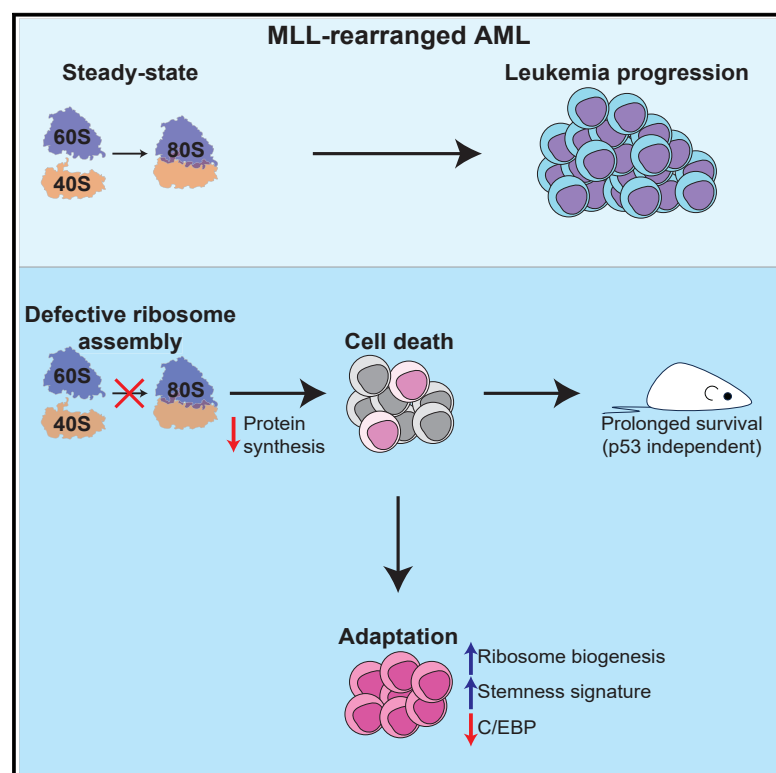


Defective ribosome assembly impairs leukemia progression in a murine model of acute myeloid leukemia

Graphical abstract



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In brief

Sjövall et al. report that defective ribosome assembly impairs leukemia progression in murine AML independent of p53. Leukemia cell adaptation to defective ribosome assembly is associated with the upregulation of ribosome biogenesis and the downregulation of the C/EBP transcription factor family. These findings highlight ribosome assembly as a potential therapeutic target in AML.

Highlights

- Immature AML cells show relatively high ribosome biogenesis rates
- Defective ribosome assembly prolongs survival in mice with MLL-AF9 AML
- Defective ribosome assembly triggers a global transcriptional adaptation response
- Defective ribosome assembly shows antileukemia efficacy in p53-deficient AML



Article

Defective ribosome assembly impairs leukemia progression in a murine model of acute myeloid leukemia

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SUMMARY

Despite an advanced understanding of disease mechanisms, the current therapeutic regimen fails to cure most patients with acute myeloid leukemia (AML). In the present study, we address the role of ribosome assembly in leukemia cell function. We apply patient datasets and murine models to demonstrate that immature leukemia cells in mixed-lineage leukemia-rearranged AML are characterized by relatively high ribosome biogenesis and protein synthesis rates. Using a model with inducible regulation of ribosomal subunit joining, we show that defective ribosome assembly extends survival in mice with AML. Single-cell RNA sequencing and proteomic analyses reveal that leukemia cell adaptation to defective ribosome assembly is associated with an increase in ribosome biogenesis and deregulation of the transcription factor landscape. Finally, we demonstrate that defective ribosome assembly shows antileukemia efficacy in p53-deficient AML. Our study unveils the critical requirement of a high protein synthesis rate for leukemia progression and highlights ribosome assembly as a therapeutic target in AML.

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous hematologic malignancy characterized by clonal expansion of aberrant myeloid precursor cells and bone marrow (BM) failure. Despite significant advances in the understanding of AML disease mechanisms, cytarabine- and anthracycline-based regimens have remained a standard of care in AML.¹ These regimens induce high rates of remission but in most cases fail to eradicate leukemia stem cells (LSCs) that ultimately underlie disease relapse. There is, therefore, an unmet clinical need for novel therapeutic strategies that target LSCs.

Manipulation of the protein synthesis machinery is emerging as a promising treatment strategy in AML. The protein synthesis inhibitor homoharringtonine has been used for decades in China in the treatment of AML, and it is also approved by the United States Food and Drug Administration in the treatment of tyrosine kinase inhibitor-resistant chronic myeloid leukemia.² More recently, the RNA polymerase I inhibitor CX-5461, which inhibits ribosomal DNA transcription, was shown to outperform standard chemotherapies in mouse models of AML and is currently being

investigated in phase 1 clinical trials.^{3,4} Finally, strategies targeting signal transduction pathway components including FLT3 receptor tyrosine kinase indirectly inhibit ribosome biogenesis and protein synthesis.⁵

Despite the promise of protein synthesis machinery as a target in AML, the cellular and molecular mechanisms that define the response of leukemia cells to ribosomal insults remain incompletely understood. In particular, the difficulty of modeling ribosomal dysfunction in mice has presented a significant barrier for the validation of the role of ribosome assembly in AML *in vivo*. In the present study, using a mouse model that enables inducible, graded, and stable regulation of ribosomal subunit joining, we demonstrate that defective ribosome assembly impairs leukemia progression in mouse models of AML.

RESULTS

Immature AML cells exhibit higher ribosome biogenesis rates than mature cells

To approach alterations in ribosome biogenesis in AML, we applied the ribosome biogenesis cell score (RiBi score) to a



single-cell RNA sequencing (scRNA-seq) dataset of pediatric AML.⁶ This strategy revealed that immature leukemia cells in mixed-lineage leukemia (MLL)-rearranged AML and t(8;21) and inv(16) core binding factor AMLs have higher RiBi scores compared with mature leukemia cells (Figure 1A). Next, we applied the RiBi score to an independent scRNA-seq dataset, which allowed us to compare healthy and malignant hematopoietic stem and progenitor cells (HSPCs) within individual patients with AML.⁷ We observed that malignant progenitor and granulocyte-monocyte progenitor (GMP) cells, but not HSCs, show significantly higher RiBi scores compared with their healthy counterparts in approximately half of the cases, including three out of four cases with *FLT3* mutations (Figure 1B). When pooled together, these data suggest that healthy progenitor cells exhibit overall higher RiBi scores compared with healthy HSCs and GMPs, while GMP-like cells exhibit the highest RiBi scores among the malignant cells (Figure 1C).

We next applied a clinically relevant retroviral mouse model of MLL-AF9 AML to directly measure protein synthesis rates in AML.⁹ While the immunophenotype and relative frequency of LSCs in this model remain a topic of scientific discussion, it is known that immature leukemia cells can be enriched based on the presence of the c-Kit receptor.^{10,11} Consistently, c-Kit+ leukemia cells produced more colonies and showed higher proliferation compared with c-Kit− cells (Figures S1A and S1B). To quantify the protein synthesis rate in MLL-AF9 AML, we performed O-propargyl-puromycin (OP-Puro) labeling experiments in leukemia-engrafted mice (Figure 1D). OP-Puro is a puromycin analog that is incorporated into nascent polypeptides, which can be quantified using flow cytometry.¹² These experiments demonstrated that c-Kit+ leukemia cells represent one of the most highly translating cell types in the BM, incorporating six and three times more OP-Puro compared with c-Kit− leukemia cells and normal CD11b+ myeloid cells, respectively (Figures 1E and 1F). In contrast to c-Kit+ leukemia cells, c-Kit− leukemia cells displayed a bimodal distribution of OP-Puro incorporation, comprising a major OP-Puro^{low} subset and a minor OP-Puro^{high} subset.

Defective ribosome assembly prolongs survival in leukemia-engrafted mice

To experimentally assess the significance of a high protein synthesis rate on leukemia cell function, we took advantage of a transgenic eukaryotic initiation factor 6 (eIF6) mouse strain.¹³ These mice express human *EIF6* transgene by a doxycycline (Dox)-responsive promoter, allowing inducible overexpression of eIF6 (Figure 2A). Given its ability to bind the intersubunit face of 60S ribosomal subunits, eIF6 functions as a ribosome anti-association factor that prevents the joining of 60S and 40S subunits into translation-competent 80S ribosomes.^{13,14} eIF6 overexpression therefore sequesters 60S subunits in the cytoplasm, disrupts ribosomal subunit joining, and attenuates protein synthesis.¹³

We isolated BM cells from mice with two copies of the *M2-reverse tetracycline transactivator* (*M2-rtTA*) transgene and one copy of the *EIF6* transgene and transduced them with MLL-AF9-GFP retrovirus to generate AML where the expression of *EIF6* transgene is Dox inducible. To evaluate *EIF6* transgene

expression, we isolated c-Kit+ leukemia cells from Dox-treated leukemia-engrafted mice and performed quantitative reverse-transcription PCR (RT-qPCR) to measure *EIF6* mRNA. Using two sets of primers to distinguish endogenous mouse *Eif6* mRNA from total *Eif6+EIF6* mRNA, we observed a 7.1-fold increase in *EIF6* mRNA compared with controls (Figure 2B). To assess the impact of eIF6 overexpression on ribosome assembly, we cultured c-Kit+ leukemia cells in the presence of Dox and profiled cell extracts by sucrose gradient sedimentation. Consistent with a ribosomal subunit joining defect, eIF6 overexpression led to increased levels of free ribosomal subunits, with a concomitant shift in polysome distribution toward the lighter polysomes (Figures 2C–2E). Immunoblotting of fractionated cell extracts confirmed an increase in eIF6 protein abundance, both in the ribosome-free fraction and in association with 60S subunits (Figure 2F).

Next, we assessed the impact of defective ribosome assembly on leukemia progression. We transplanted irradiated recipient mice with leukemia cells and administered them Dox to induce eIF6 overexpression (Figure 2G). Of note, Dox administration was initiated 1 day after transplantation to avoid the confounding effects of eIF6 overexpression on leukemia cell engraftment. At 2 weeks after transplantation, Dox-treated mice showed, on average, a 65% decrease in white blood cell count in the peripheral blood compared with controls (Figure 2H). This outcome was associated with a 30% decrease in the frequency of GFP+ leukemia cells (Figure 2I). Consistent with a reduced leukemia burden, eIF6 overexpression significantly prolonged survival in leukemia-engrafted mice (median survival of 15.5 and 24 days in control and Dox groups, respectively) (Figure 2J). Similar experiments using MLL-AF9 AML without the *EIF6* transgene revealed that Dox alone had a significant but considerably milder effect on leukemia progression (Figures S1C and S1D).

To assess the impact of eIF6 overexpression on c-Kit+ leukemia cells and their progeny, we harvested BM cells from mice that were administered Dox for 5 days and quantified leukemia cells by flow cytometry (Figure S1E). In the BM, eIF6 overexpression had no significant impact on cellularity or the frequency of GFP+ leukemia cells but led to a significant reduction in the frequency of c-Kit+ cells within the GFP+ leukemia cell compartment (Figures 2K and 2L). Spleen analysis revealed a significant decrease in spleen weight in Dox-treated mice and a reduction in both GFP+ leukemia cell and c-Kit+ leukemia cell fractions, although not statistically significant (Figures S1F and S1H).

