (Sigma). Cells were used for experiments within 15 to 20 passages from thawing.

**CRISPR Screen**

Cas9-expressing MOLM-13 cells were transduced with the Brunello sgRNA library (18 virus) at a low multiplicity of infection (~0.3). On day 2 after transduction, GFP percentage was assessed to determine infection efficiency and sgRNA coverage (~1,000x). Then, puromycin (1 μg/mL) was added for 5 days to select infected (GFP+) cells. After selection, viable infected cells were isolated by Histopaque 1077 (Sigma-Aldrich) and grown without antibiotics for 2 days. After recovery, 100 × 10^6 infected cells were cultured with 10 nmol/L venetoclax (Selleckchem) or DMSO. Another 100 × 10^6 cells were used for genomic DNA (gDNA) extraction and served as an initial reference (day 0 of drug treatment). The concentration of venetoclax was increased during the screen (from 10 nmol/L to 2 μmol/L) to avoid spontaneous gain of drug resistance. For each passage, 100 × 10^6 cells were placed back into culture until 16 days after drug treatment. gDNA of cells containing ~1,000X coverage was harvested on days 8 and 16 after drug treatment using a Qiagen DNA kit according to the manufacturer’s protocol. Further details on library construction and data analysis can be found in the Supplementary Data.

**Drug Treatment and IC_{50} Measurements**

Cells were plated in 96-well plates and exposed to venetoclax (Selleckchem) at concentrations ranging from 0.4 to 300 nmol/L (for MOLM-13 and MV4-11) or 150 nmol/L to 10 μmol/L (for venetoclax-resistant cell lines and THP-1) with a minimum of three technical replicates per concentration per cell line. Cell viability was measured with the CellTiter-Glo reagent (Promega) according to the manufacturer’s instructions. Absolute viability values were converted to percentage viability versus DMSO control treatment, and then nonlinear fit of log(inhibitor) versus response (three parameters) was performed in GraphPad Prism v7.0 to obtain the IC_{50} values. For venetoclax and azacitidine combined treatment, two drugs were mixed at the fixed ratio (ven:aza = 1:4) before diluting to certain concentrations.

**Transmission Electron Microscopy**

Cultured cells were fixed in 0.1 mol/L sodium cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde and 2% paraformaldehyde for 2 hours and post-fixed with 1% osmium tetroxide and 1% potassium ferrocyanide for 1 hour at 4°C. Then, block was stained in 0.25% aqueous uranyl acetate, processed in a standard manner, and embedded in EMbed 812 (Electron Microscopy Sciences). Ultrathin

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sections (60 nm) were cut, mounted on copper grids, and stained with uranyl acetate and lead citrate. Stained grids were examined under Phillips CM-12 electron microscope and photographed with a Gatan (4k × 2.7k) digital camera. For morphometric analysis, maximal cristae width was measured using ImageJ (NIH) as shown in Supplementary Fig. S2A in at least 50 randomly selected mitochondria from a minimum of 15 cells/experiment.

Mitochondrial Respiration
Oxygen consumption rate (pmol/min) was determined using the XFe24 Extracellular Flux Analyzer and the XF CitoMito Stress Test kit (Agilent). Details can be found in the Supplementary Data.

Mitochondrial Membrane Potential
Mitochondrial membrane potential was monitored cytofluorometrically using TMRM. Cells were loaded with 10 nmol/L TMRM in the presence of 2 μmol/L cyclosporin H in Hank’s Balanced Salt Solution supplemented with 10 mmol/L HEPES, 30 minutes at 37°C. When indicated, the experiments were performed in cells pretreated with venetoclax for 16 hours at the indicated concentrations.

Metabolomics
Cells (7 × 10⁶) were flash-frozen as pellets and processed using the LC/MS-MS with the hybrid metabolomics method. Details can be found in the Supplementary Data.

Animal Experiments
For in vivo experiments, Cas9 and luciferase-expressing parental and venetoclax-resistant MOLM-13 cells were transduced with sgRosa or sgCLBP #1 constructs. At day 2 after transduction, sgRNA-positive cells (GFP-marked) were sorted by FACS. A half million sgRNA-expressing leukemia cells were intravenously injected into recipient NSG mice. For experiments that required drug administration, recipient mice were treated with vehicle or venetoclax (100 mg/kg; Selleckchem) daily by oral gavage from days 6 to 20. Venetoclax was prepared in 50 propylene glycol (Lipoid). Whole-body bioluminescent imaging was performed after 5 minutes using an IVIS imager. Bioluminescent Microscopy Lab for their help in acquiring and analyzing the data presented; and NYU High Throughput Biology Laboratory, which is partially supported by the Laura and Isaac Perlmutter Cancer Center Support Grant (NIH/NCI P30CA16087) and NYSSTEM Contract C026719. The proteomics studies were supported in part by the NYU School of Medicine and the Laura Isaac Perlmutter Cancer Center Support Grant P30CA16087 from the NCI. The Orbitrap Fusion Lumos mass spectrometer used in this study was purchased with a shared instrumentation grant (1S10OD010582-01A1) from the NIH. Also, we thank C. Quinn and L. Scorrano laboratory for the recombinant eBD. Finally, we thank L. Pernas and M.E. Somano for critical reading of the manuscript. I. Aifantis was supported by the NIH and the NCI (R01CA173636, R01CA216421, R01CA133379, and R01CA169784), the Leukemia and Lymphoma Society (TRP#6340-11 and TRP#6340-12) and NCI grant R01-CA17897. R. Tibes was supported by the New York State Department of Health (#CO030132, C32587GG, and C32563GG). R. Tibes was a principal investigator in the work; K. Dancel-Manning and K. Petzold, and S. S. Zhou, and Y. Gong, E. Gavathiotis, R. Tibes, I. Aifantis. Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Chen, C. Glytsou, H. Zhou, Y. Gong. Study supervision: X. Chen, C. Glytsou, I. Aifantis.

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Targeting Mitochondrial Structure Sensitizes Acute Myeloid Leukemia to Venetoclax Treatment

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