Severe ribosome assembly defect eradicates most leukemia cells

The significant impact of eIF6 overexpression on leukemia progression prompted us to assess whether a more severe ribosome assembly defect was sufficient to eradicate the disease. To this end, we generated MLL-AF9 AML using BM from mice with two copies of *M2-rtTA* and *EIF6* transgenes. The additional copy of the *EIF6* transgene in this AML model increased the level of total *EIF6* mRNA overexpression to 12.2-fold compared with 7.1-fold in the model with one copy (Figures S2A and 2B). Consistent with a dose-dependent effect of eIF6 on ribosomal subunit joining,¹³ high eIF6 overexpression caused a rapid collapse of polysomes (Figures S2B and S2C). The induction of

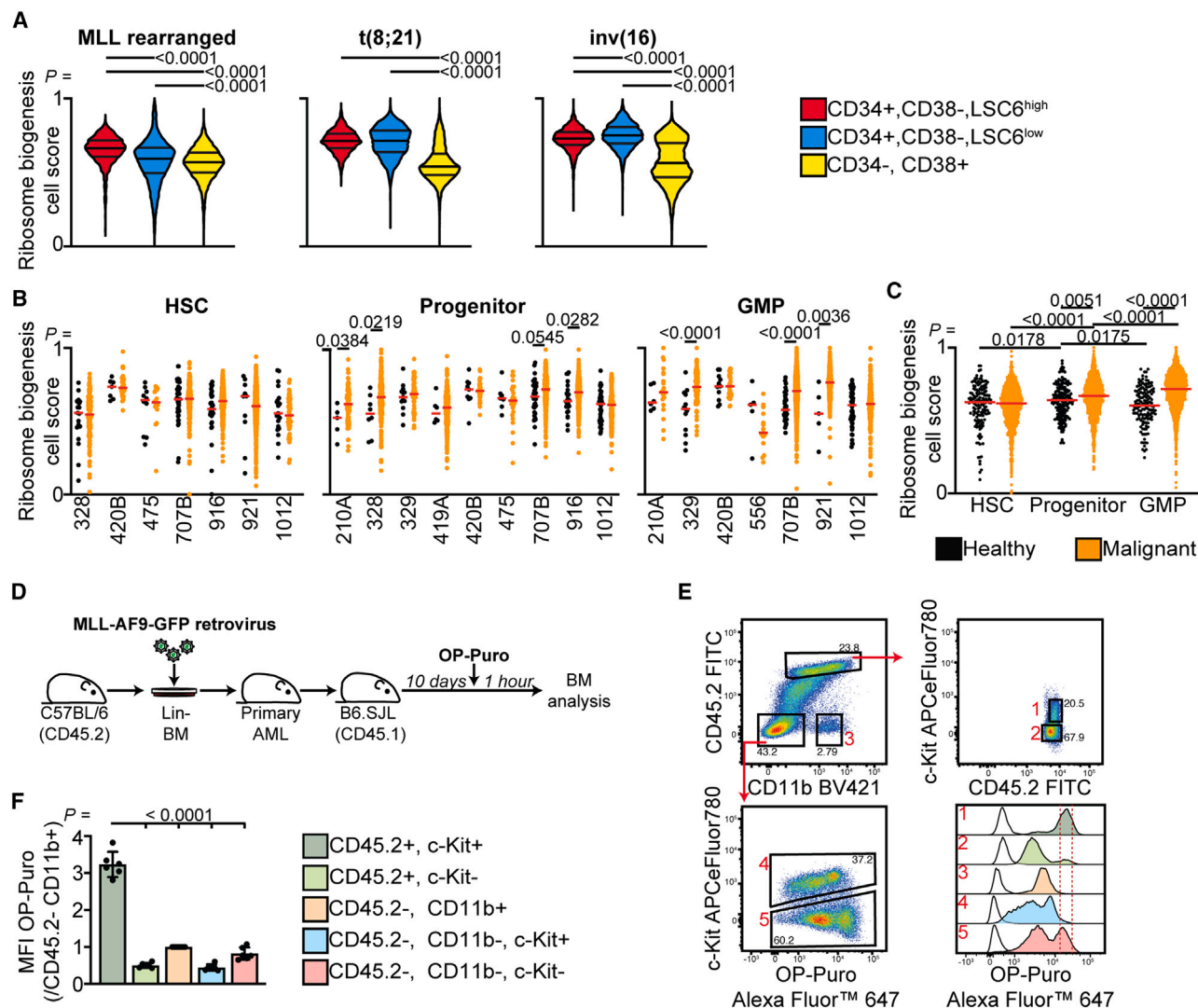


Figure 1. Immature leukemia cells in AML exhibit relatively high ribosome biogenesis rates

(A) Ribosome biogenesis cell scores in distinct leukemia cell subsets in scRNA-seq dataset of human pediatric AML.⁶ LSCs, which were identified using the LSC6 score as previously described,^{6,8} colocalized with CD34⁺CD38⁻ cells across the three AML subgroups. The ribosome biogenesis cell score, which reflects the ribosome biogenesis rate, was calculated using the average expression of 307 ribosome biogenesis genes (see Table S6). The center line represents the median, and the outer lines represent the two quartiles.

(B) Ribosome biogenesis cell scores in healthy and malignant HSCs, progenitors, and GMPs within individual patients in scRNA-seq dataset of human AML.⁷ The cell type and disease state annotations were obtained from the original study, where healthy and malignant cells were distinguished from each other by integrating scRNA-seq and genotyping data using a machine learning classifier.⁷ Cell type annotation was then performed using unsupervised clustering of scRNA-seq data and merging of clusters based on marker gene expression, including but not limited to CD34⁺CD38⁻ (HSCs), CD34⁺CD38⁺ (progenitors), and CD34^{low}CD38⁺MPO⁺ELANE⁺ (GMPs).⁷ Patients 210A, 328, 329, and 419A harbor mutations in the *FLT3* gene. The central red bar represents the median, and the dots represent individual cells.

(C) Ribosome biogenesis cell scores in healthy and malignant HSCs, progenitors, and GMPs across the cases shown in (B). The central red bar represents the median, and the dots represent individual cells.

(D) Schematic overview of the O-propargyl puromycin (OP-Puro) labeling experiments.

(E) Representative flow cytometry gating strategy to quantify OP-Puro incorporation in the BM of leukemia-engrafted mice. CD45.2 was used to identify leukemia cells due to the loss of GFP fluorescence during cell fixation and permeabilization. The white curves represent BM of PBS-injected control mouse.

(F) Median fluorescence intensity (MFI) of OP-Puro normalized against CD45.2⁻/CD11b⁺ host myeloid precursor cells. The data represent two independent experiments with a total of six animals per group. The data represent mean $\pm$ standard deviation.

Student's t test was used to determine statistical significance in (A), (B), (C), and (F). Two-tailed *p* values are shown. HSC, hematopoietic stem cell; GMP, granulocyte-monocyte progenitor; BM, bone marrow.

See also Figure S1.

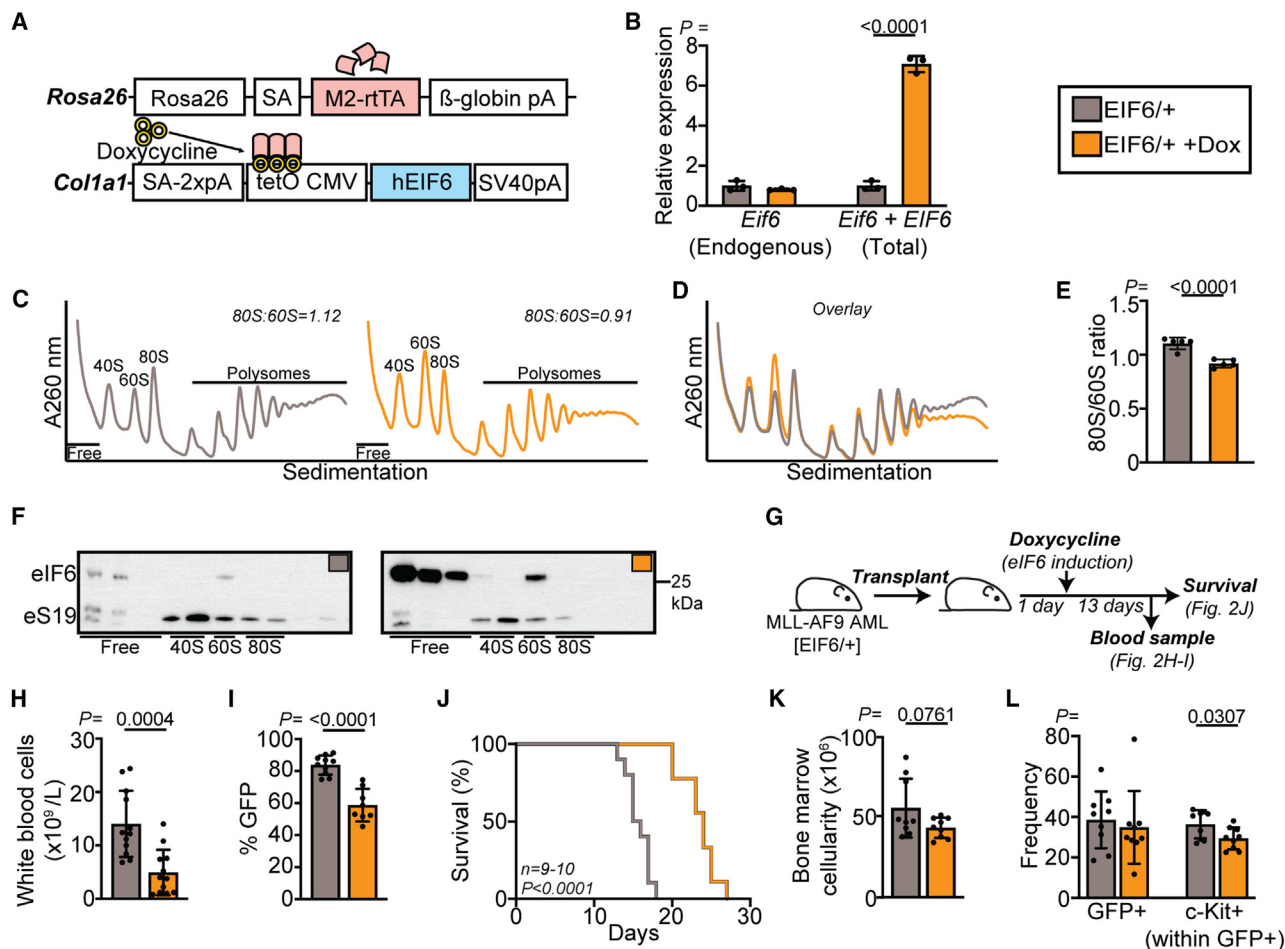


Figure 2. Enforced expression of eIF6 impairs ribosomal subunit joining and prolongs survival in mice with MLL-AF9 AML

(A) Schematic overview of transgenic eIF6 mouse strain. *M2-reverse tetracycline transactivator (M2-rtTA)* is constitutively expressed at the *Rosa26* locus. This permits doxycycline (Dox)-inducible expression of the human *EIF6* transgene downstream of the *Col1a1* locus.

(B) RT-qPCR analysis of endogenous murine *Eif6* and total murine *Eif6* + transgenic *EIF6* mRNA in c-Kit⁺ leukemia cells. Cells were harvested from leukemia-engrafted mice that were administered Dox for 5 days. $n = 3$ biologically independent samples per group. The data represent mean $\pm$ standard deviation.

(C and D) Sucrose gradient sedimentation of extracts from cultured c-Kit⁺ leukemia cells. Dox induction, 16 h. Data shown are representative of five independent experiments.

(E) Quantification of the 80S:60S ratios shown in (C) ($n = 5$ per group). The data represent mean $\pm$ standard deviation.

(F) Immunoblot analysis of eIF6 and eS19 (RPS19) in fractionated extracts. The image has been cropped to show only the lanes corresponding to free fractions, 40S and 60S subunits, 80S monosomes, and light polysomes. Data shown are representative of two independent experiments.

(G) Experimental strategy to assess the impact of eIF6 overexpression on leukemia progression. 50,000 serial transplanted leukemic BM cells were transplanted into sublethally irradiated recipient mice. One day after transplantation, half of the engrafted mice were administered Dox to induce *EIF6* transgene expression.

(H) Number of white blood cells in the peripheral blood. $n = 12$ animals per group. The data represent mean $\pm$ standard deviation.

(I) Frequency of GFP⁺ cells within the total CD45⁺ fraction in the peripheral blood. $n = 10$ and 8 animals per group. The data represent mean $\pm$ standard deviation.

(J) Survival of leukemia-engrafted mice. $n = 10$ and 9 animals per group.

(K) BM cellularity. 50,000 serial transplanted leukemic BM cells were transplanted into sublethally irradiated recipient mice. Five days after transplantation, half of the engrafted mice were administered Dox, and all mice were analyzed on day 10. $n = 9$ animals per group. The data represent mean $\pm$ standard deviation.

(L) Frequencies of GFP⁺ leukemia cells and of c-Kit⁺ cells within the GFP⁺ leukemia cells in the BM. $n = 9$ animals per group. The data represent mean $\pm$ standard deviation.

Student's *t* test was used to determine statistical significance in (B), (H), (I), (K), and (L). A paired samples *t* test was used in (E). Two-tailed *p* values are shown. Log-rank (Mantel-Cox) test was used to determine statistical significance in (J). See also Figure S1.

high eIF6 overexpression in leukemia-engrafted mice further extended the median survival to 31.5 days compared with 24 days in the model with one *EIF6* transgene (Figure 3A). A short-term induction of high eIF6 overexpression led to a significant reduction in BM cellularity that was associated with an 83%

decrease in the frequency of GFP⁺ leukemia cells (Figures 3B–3D). The frequency of c-Kit⁺ cells within the GFP⁺ compartment was reduced by 62% (Figure 3D). Moreover, the frequency of GMP-like leukemia cells (L-GMPs), which are reported to contain a population of LSCs,¹¹ was reduced by 89% (Figures S2D and

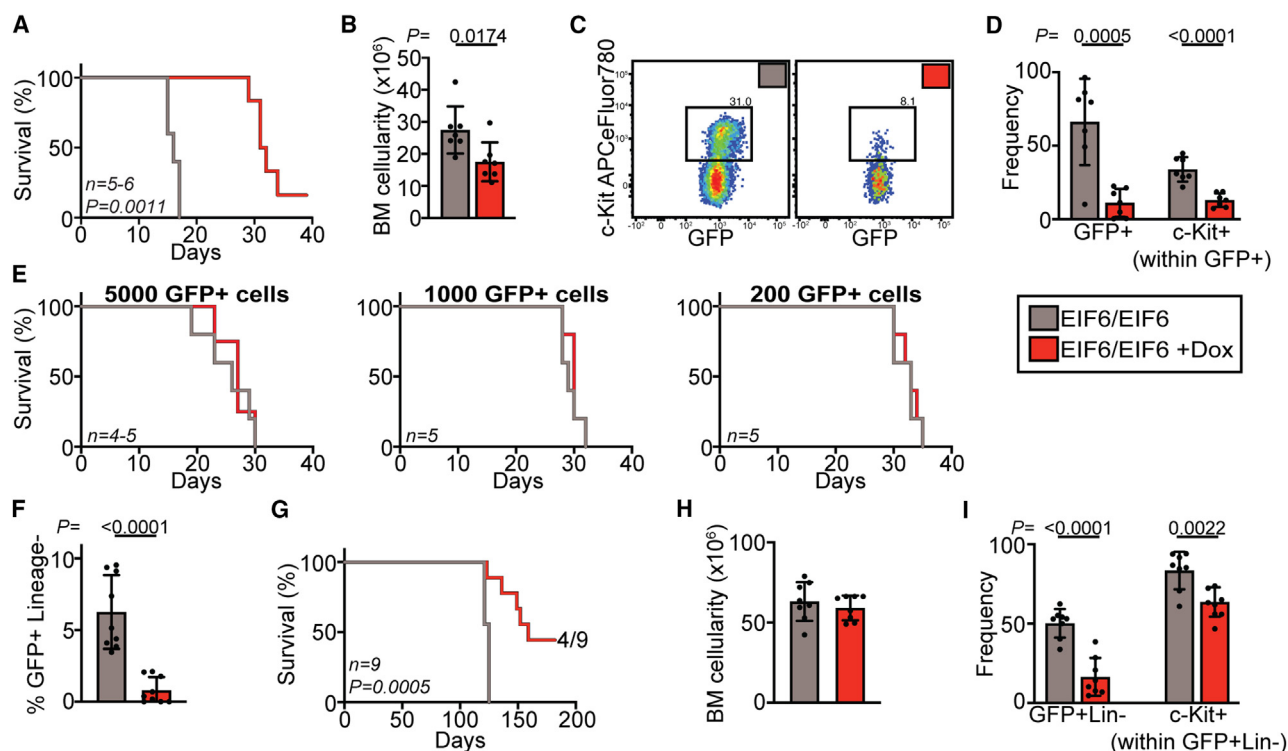


Figure 3. A severe ribosome assembly defect eradicates most leukemia cells

(A) Survival of leukemia-engrafted mice. 100,000 serial transplanted leukemic BM cells were engrafted into sublethally irradiated recipient mice. One day after transplantation, half of the mice were administered Dox to induce *EIF6* transgene expression. $n = 5$ and 6 animals per group.

(B) BM cellularity. 100,000 serial transplanted leukemic BM cells were engrafted into sublethally irradiated recipient mice. Five days after transplantation, half of the mice were administered Dox, and all mice were analyzed on day 10. $n = 7$ animals per group. The data represent mean $\pm$ standard deviation.

(C) Representative flow cytometry plots of GFP and c-Kit.

(D) Frequencies of GFP+ leukemia cells and of c-Kit+ cells within the GFP+ leukemia cells in the BM. $n = 7$ animals per group. The data represent mean $\pm$ standard deviation.

(E) Survival of mice transplanted with 200, 1,000, or 5,000 GFP+ leukemia cells. GFP+ leukemia cells were isolated from the BM of primary recipients that were induced with Dox for 5 days as in (B)–(D), followed by transplantation into sublethally irradiated secondary recipient mice in the absence of Dox.

(F) Frequency of GFP+ lineage- cells in the peripheral blood in mice with AML1-ETO9a AML. 650,000 BM cells isolated from a primary recipient mouse with terminal AML1-ETO9a AML were transplanted into sublethally irradiated secondary recipient mice. Fifteen days after transplantation, half of the recipient mice were administered Dox to induce *EIF6* transgene expression. Peripheral blood analysis was performed 9 weeks after Dox administration. $n = 9$ animals per group. The data represent mean $\pm$ standard deviation.

(G) Survival of mice with AML1-ETO9a AML. These mice represent the same cohort as described in (F). Three out of four mice that survived until termination of the experiment on day 182 showed no GFP+ cells in the BM (data not shown).

(H) BM cellularity in mice with AML1-ETO9a AML. 650,000 BM cells isolated from a primary recipient mouse with terminal AML1-ETO9a AML were transplanted into sublethally irradiated secondary recipient mice. Nine weeks after transplantation, half of the recipient mice were administered Dox, and all mice were analyzed 5 days later. $n = 10$ animals per group. The data represent mean $\pm$ standard deviation.

(I) Frequencies of GFP+ lineage- leukemia cells and of c-Kit+ cells within the GFP+ lineage- leukemia cells in the BM in mice with AML1-ETO9a AML. The data represent mean $\pm$ standard deviation.

Student's *t* test was used to determine statistical significance in (B), (D), (F), (H), and (I), and two-tailed *p* values are shown. Log-rank (Mantel-Cox) test was used to determine statistical significance in (A), (E), and (G). See also Figures S2–S5.

S2E). No differences were observed in the expression of the Gr-1 surface molecule (Figures S2F and S2G). Spleen analysis revealed a significant reduction in spleen weight that was associated with a decrease in GFP+ and c-Kit+ leukemia cells (Figures S2H and S2I). Consistent with the reduced leukemia burden in response to *EIF6* overexpression, transplantation of 100,000 unfractionated BM cells isolated from Dox-treated mice generated the disease in secondary hosts with significantly longer latency compared with controls (Figures S3A and S3B). Moreover, we observed no difference in the ability of persistent

c-Kit+ leukemia cells to form colonies compared with controls (Figures S3C–S3E). However, despite the reduced frequencies of c-Kit+ leukemia cells and L-GMPs within GFP+ leukemia cells in response to *EIF6* overexpression, we observed no differences in the proportion of leukemic mice or in leukemia latencies when transplanting 200, 1,000, or 5,000 prospectively purified GFP+ leukemia cells into secondary hosts, suggesting that the frequency of LSCs within GFP+ leukemia cells is not altered in response to defective ribosome assembly (Figure 3E). Finally, *EIF6* overexpression also significantly decreased the frequency

of leukemia cells in the peripheral blood and BM and extended survival in a mouse model of t(8;21) AML, the retroviral AML1-ETO9a model,¹⁵ demonstrating that the antileukemia effect of defective ribosome assembly is not restricted to MLL-AF9 AML (Figures 3F–3I).

To assess the impact of eIF6 overexpression on normal hematopoiesis, we performed a non-competitive transplantation experiment using BM isolated from mice with two copies of the *M2-rtTA* and *EIF6* transgenes (Figure S4A). eIF6 overexpression resulted in macrocytic anemia and decreased white blood cell and platelet counts (Figures S4B–S4E). The decrease in the number of white blood cells was associated with a relative decrease in CD19⁺ cells and increase in CD11b⁺ cells (Figure S4F). Moreover, and similar to our previous study,¹³ we also observed a significant decrease in donor reconstitution of CD3⁺ cells in response to eIF6 overexpression (Figure S4G). BM analysis of the recipient mice revealed an increase in total cellularity and a decrease in the frequency of lineage[–] cells (Figures S4H and S4I), presumably due to abnormal accumulation of erythroblasts upon eIF6 overexpression.¹³ Analysis of the immature HSPCs revealed a significant decrease in lineage[–]/Sca-1⁺/c-Kit⁺ (LSK) cells but not in HSCs (Figures S4J–S4M). The number of pre-GM and GMP cells was significantly increased (Figure S4K). To further address the impact of eIF6 overexpression on HSC function, we performed a competitive transplantation experiment by reconstituting lethally irradiated recipient mice with an equal ratio of transgenic eIF6 BM and congenic wild-type BM (Figure S5A). As expected, peripheral blood analysis of untreated recipient mice at 4 weeks demonstrated an average donor reconstitution of approximately 50% (Figures S5B and S5C). Consistent with the non-competitive transplantation experiment, Dox administration led to a significant decrease in donor white blood cell reconstitution compared with non-treated controls (Figures S5D and S5E). However, in contrast to the non-competitive transplantation experiment, Dox-treated mice also showed a significant reduction in the frequency and donor reconstitution of HSCs (Figures S5F and S5G). Taken together, these results indicate that while eIF6 overexpression has a negative effect on HSC donor reconstitution, it does not fully deplete HSCs, suggesting that there is a potential therapeutic window for targeting ribosome assembly in AML.

Defective ribosome assembly triggers global transcriptional adaptation in LSCs

Despite the robust depletion of leukemia cells in response to eIF6 overexpression (Figures 3C and 3D), persistent GFP⁺ leukemia cells were able to propagate the disease in secondary hosts with similar kinetics compared to controls (Figure 3E). These results demonstrate that a subset of leukemia cells can adapt to defective ribosome assembly. To address whether this outcome was due to the selection or adaptation of pre-existing leukemia cell transcriptional states, we performed scRNA-seq of GFP⁺ leukemia cells freshly isolated from leukemia-engrafted mice (*n* = 2 and 3 for control and Dox groups, respectively). We obtained 18,426 high-quality transcriptomes with an average of 4,733 genes detected per cell that were optimally separated into eight cell populations using unsupervised clustering (Figure 4A). Control and eIF6^{high} cells showed overall non-overlap-

ping cluster-association patterns, with clusters 0 and 2 consisting of control cells and clusters 1 and 3 of eIF6^{high} cells (Figures 4A, S6A, and S6B). Together with the uniform manifold approximation and projection (UMAP) visualization, cluster marker analysis suggested that clusters 0 and 1 and clusters 2 and 3 represent pairs of control and eIF6^{high} leukemia cells of two distinct transcriptional states, respectively (Figures 4A and 4B). Profiling of *Kit* and gene signatures that correlate with LSC frequency in murine *MLL*-rearranged AML¹⁶ indicated that these transcriptional states contain LSCs (hereafter, LSC-I for clusters 0 and 1 and LSC-II for clusters 2 and 3), while clusters 4, 5, and 6 represent more differentiated leukemia cells (Figures 4C and 4D). Notably, eIF6^{high} clusters 1 and 3 showed higher LSC scores compared with control clusters 0 and 2, suggesting that adaptation to defective ribosome assembly leads to the upregulation of genes that associate with the LSC identity. Cluster 7 displayed a high LSC score but was also enriched for the chromosome segregation Gene Ontology (GO) category, suggesting that it represents a collection of mitotic cells rather than a distinct LSC state (Figure S6C).

Previous studies have established that MLL-AF9 transforms GMPs by preserving their gene expression program and thus perpetuates this cellular state.^{17–19} The GMP compartment can be resolved into distinct developmental stages based on Ly6C (*Ly6c2*) and CD115 (*Csf1r*) surface markers, including granulocyte progenitors (GP; Ly6C⁺ CD115^{low}) and monocyte progenitors (MPs; Ly6C⁺ CD115^{high}).^{19,20} While in our dataset both LSC states expressed high levels of *Ly6c2*, LSC-II showed higher expression of *Csf1r* compared with LSC-I (Figure 4E), suggesting that LSC-I and LSC-II resemble GPs and MPs, respectively. Consistently, differential gene expression analysis identified genes encoding granulocyte transcription factors (TFs) CCAAT/enhancer binding protein ϵ (C/EBP ϵ) and GFI1 in LSC-I and monocyte TF IRF8 in LSC-II (Figure 4F; Table S1).²¹ Moreover, genes whose expression correlates with *Gfi1* and *Irf8* in normal GMPs, including *S100a8*, *Elane*, *Myb*, *Rassf4*, *Cd34*, and *Ctss*, were also differentially expressed (Figure 4F).²¹ However, the overall number of differentially expressed genes between the two LSC states was limited (81 upregulated and 82 downregulated; adjusted *p* < 0.05, at least 1.5-fold change [FC]). Of note, the proportion of LSC-I cells was reduced from 17% to 11% in response to eIF6 overexpression, suggesting that they may be particularly vulnerable to defective ribosome assembly (Figures 4G and S6D). Taken together, our data demonstrate that adaptation to defective ribosome assembly is due to the global transcriptional reprogramming of LSC states rather than selection for a specific LSC transcriptional state.

Adaptation to defective ribosome assembly associates with increased ribosome biogenesis

To characterize the adaptive transcriptional program induced by defective ribosome assembly, we performed differential gene expression analysis between control and eIF6^{high} cells within the two LSC states (cluster 0 vs. 1 and cluster 2 vs. 3). The transcriptional responses of both LSC states to eIF6 overexpression were highly similar, comprising an upregulation of genes belonging to ribosome biogenesis, translation, peptide biosynthetic process, and RNA processing GO categories (Figures 5A and 5B;

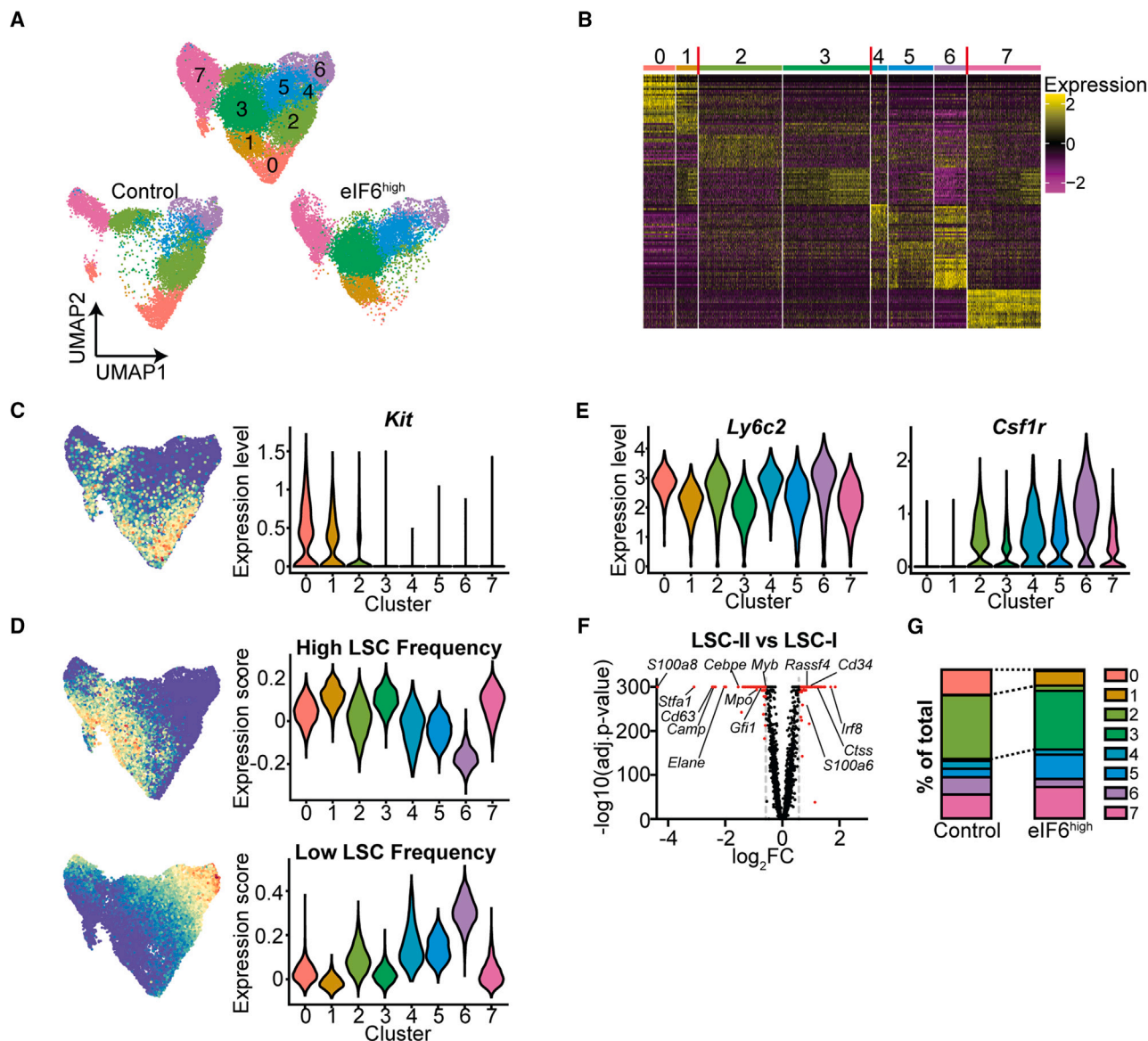


Figure 4. Single-cell transcriptional landscape of MLL-AF9 AML

(A) UMAP plots of unsupervised clustering of GFP+ leukemia cells.

(B) Heatmap of the top twenty marker genes for each cell cluster.

(C) UMAP and violin plots showing the average expression of *Kit*. See Table S7 for statistical significances.

(D) UMAP and violin plots of gene signatures that associate with high or low LSC frequency in mouse models of MLL-rearranged AML.¹⁶ See Table S6 for LSC gene signatures and Table S7 for statistical significances.

(E) Violin plots showing the average expression of *Ly6c2* and *Csf1r*. See Table S7 for statistical significances.

(F) Volcano plot of differential gene expression between LSC-II (clusters 2 + 3) and LSC-I (clusters 0 + 1). Red dots depict differentially expressed genes (adjusted $p < 0.05$, at least 1.5-fold change). See also Table S1.

(G) Average cluster frequency in control and eIF6^{high} groups.

One-way ANOVA with Tukey's multiple comparisons test was used to calculate statistical significances in (C)–(E). In (F), adjusted p values are shown. UMAP, uniform manifold approximation and projection for dimension reduction; FC, fold change. See also Figure S6.

Tables S2 and S3). We therefore combined the two LSC states together to characterize a global LSC transcriptional response to eIF6 overexpression (clusters 0 + 2 vs. 1 + 3). This strategy identified a total of 118 differentially expressed genes (adjusted $p < 0.05$, at least 1.5 FC) (Figure 5C; Table S4). Consistent with

the enrichment of ribosome biogenesis GO category, 18% (10/55) of the significantly upregulated genes encoded ribosomal proteins (Figure 5C). Quantitative proteomics of c-Kit+ leukemia cells confirmed the increase in ribosomal protein abundance in response to eIF6 overexpression (Figures S6E and S6F; Table S5).

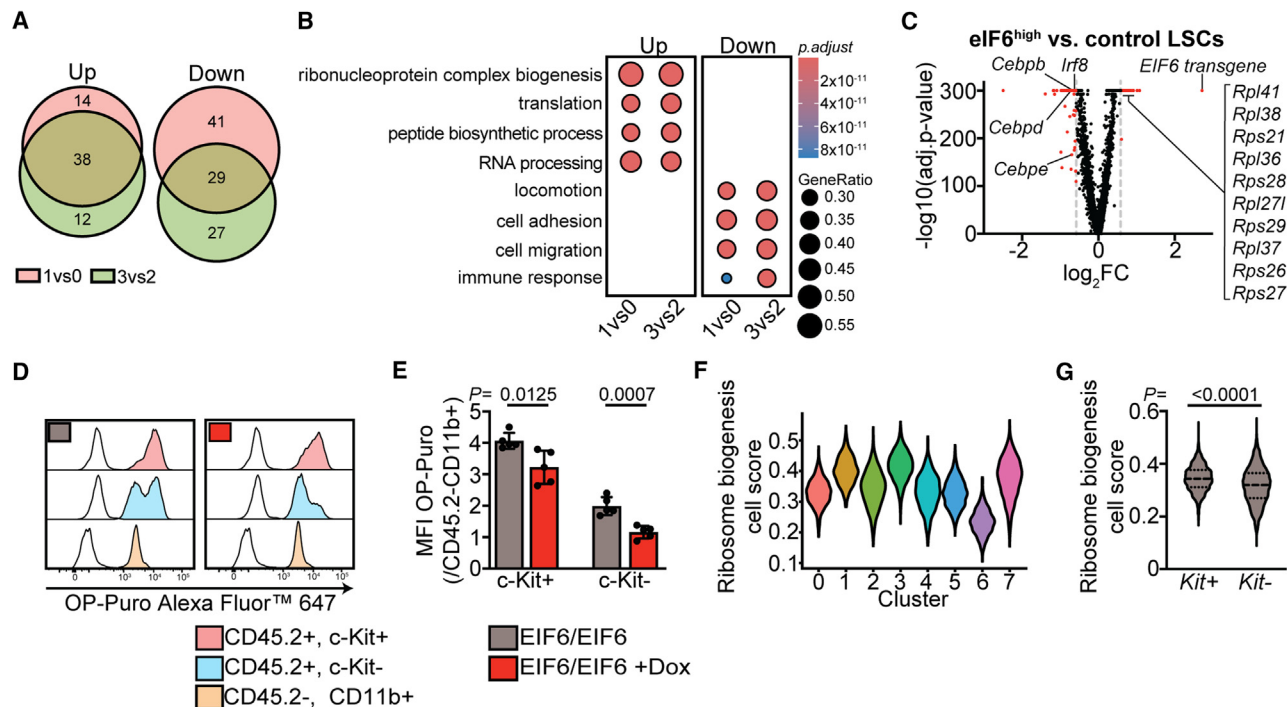


Figure 5. Resistant LSCs exhibit high ribosome content

(A) Venn diagram of the differentially expressed genes between depicted clusters. See also Tables S2 and S3.
(B) Gene Ontology analysis of differentially expressed genes. Shown are the most significantly up- and downregulated biological process categories.
(C) Volcano plot of differential gene expression between *eIF6*^{high} LSCs (clusters 1 + 3) and control LSCs (clusters 0 + 2). Red dots depict differentially expressed genes (adjusted $p < 0.05$, at least 1.5-fold change). See also Table S4.
(D) Representative flow cytometry plot of OP-Puro incorporation in the BM. 100,000 serially transplanted leukemic BM cells were engrafted into sublethally irradiated recipient mice. Five days after transplantation, half of the mice were administered Dox, and OP-Puro labeling was performed on day 9 or 10. CD45.2 was used to identify leukemia cells due to the loss of GFP fluorescence during cell fixation and permeabilization. The white curves represent BM of PBS-injected control mouse.
(E) Median fluorescence intensity (MFI) of OP-Puro normalized against the CD45.2⁺/CD11b⁺ host myeloid precursor cells. The data represent two independent OP-Puro labeling experiments with a total of five animals per group. The data represent mean $\pm$ standard deviation.
(F) Ribosome biogenesis cell scores in different clusters. See Table S6 for gene signature and Table S7 for statistical significances.
(G) Ribosome biogenesis cell scores in *Kit*⁺ and *Kit*⁻ control leukemia cells. The center line represents the median, and the outer lines represent the two quartiles. Student's *t* test was used to determine statistical significance in (E) and (G), and two-tailed *p* values are shown. In (B) and (C), adjusted *p* values are shown. One-way ANOVA with Tukey's multiple comparisons test was used to calculate statistical significances in (F).
See also Figure S6.

To address whether the increased ribosome content was sufficient to rescue the protein synthesis rate in these cells, we performed OP-Puro labeling experiments in leukemia-engrafted mice. Similar to before (Figure 1F), c-Kit⁺ leukemia cells in control mice incorporated four times more OP-Puro compared with normal CD11b⁺ myeloid cells, and c-Kit⁻ leukemia cells could be divided into OP-Puro^{high} and OP-Puro^{low} subsets (Figures 5D and 5E). Importantly, *Kit*⁺ cells in control samples showed higher RiBi scores compared with *Kit*⁻ cells, with the most differentiated cells (cluster 6) displaying the lowest RiBi scores (Figures 5F and 5G). These data are consistent with the high protein synthesis rates in c-Kit⁺ leukemia cells (Figures 5D and 5E) and in line with our observation that immature leukemia cells in human *MLL*-rearranged AML show higher ribosome biogenesis rates compared with more mature leukemia cells (Figure 1A). *eIF6* overexpression led to 21% and 42% decreases in OP-Puro incorporation in c-Kit⁺ and c-Kit⁻ leukemia cells, respec-

tively, demonstrating a persistent reduction in the protein synthesis rate despite the increase in ribosome content (Figure 5E). The more pronounced reduction in OP-Puro incorporation in c-Kit⁻ leukemia cells was associated with a profound loss of the OP-Puro^{high} subset. Consistent with the reduced protein synthesis rate in c-Kit⁺ cells, the proteome dataset revealed a particular increase in the abundance of ZNF622, NMD3, and RSL24D1 ribosome assembly factors (Figure S6G). Since these factors are evicted from the nascent pre-60S ribosomal subunit in the cytoplasm, this finding suggests that *eIF6* overexpression interferes with the final maturation of pre-60S subunits, leading to an accumulation of such particles.²²

Defective ribosome assembly results in deregulated TF landscape

Next, we set out to address whether the adaptive transcriptional program was associated with an altered TF landscape. We

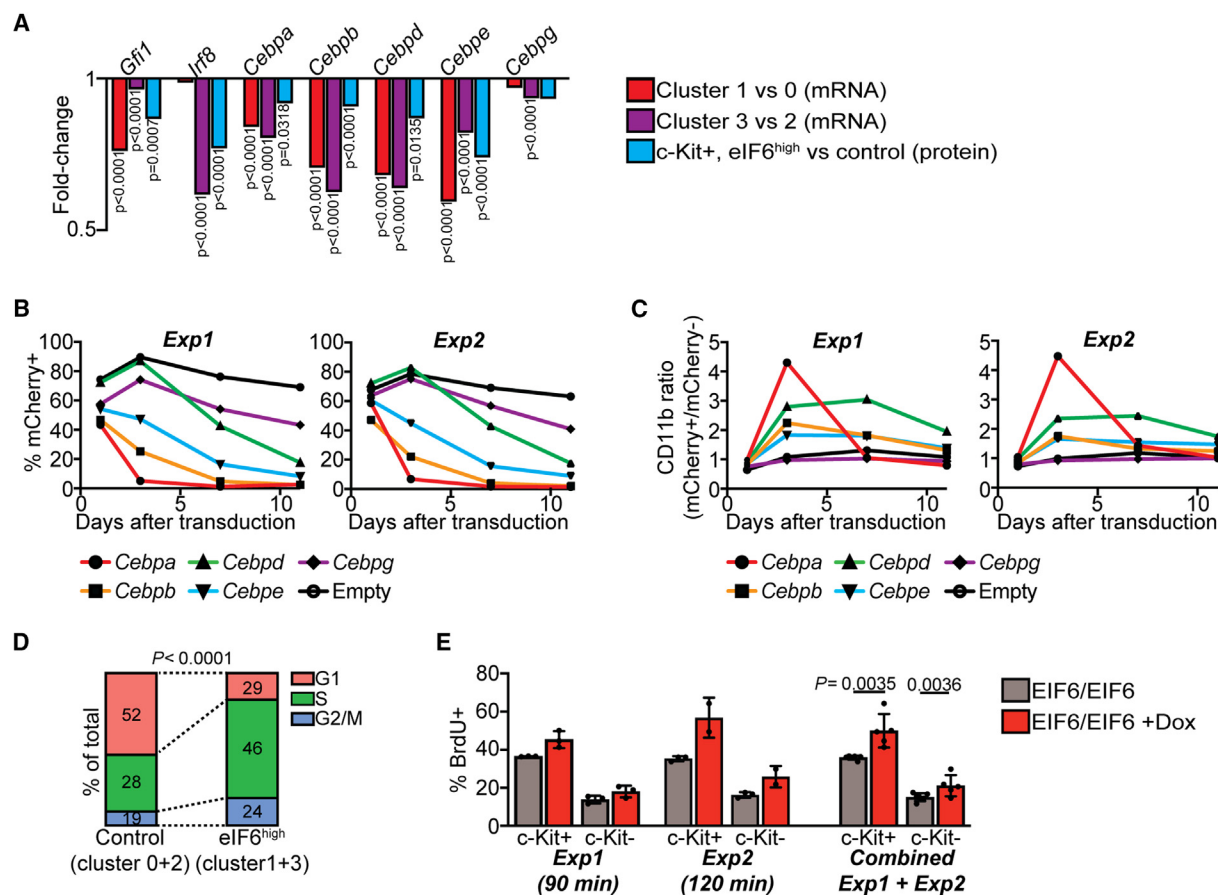


Figure 6. LSC adaptation to defective ribosome assembly is associated with deregulated transcription factor landscape

(A) Differential gene expression of selected transcription factors in scRNA-seq and proteome datasets.
(B) Frequency of transduced cells (mCherry+) in bulk transduced leukemia cell culture over time. Empty control represents the MSCV-IRES-mCherry retroviral backbone. Shown are two independent experiments.
(C) Ratio of CD11b median fluorescence intensity between transduced (mCherry+) and untransduced (mCherry-) cells in bulk transduced leukemia cell culture over time. Shown are two independent experiments.
(D) Seurat classification of control and eIF6^{high} LSCs in cell cycle phases. Control, clusters 0 + 2. eIF6^{high}, clusters 1 + 3.
(E) Frequency of BrdU-labeled c-Kit+ and c-Kit- leukemia cells in the BM of leukemia-engrafted mice. 100,000 serial transplanted leukemic BM cells were engrafted into sublethally irradiated recipient mice. Five days after transplantation, half of the mice were administered Dox, and all mice were analyzed on day 10. The data represent two independent experiments with 90 or 120 min BrdU labeling and a total of six and five animals per group. The data represent mean $\pm$ standard deviation.
Chi-squared test was used to determine statistical significance in (D). Student's t test was used to determine statistical significance in (E), and two-tailed p values are shown. In (A), adjusted p values are shown.

observed a significant downregulation of *Irf8*, *Cebpb*, *Cebpd*, and *Cebpe* in LSCs in response to eIF6 overexpression (Figure 5C). Individual analyses of the two LSC states revealed a significant downregulation of *Gfi1* and *Irf8* in LSC-I and LSC-II, respectively, as well as at the protein level in c-Kit+ leukemia cells (Figure 6A). A similar analysis of C/EBP TFs identified a significant downregulation of *Cebpa*, *Cebpb*, *Cebpd*, and *Cebpe*, while *Cebpg* was not consistently altered (Figure 6A). This suggests that TF downregulation constitutes a mechanism of leukemia resistance by inhibiting differentiation and thus maintaining the LSC pool. Consistently, ectopic expression of *Cebpa*, *Cebpb*, *Cebpd*, and *Cebpe*, but not *Cebpg*, in leukemia cells led to a proliferative disadvantage and terminal differentiation as judged by the upregulation of CD11b (Figures 6B and 6C).

This is of interest since C/EBP TFs hetero- or homodimerize to promote myeloid differentiation apart from C/EBP γ (*Cebpg*), which is thought to act as a dominant negative inhibitor of other C/EBPs.^{23,24} These data suggest that both the overall reduction and the altered ratio of distinct C/EBP TFs likely contribute to adaptation to defective ribosome assembly.

IRF8, GFI1, and C/EBP TFs drive myeloid differentiation in part by promoting cell cycle withdrawal.^{20,21,23,24} We therefore hypothesized that reduced levels of these factors in response to eIF6 overexpression should increase the LSC cell division rate. Consistent with the high cell division rate of normal GMPs,¹⁹ prediction of cell cycle identities in the scRNA-seq dataset suggested that 48% of LSCs in the control mice were undergoing cell division (Figure 6D). Strikingly, this fraction was further increased to 71%

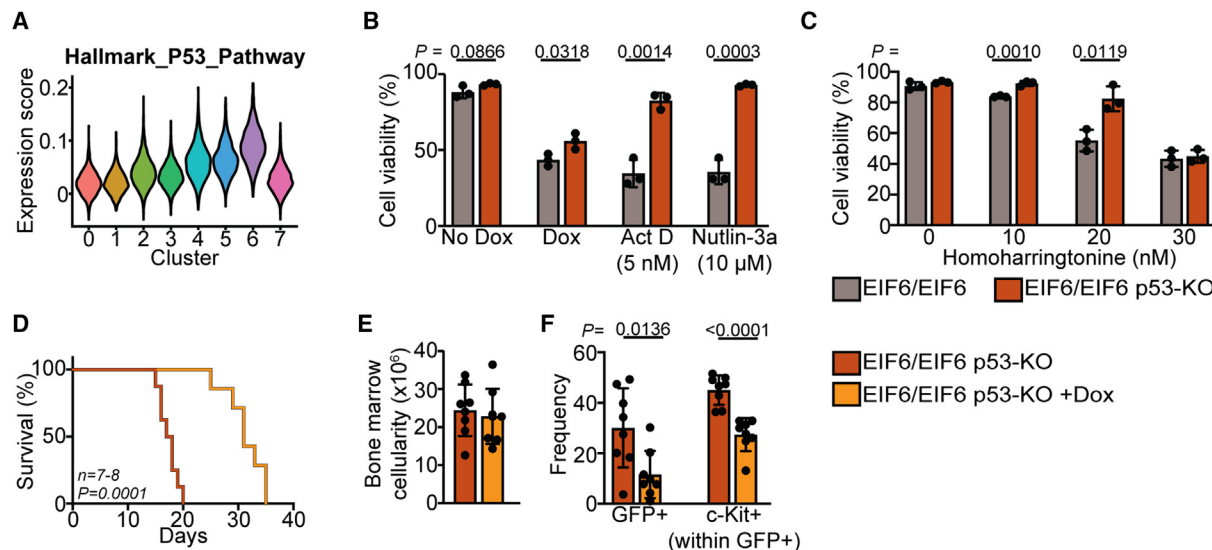


Figure 7. Defective ribosome assembly shows antileukemia efficacy in p53-deficient AML

(A) Violin plot of p53 cell scores in different clusters. See Table S6 for gene signature and Table S7 for statistical significances.

(B) Cell viability. Leukemia cells were cultured in the presence of Dox, actinomycin D (5 nM), or Nutlin-3a (10 μ M) for 24 h, and cell viability was measured using trypan blue exclusion assay. $n = 3$ independent experiments. The data represent mean $\pm$ standard deviation.

(C) Cell viability. Leukemia cells were cultured in the presence of increasing concentrations of homoharringtonine for 24 h, and cell viability was measured using trypan blue exclusion assay. $n = 3$ independent experiments. The data represent mean $\pm$ standard deviation.

(D) Survival of leukemia-engrafted mice. 100,000 serial transplanted leukemic BM cells were engrafted into sublethally irradiated recipient mice. Dox administration was initiated 1 day after transplantation. The graph represents two individual experiments with a total of eight and seven animals per group.

(E) BM cellularity. 100,000 serial transplanted leukemic BM cells were engrafted into sublethally irradiated recipient mice. Five days after transplantation, half of the mice were administered Dox, and all mice were analyzed on day 10. The data represent two independent experiments with a total of eight animals per group. The data represent mean $\pm$ standard deviation.

(F) Frequencies of GFP+ leukemia cells and of c-Kit+ cells within the GFP+ leukemia cells in the BM. The data represent two independent experiments with a total of eight animals per group. The data represent mean $\pm$ standard deviation.

Student's *t* test was used to determine statistical significance in (B), (C), (E), and (F), and two-tailed *p* values are shown. Log-rank (Mantel-Cox) test was used to determine statistical significance in (D). One-way ANOVA with Tukey's multiple comparisons test was used to calculate statistical significances in (A).

See also Figure S6.

following eIF6 overexpression. *In vivo* BrdU labeling experiments functionally validated these results (Figure 6E).

Defective ribosome assembly shows antileukemia efficacy in p53-deficient AML

Perturbations of ribosome biogenesis in the nucleolus are known to trigger a p53-dependent ribosome biogenesis stress pathway.^{25–28} We therefore set out to assess the role of p53 in response to defective ribosome assembly. Quantitative proteomics of c-Kit+ leukemia cells indicated that eIF6 overexpression leads to a significant increase in the abundance of p53 and p19ARF proteins (Figure S6E). However, the scRNA-seq dataset revealed no change and a modest but significant decrease in p53 transcriptional signature²⁹ within the LSC-I and LSC-II states, respectively, in response to eIF6 overexpression (Figure 7A). This finding indicates that the observed increase in p53 protein abundance is due to an enrichment of LSC-II cells that show higher p53 signatures compared with LSC-I cells within the c-Kit+ cells (Figure 4G) rather than a genuine activation of the p53 pathway. Consistently, the proteome dataset revealed a significant reduction of several LSC-I-associated proteins including STFA1, CHIL3, S100A8, and S100A9 and a significant

increase in LSC-II-associated protein S100A6 in response to eIF6 overexpression (Figure S6E).

To further assess the role of p53 in the depletion of leukemia cells upon defective ribosome assembly, we applied CRISPR-Cas9 to generate a p53-deficient AML subclone. We observed a robust induction of apoptosis in response to eIF6 overexpression regardless of p53 status (Figure 7B). By contrast, treatment of these cells with the Mdm2 inhibitor Nutlin-3a or actinomycin D, a known inducer of the ribosome biogenesis stress pathway,²⁶ triggered apoptosis in p53-proficient, but not in p53-deficient, cells (Figure 7B). A somewhat higher concentration of homoharringtonine was required to induce apoptosis in p53-deficient leukemia cells (Figure 7C). In line with these findings, loss of p53 did not alter the impact of eIF6 overexpression on leukemia progression *in vivo* compared with p53-proficient parental cells (Figures 7D and 3A). Although BM analysis of mice transplanted with these cells revealed no significant difference in cellularity (Figure 7E), we observed a 61% reduction in GFP+ leukemia cells and a 39% reduction in c-Kit+ cells within this fraction (Figure 7F), similar to p53-proficient cells (Figure 3D).

DISCUSSION

Our study demonstrates that the induction of defective ribosome assembly significantly extends survival in mice with *MLL*-rearanged AML. At the same time, the normal hematopoietic system was able to tolerate defective ribosome assembly for at least 4 months, despite the negative effect on HSCs over time. Together with clinical studies on homoharringtonine and CX-5461,^{2,4} these data suggest the existence of a therapeutic window for targeting ribosome assembly in AML.

The induction of defective ribosome assembly led to a profound decrease in GFP+ leukemia cells in leukemia-engrafted mice that was associated with a significant reduction in the frequency of c-Kit+ cells and L-GMPs among these cells. However, despite this impact on leukemia cell immunophenotype, serial transplantation of prospectively purified GFP+ leukemia cells demonstrated no difference in leukemia latencies between control and eIF6^{high} groups, suggesting that the frequency of LSCs within GFP+ leukemia cells is not altered in response to defective ribosome assembly. This outcome highlights the difficulty in identifying LSCs using flow cytometry and is consistent with the relatively similar frequencies of distinct leukemia cell transcriptional states in our scRNA-seq data. However, given the drastic overall 83% reduction of GFP+ leukemia cells in response to eIF6 overexpression, it can be estimated that defective ribosome assembly results in a similar decrease in the absolute number of LSCs. Consistently, transplantation of an equal number of unfractionated BM cells regenerated leukemia in secondary hosts with significantly longer latency compared with controls. Finally, consistent with the recent identification of RIO-kinase 2 as a target in AML,³⁰ defective ribosome assembly showed an antileukemia effect in p53-deficient AML. However, further validation is needed to prove whether this approach can overcome the adverse risk effects in AML with *TP53* mutations.³¹

Our scRNA-seq data indicate that leukemia cell adaptation to defective ribosome assembly is due to global transcriptional reprogramming of LSC states rather than selection for a pre-existing LSC transcriptional state. Most notably, defective ribosome assembly triggered a significant upregulation of ribosome biogenesis genes, suggesting that the ability of LSCs to adapt to this insult may depend on maintaining a critical threshold level of protein synthesis. Consistent with this concept, the low ribosome biogenesis rate in *RUNX1*-mutated AML was shown to confer increased sensitivity of leukemia cells to homoharringtonine.³² Our scRNA-seq data also show that persistent LSCs have reduced expression of TFs that promote myeloid differentiation, leading to the adoption of a more immature and proliferative cellular state. Many of these TFs, including members of C/EBP and IRF families, have been directly implicated in the transcriptional regulation of ribosome biogenesis, thus linking protein synthesis to LSC self-renewal and therapy response.³³

Limitations of the study

Our study applies a RiBi score as an indirect proxy to estimate ribosome biogenesis rates in distinct leukemia cell fractions and their normal counterparts. While confirmation of these findings using a more direct method would benefit our study, such

analyses are not feasible due to the lack of surface markers that would enable prospective isolation of these cellular fractions from primary patient material. Our study takes advantage of a transgenic eIF6 mouse strain to assess the role of cytoplasmic ribosome assembly as a target in AML. However, the lack of small molecules that modulate the binding of eIF6 to the 60S subunit has prevented the direct pharmacological verification of our findings in murine and human AML cells.

RESOURCE AVAILABILITY

Lead contact

Further information and request for resources and reagents should be directed to and will be fulfilled by the lead contact, Pekka Jaako (pekka.jaako@gu.se).

Materials availability

The newly generated plasmids listed in the [key resources table](#) are available upon request.

Data and code availability

- scRNA-seq data have been deposited at ArrayExpress under accession number E-MTAB-13567. Proteomic data have been deposited to the ProteomeXchange Consortium via PRIDE³⁴ with the identifier PXD038318. These datasets are publicly available as of the date of publication. Additionally, this paper utilizes existing, publicly available data. Accession numbers for the datasets are listed in the [key resources table](#). Additional data reported in this study will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

P.J. conceived the study. D.S., A.H., C.G., and P.J. designed, performed, and analyzed experiments. S.G. and J.H. performed mass spectrometry and protein quantification. N.F.-F., T.V.-H., P.M., and P.J. performed the analysis of human AML scRNA-seq datasets. P.J. wrote the manuscript with feedback from all authors.

DECLARATION OF INTERESTS

P.M. is co-founder of OneChain Immunotherapeutics, a spin-off company from the Josep Carreras Leukemia Research Institute with scientific interests unrelated to this work.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD11b – BV421 (M1/70)	Biolegend	Cat#101235; RRID:AB_10897942
c-Kit – APC-eFluor780 (2B8)	Thermo Fisher Scientific	Cat#47-1171-82; RRID:AB_1272177
Sca1 – Pacific Blue (E13–161.7)	Sony Biotechnology	Cat#1212600; RRID:N/A
CD48 – PE (HM48-1)	Sony Biotechnology	Cat#1117025; RRID:N/A
CD150 – APC (TC15-12F12.2)	Sony Biotechnology	Cat#1179550; RRID:N/A
CD3 – PE/Cy5 (145-2C11)	Sony Biotechnology	Cat#1101550; RRID:N/A
CD11b – PE/Cy5 (M1/70)	Sony Biotechnology	Cat#1106050; RRID:N/A
B220 – PE/Cy5 (RA3-6B2)	Sony Biotechnology	Cat#1116050; RRID:N/A
Gr-1 – PE/Cy5 (RB6-8C5)	Sony Biotechnology	Cat#1142050; RRID:AB_2935668
TER-119 – PE/Cy5 (TER-119)	Sony Biotechnology	Cat#1181050; RRID:N/A
Gr-1 – BV421 (RB6-8C5)	Sony Biotechnology	Cat#1142225; RRID:N/A
CD45.1 – BUV395 (A20)	BD Biosciences	Cat#565212; RRID:AB_2722493
CD45.2 – BUV737 (104)	BD Biosciences	Cat#612778; RRID:AB_2870107
CD45.2 – FITC (104)	Sony Biotechnology	Cat#1149030; RRID:N/A
CD45.1 – PE (A20)	Sony Biotechnology	Cat#1153540; RRID:N/A
CD19 – PE/Cy7 (1D3)	Thermo Fisher Scientific	Cat#25-0193-82; RRID:AB_657663
CD11b – APC (M1/70)	Sony Biotechnology	Cat#1106060; RRID:N/A
CD3 – Alexa Fluor 700 (17A2)	Sony Biotechnology	Cat#1101080; RRID:AB_2935667
TER-119 – Biotin (TER-119)	Sony Biotechnology	Cat#1181020; RRID:AB_3083430
CD4 – Biotin (GK1.5)	Sony Biotechnology	Cat#1102020; RRID:N/A
CD8a – Biotin (53–6.7)	Sony Biotechnology	Cat#1103520; RRID:AB_3083433
B220 – Biotin (RA3-6B2)	Sony Biotechnology	Cat#1116020; RRID:N/A
Gr-1 – Biotin (RB6-8C5)	Sony Biotechnology	Cat#1142020; RRID:AB_3083431
CD11b – Biotin (M1/70)	Sony Biotechnology	Cat#1106020; RRID:N/A
Sca-1 – Biotin (D7)	BioLegend	Cat#108103; RRID:AB_313340
CD127 – Biotin (A7R34)	Sony Biotechnology	Cat#1275030; RRID:N/A
CD34 – BV711 (RAM34)	BD Biosciences	Cat#751621; RRID:AB_2875614
EIF6 (rabbit)	GeneTex	Cat#GTX117971; RRID:AB_11173506
GAPDH (rabbit)	Merck	Cat#G9545; RRID:AB_796208
eS19 (rabbit)	Proteintech	Cat#15085-1-AP; RRID:AB_2180202
IgG – HRP (goat)	Cell Signaling Technology	Cat#7074; RRID:AB_2099233
Bacterial and virus strains		
Competent E. coli DH5a	New England BioLabs	Cat#C2987H
Chemicals, peptides, and recombinant proteins		
Recombinant Murine SCF	Peptotech	Cat#250-03-250
Recombinant Murine IL-3	Peptotech	Cat#213-13-100
Recombinant Murine TPO	Peptotech	Cat#315-14
RetroNectin	Takara	Cat#T100B
ACK lysis buffer	Thermo Fisher Scientific	Cat#A1049201
Propidium Iodide	Thermo Fisher Scientific	Cat#P3566
Alt-R® S.p. Cas9Nuclease V3	IDT	Cat#1081059
Lipofectamine LTX reagent	Thermo Fisher Scientific	Cat#15338030
NuPAGE Bis-Tris polyacrylamide gels	Thermo Fisher Scientific	Cat#NP0302BOX

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
NuPAGE MOPS SDS running buffer	Thermo Fisher Scientific	Cat#NP0001
NuPAGE LDS Sample Buffer	Thermo Fisher Scientific	Cat#NP0007
SuperSignal West Pico PLUS reagents	Thermo Fisher Scientific	Cat#34577
SsoFast EvaGreen supermix	Bio-Rad	Cat#1725201
Nutlin-3a	Merck	Cat#N6287-1MG
Puromycin	InvivoGen	Cat#ant-pr-1
Cycloheximide	Merck	Cat#01810-1G
cComplete™ EDTA-free Protease Inhibitor Cocktail	Merck	Cat#11873580001
OP-Puro	MedChemExpress	Cat#HY-15680
OP-Puro	Jena Bioscience	Cat#NU-931-5
Alexa Fluor™ 647 Azide	Thermo Fisher Scientific	Cat#A10277
SpectraMulticolor Broad Range Protein Ladder	Thermo Fisher Scientific	Cat#26634
Doxycycline food pellets (2000 mg/kg)	Ssniff spezialdiäten GmbH	Cat#A112D72003
MethoCult M3231	STEMCELL Technologies	Cat#03231
Anti-Biotin MicroBeads	Miltenyi Biotec	Cat#130-090-485
LS Columns	Miltenyi Biotec	Cat#130-042-401
Qdot™ 605 Streptavidin	Thermo Fisher Scientific	Cat#Q10101MP
Homoharringtonine	Tocris	Cat#1416
Actinomycin D	Enzo	Cat#BML-GR300-0005
Doxycycline hyclate	Merck	Cat#D9891
HEPES	Merck	Cat# H3375
Rapigest SF Surfactant	Waters Corp.	Cat#186001861
DTT	Merck	Cat#D0632
Iodoacetamide	Merck	Cat#I1149
Trypsin	Promega	Cat#V5111
Trifluoroacetic acid	Thermo Fisher Scientific	Cat#A11650
Acetonitrile	Thermo Fisher Scientific	Cat#A955212

Critical commercial assays

BD Pharmingen™ APC BrdU Kit	BD Biosciences	Cat#552598
RNeasy mini kit	Qiagen	Cat#74104
Human CD34 ⁺ Cell Nucleofector™ Kit	Lonza	Cat#VPA-1003
Click-iT™ Cell Reaction Buffer Kit	Thermo Fisher Scientific	Cat#C10269
SuperScript III first-strand synthesis system	Thermo Fisher Scientific	Cat#18080051
Chromium Next GEM Single Cell 3' Kit v3.1	10x Genomics	Cat#1000268
Gibson Assembly® Cloning Kit	New England BioLabs	Cat#E5510S
TMT10plex reagents	Thermo Fisher Scientific	Cat#90110
Sep-Pak tC18 1cc vac cartridge, 50mg	Waters Corp.	Cat#WAT054960
50cm EasySpray column	Thermo Fisher Scientific	Cat#ES903

Deposited data

scRNA-seq data	This paper	ArrayExpress: E-MTAB-13567
Raw mass spectrometry data	This paper	ProteomeXchange: PXD038318
scRNA-seq data	Velasco-Hernandez et al. ⁶	EGAS00001005980
scRNA-seq data	Van Galen et al. ⁷	GSE116256

Experimental models: Cell lines

Platinum-E retroviral packaging cell line	Cell Biolabs	Cat#RV-101
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Experimental models: Organisms/strains

Mouse: C57BL/6J	The Jackson Laboratory	RRID:IMSR_JAX:000664
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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse: B6 CD45.1	The Jackson Laboratory	RRID:IMSR_JAX:002014
Mouse: Rosa26-M2rtTA; Col1a1-tetO-EIF6	Wellcome Sanger Institute.	RRID:N/A

Oligonucleotides

See Table S8

Recombinant DNA

pMIG-FLAG-MLL-AF9	Addgene	Cat#71443
MigR1-AE9a	Addgene	Cat#12433
MSCV-IRES-GFP	Addgene	Cat#20672
MSCV-IRES-mCherry	This paper	N/A
MSCV-Cebpa-IRES-mCherry	This paper	N/A
MSCV-Cebpb-IRES-mCherry	This paper	N/A
MSCV-Cebpd-IRES-mCherry	This paper	N/A
MSCV-Cebppe-IRES-mCherry	This paper	N/A
MSCV-Cebpg-IRES-mCherry	This paper	N/A

Software and algorithms

Prism 9 and 10	Graphpad	N/A
Photoshop CS5	Adobe	N/A
Illustrator 2023	Adobe	N/A
FACSDiva	BD Biosciences	N/A
FlowJo v10.8.2	BD Biosciences	N/A
10x Genomics Cell Ranger v7.0.1	10x Genomics	N/A
R (v4.3.1)		https://www.r-project.org/
Seurat (v4.0.1 and v4.3.0)	Hao et al. ³⁵	https://satijalab.org/seurat/
SoupX (v1.6.2)	Young et al. ³⁶	https://github.com/constantAmateur/SoupX
clusterProfiler (v4.8.1)	Yu et al. ³⁷	https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html
Proteome Discoverer	Thermo Fisher Scientific	N/A
MSSStatsTMT	Huang et al. ³⁸	https://www.bioconductor.org/packages/release/bioc/html/MSSStatsTMT.html

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals

All mouse experiments were regulated by the local animal ethics committee (# 2917/20). C57BL6J (RRID:IMSR_JAX:000664) and B6 CD45.1 (RRID:IMSR_JAX:002014) mice were purchased from the Jackson Laboratory. Transgenic eIF6 mouse strain has been described previously.¹³ Mice were maintained at the University of Gothenburg Experimental Biomedicine core facility where they were housed in individually ventilated cages at a temperature of 23°C (+/– 1°C) and a relative humidity of 45–70% with free access to sterile food and water. All experiments were performed using 8–12 weeks old adult mice. The C57BL/6J MLL-AF9, [M2-rtTA/M2-rtTA][EIF6/+] MLL-AF9 and [M2-rtTA/M2-rtTA][EIF6/EIF6] AML1-ETO9a AML models were of male origin. The [M2-rtTA/M2-rtTA][EIF6/EIF6] MLL-AF9 AML was of female origin. Male donor (CD45.2) was used in non-competitive transplantation experiment. Competitive transplantation experiment was performed using BM cells from [M2-rtTA/M2-rtTA][EIF6/EIF6] female donor and CD45.1 male donor. Both female and male mice were used as hosts for transplantation experiments. *EIF6* transgene was induced *in vivo* by feeding mice with doxycycline food pellets (2000 mg/kg; Ssniff spezialdiäten GmbH).

Cell culture

Platinum-E retroviral packaging cell line (Cell Biolabs) were cultured according to manufacturer's instructions in DMEM GlutaMAX media (Thermo Fisher Scientific), supplemented with 10% fetal calf serum (FCS, Merck), penicillin/streptomycin (P/S, Life Technologies), 1 µg/mL puromycin (InvivoGen) and 10 µg/mL blasticidin (InvivoGen). Following transfection with retroviral plasmids, puromycin and blasticidin were removed from the media. For retroviral transduction, lineage-depleted mouse BM cells were cultured in OptiMEM I reduced Serum Media (Thermo Fisher Scientific), supplemented with 10% FCS, P/S, 25 ng/mL murine stem cell factor (mSCF; PeproTech), 10 ng/mL murine interleukin 3 (mIL-3; PeproTech) and 50 ng/mL mouse thrombopoietin (PeproTech). Mouse

leukemia cells were cultured in OptiMEM I media supplemented with 10% FCS, P/S, 25 ng/mL mSCF, 10 ng/mL mIL-3. Cells were maintained in an incubator at 37°C and 5% CO₂.

METHOD DETAILS

Tissue harvest

Peripheral blood was collected from the tail vein into Microvette tubes (Sarstedt) and cellularity was analyzed using Sysmex KX-21 or Sysmex XP-300 hematology analyzers. Bone marrow was harvested by crushing the hips, femurs and tibias in ice-cold PBS with 2% FCS. Isolated cells were passed through a 70 µm cell strainer (Thermo Fisher Scientific). Spleens were collected and dissociated into single cell suspensions using a plunger from a sterile 1 mL syringe and 70 µm cell strainer in ice-cold PBS with 2% FCS.

Cell counting

Unless stated otherwise, cells were counted on TC20 Automated Cell Counter (Bio-Rad) using trypan blue. A cell size gate of ≥ 6 µm was applied to exclude red blood cells when counting bone marrow cellularity.

Retrovirus production

The MLL-AF9-GFP, AML1-ETO9a and MSCV-IRES-GFP retroviral vectors were obtained from Addgene (#71443, pMIG-FLAG-MLL-AF9, a gift from Daisuke Nakada; #12433, MigR1-AE9a, a gift from Dong-Er Zhang; #20672, MSCV-IRES-GFP, a gift from Tannishtha Reya). The MSCV-IRES-mCherry retroviral vector was generated by replacing the GFP in MSCV-IRES-GFP with mCherry using Nco1 and Not1 restriction enzymes and Gibson assembly. *Cebpa*, *Cebpb*, *Cebpd*, *Cebpe* and *Cebpg* genes were PCR amplified from genomic DNA isolated from transgenic eIF6 mouse strain and inserted into EcoRI-XhoI digested MSCV-IRES-mCherry backbone using Gibson assembly. Lipofectamine LTX reagent (ThermoFisher Scientific) was used to transiently transfect ecotropic Platinum-E retroviral packaging cell line (Cell Biolabs). Retroviral supernatant was harvested 48 h after transfection, passed through a 0.45 µm filter and stored at -80°C .

Retroviral transduction

Freshly harvested bone marrow cells were stained with biotin-conjugated antibodies against TER-119, CD4, CD8a, B220, Gr-1 and CD11b, and Anti-Biotin MicroBeads (Miltenyi) were used for lineage depletion. The lineage-depleted cells were cultured for 24 h and retroviral transduction was performed using retronectin-coated plates (Takara) according to manufacturer's instructions.

Transplantation assays

MLL-AF9 and AML1-ETO9a AML model systems were generated by transplanting one million bulk-transduced cells (CD45.2) together with 0.5 million CD45.1 BM cells into lethally irradiated (2×475 rad, X-ray source) CD45.1 recipient mice. Following the generation of primary AML model systems, all experiments were performed by transplanting 50,000 or 100,000 (MLL-AF9) or 600,000 (AML1-ETO9a) BM cells from terminally ill mice into sublethally irradiated (475 rad) recipient mice. For serial dilution experiment, 200, 1000 or 5000 FACS-purified viable GFP+ leukemia cells were transplanted into sublethally irradiated C57BL/6J and CD45.1 female recipient mice. Non-competitive transplantation experiment was performed by injecting three million unfractionated [M2-rTA/M2-rTA][EIF6/EIF6] BM cells from a male donor (CD45.2) into lethally irradiated (2×475 rad) female recipient mice (CD45.1). Competitive transplantation experiment was performed by injecting 500,000 unfractionated [M2-rTA/M2-rTA][EIF6/EIF6] BM cells from a female donor together with 500,000 unfractionated BM cells from a male CD45.1 donor into lethally irradiated (2×475 rad) female recipient mice (CD45.1).

Protein synthesis rate measurement

Mice were injected intraperitoneally with 200 µL of OP-Puro solution (3.4 mM; MedChemExpress or Jena Bioscience) and sacrificed 1 h later. Three million BM cells were stained with antibodies against cell surface markers and fixed with 2% formaldehyde (Alfa Aesar) for 15 min on ice. Stained cells were permeabilized using PBS supplemented with 0.1% saponin (Merck) and 3% bovine serum albumin (BSA; Merck). The azide-alkyne reaction was performed using the Click-iT Cell Reaction Buffer Kit with Alexa Fluor 647 Azide (Thermo Fisher Scientific) at a final concentration of 2.5 µM.

BrdU labeling

BrdU labeling was performed by intraperitoneal injection of 200 µL of 10 mg/mL BrdU solution using the BD Pharmingen APC BrdU Kit (BD Biosciences).

Flow cytometry

Fluorescently labeled and biotinylated antibodies used in this study are listed in the [key resources table](#). For peripheral blood analysis, erythrocytes were first sedimented with 2% Dextran (Merck) for 30 min at 37°C and then lysed using ACK lysis buffer (Thermo Fisher Scientific) for 2 min at room temperature. The cells were stained with antibodies in ice-cold PBS with 2% FCS for 30 min on ice and in dark. When using primary biotinylated antibodies, a secondary staining with Qdot 605 Streptavidin was performed (Thermo

Fisher Scientific). Propidium iodide (Thermo Fisher Scientific) was used to label dead cells. Flow cytometry was performed using BD LSRFortessa, BD LSR II and BD FACS Aria Fusion flow cytometers (BD Biosciences). Data analysis was performed using the FlowJo software (v10.8.2; BD Biosciences).

Quantitative reverse transcription PCR

Total RNA was isolated using RNeasy mini kit (Qiagen) and reverse transcription was performed using SuperScript III first-strand synthesis system (Thermo Fisher Scientific). Real-time PCR was performed using SsoFast EvaGreen supermix (Bio-Rad) on Applied Biosystems 7900HT Real-Time PCR System. Primers are listed in [Table S8](#).

Polysome profiling

Freshly harvested bone marrow cells were first stained with biotin-conjugated c-Kit antibody and then enriched for c-Kit⁺ cells using Anti-Biotin MicroBeads (Miltenyi). The c-Kit-enriched cells were cultured for 16 h in the presence of 1 μ g/mL Dox, and then treated with cycloheximide (CHX; 100 μ g/mL; Merck) for 5 min at 37°C before harvesting by centrifugation. Cells were washed twice with ice-cold PBS supplemented with CHX (100 μ g/mL), and lysed for 30 min on ice in lysis buffer (20 mM HEPES pH 7.5, 50 mM KCl, 10 mM Mg(CH₃COO)₂, 1.5 mM Dithiothreitol (DTT; Merck), 100 μ g/mL CHX, cOmplete EDTA-free Protease Inhibitor Cocktail (Merck), RNaseOUT recombinant ribonuclease inhibitor (200 U/mL; Thermo Fisher Scientific) and IGEPAL CA-630 (0.7%; Merck)). The lysate was cleared by centrifugation (14,000g for 5 min at 4°C), and RNA content was quantified using NanoDrop spectrophotometer (Thermo Fisher Scientific). Equal A₂₆₀ units (typically 3–5 units) of lysate were loaded onto a 10–50% (w/v) sucrose gradient (prepared in 20 mM HEPES pH 7.5, 50 mM KCl, 10 mM Mg(CH₃COO)₂, 1.5 mM DTT, 100 μ g/mL CHX, cOmplete EDTA-free Protease Inhibitor Cocktail, RNaseOUT recombinant ribonuclease inhibitor (4 U/mL)) prepared in open-top polyclear tubes (#7030; Seton Scientific). A Gradient Master (Biocomp) was used to prepare the sucrose gradients. After centrifugation at 36,000 rpm for 2 h at 4°C using a Thermo Scientific TH-641 rotor, sucrose gradients were profiled and fractionated using Biocomp gradient station (Biocomp). Proteins were precipitated with trichloroacetic acid (Merck; 25% (v/v); 15 min on ice). Following centrifugation at 14,000 g for 5 min at 4°C, protein precipitates were washed with ice-cold acetone. After drying, protein pellets were resuspended in 1x NuPAGE LDS Sample Buffer (Thermo Fisher Scientific).

Immunoblot

Proteins in 1x NuPAGE LDS sample buffer supplemented with 50 mM DTT were incubated at 85°C for 10 min and run on NuPAGE Bis-Tris polyacrylamide gels in NuPAGE MOPS SDS running buffer (Thermo Fisher Scientific). The iBlot 2 gel transfer device (Thermo Fisher Scientific) was used to transfer proteins to nitrocellulose membranes. Membranes were first blocked for 1 h in 5% milk (Semper) in PBS supplemented with 0.1% Tween (Merck), and then stained with primary antibodies overnight at 4°C on a tube roller. The blots were washed three times with PBS-tween and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody. Following five washes with PBS-tween, the blots were detected using the SuperSignal West Pico PLUS reagents (Thermo Fisher Scientific) and Fujifilm LAS-1000 Intelligent Dark Box II. Antibodies are listed in the [key resources table](#).

CRISPR/Cas9 targeting

To inactivate the *Trp53* gene, ribonucleoprotein complexes containing crRNA, tracrRNA and recombinant Cas9 nuclease were assembled according to manufacturer's (IDT) guidelines and delivered into leukemia cells using Human CD34⁺ Cell Nucleofector Kit and Nucleofector 2b Device with program U-008 (Lonza; see [Table S8](#) for RNA sequences). After 48 h, the transfected cells were treated with 10 μ M Nutlin-3a to select for p53-deficient clones, and individual subclones were selected by plating these cells on semi-solid media (MethoCult M3231, STEMCELL Technologies) supplied with 50 ng/mL mSCF and 10 ng/mL mL-3. A similar strategy was applied to inactivate the two *pac* puromycin resistance genes in [M2-rtTA/M2-rtTA][EIF6/EIF6] leukemia cells ([Table S8](#)), followed by *in vitro* selection of colonies using puromycin (1 μ g/mL). These cells were used in OP-Puro labeling experiments shown in [Figures 5D](#) and [5E](#). Sanger sequencing was used to verify biallelic frameshift mutations in the *Trp53* and *pac* genes (data not shown).

Mass spectrometry

Sample preparation for mass spectrometry analysis

300,000 FACS-sorted cells were lysed in 0.1% RapiGest (Waters) in 200 mM HEPES. Cysteine disulfide bonds were reduced with 5 mM DTT for 30 min at 56°C, alkylated with 10 mM iodoacetamide for 30 min at room temperature in the dark, and proteins were digested with sequencing grade modified trypsin (enzyme:protein ratio 1:50, Promega) at 37°C overnight. Digested peptides were labeled using TMT10plex reagents (Thermo Scientific) according to the manufacturer's protocol. Briefly, vials containing 0.8 mg of TMT label were dissolved in 41 μ L of anhydrous acetonitrile (ACN). Labeling was performed by addition of 5 μ L dissolved TMT10plex reagents to each sample and incubating for 30 min at room temperature in a thermomixer (Eppendorf) under constant shaking at 400 rpm, followed by another 5 μ L TMT reagent and incubating for 30 min. The reaction was stopped by adding trifluoroacetic acid to a final concentration of 0.5% (v/v), and RapiGest was precipitated by further incubation at 37°C for 45 min. Following centrifugation, the supernatants were collected, and the labeled peptides were pooled, desalted with SepPak C18 cartridges (Waters), and dried by vacuum centrifugation. High-pH reverse phase (HpH-RP) fractionation was carried out as previously described.³⁹

Fractionated samples were dried by vacuum centrifugation and stored at -80°C until MS analysis. Prior to LC-MS analysis, samples were dissolved in 4% ACN/0.1% formic acid (FA).

Mass spectrometry data acquisition

TMT-labeled peptide fractions were loaded onto a trap cartridge (Thermo Fisher Scientific Acclaim PepMap C18, 5 μm , 300 μm $\times$ 5 mm) followed by EASY-Spray PepMap Neo analytical column, 2 μm , 75 μm $\times$ 50 cm (Thermo Fisher Scientific). Each fraction was injected twice and eluted over 180 min gradients (UltiMate 3000 UHPLC, Thermo Fisher Scientific) ranging from 4 to 19% Solvent B (0.1% FA in 80% ACN) over 83 min, 19–29% B over 40 min, 29–41% B over 8 min, 41–95% B over 2 min and held at 95% B for 8 min at a constant flow rate of 300 nL/min at 450C. MS/MS were acquired on a Thermo Orbitrap Exploris 480 equipped with FAIMS Pro interface (Thermo Fisher Scientific) in data-dependent acquisition. FAIMS compensation voltages (CV) were set to -45 , -60 , and -80 with a cycle time of 1 s per FAIMS experiment. The spray voltage was set at 2.1 kV and the ion transfer tube temperature was set at 275°C . The full MS scan was performed in the Orbitrap in the range of 400–1400 m/z at a resolution of 60,000 with automatic gain control (AGC) set to standard and maximum ion injection time set to auto. The intensity threshold was set to $5.0\text{e}4$ and mass tolerance to 10 ppm. The most intense ions selected in the first MS scan were isolated for higher-energy collision-induced dissociation (HCD) at a precursor isolation window width of 0.7 m/z, and normalized AGC of 200%. Maximum ion injection time for MS2 was set to auto with MS2 resolution set to 45,000 FWHM. The first mass and the normalized collision energy were set to 110 m/z and 35%, respectively.

Protein quantification and statistical analysis

MS raw files were searched in Proteome Discoverer (version 2.5; Thermo Scientific) against the Swissprot mouse database (downloaded June 2021) together with human eIF6 protein sequence, GFP, MLL-AF9, as well as commonly observed contaminants using the Sequest HT node. The enzyme was set to trypsin with up to two missed cleavages, Cysteine carbamidomethylation and TMT6plex (+229.163 Da) were set as static modifications whereas methionine oxidation, N-terminal acetylation, and Met loss (protein N-terminal Met) were set as dynamic modifications. TMT batch-specific isotopic correction factor was applied in the reporter ion quantification. MS1 mass tolerance was set to 10 ppm and MS2 to 0.02 Da. The false discovery rate for peptide-spectrum matches (PSMs) was set to 0.01 using the Percolator node. The co-isolation threshold was set to 50. Statistical analysis of the TMT quantification was performed using MSstatsTMT package (2.4.1)³⁸ in R. PSMs results from Proteome Discoverer were exported and converted into MSstatsTMT-compatible format using ‘PDtoMSstatsTMTFormat’ function. PSMs were filtered with peptide percolator q value <0.01 . Only unique peptides were used for protein quantifications. Protein summarization was performed using the ‘msstats’ method and global median normalization was performed. Differential expression analysis was performed using moderated *t*-tests with Benjamini–Hochberg multiple hypothesis correction.

Single-cell RNA sequencing

Single-cell RNA sequencing analysis of the human AML datasets

The Velasco-Hernandez dataset represents prospectively purified $\text{CD}33^{+}\text{CD}34^{+}\text{CD}38^{-}$ and $\text{CD}33^{+}\text{CD}34^{-}\text{CD}38^{+}$ BM cells from 11 pediatric patients at diagnosis: inv(16) ($n = 4$), t(8; 21) ($n = 3$) and MLL-rearranged AML ($n = 4$) (European Genome-Phenome Archive repository: EGAS00001005980).⁶ LSCs were identified using the LSC6 score.^{6,8} The raw counts and annotations of AML samples from the Van Galen scRNA-seq dataset⁷ were downloaded from the GEO repository (GSE116256). For each sample a Seurat object was created by processing both counts and annotations files using Seurat (v4.0.1) including pre-processing, normalization and identification of highly variable genes. Subsequently, a ribosome biogenesis cell score, which is based on the expression of 307 ribosome biogenesis genes (Table S6), was computed using the AddModuleScore function. Finally, pre-treatment healthy and malignant cells annotated as HSC, Progenitor and GMP were selected for further analysis. Only those cell types that contained five or more cells per each condition were included in the analysis.

Single-cell RNA sequencing and data analysis of murine MLL-AF9 leukemia

scRNA-seq was performed using the 10x Chromium platform (10x Genomics). BM cells were harvested from leukemia-engrafted mice that were administered Dox for six days and viable GFP+ leukemia cells were sorted into PBS containing 0.05% BSA following the manufacturer’s protocol. For each sample, 15,000 cells were loaded in a 10x Genomics Chromium Next GEM Chip G channel, libraries were constructed using 10x Genomics Chromium Next GEM Single Cell 3’ v3.1 kit and the quality of sequencing libraries was determined using Agilent 4200 TapeStation system according to manufacturer’s instructions. Libraries were pooled at equimolar ratios and sequenced on the Novaseq 6000 system (Illumina) on one SP and one S1 flowcells, using paired-end dual indexing (R1: 28 cycles; i7: 10 cycles; i5: 10 cycles; R2: 90 cycles), obtaining an average of 43,000 reads per cell.

Raw data files (fastq) were processed using 10x Genomics Cell Ranger (v7.0.1) and reads were mapped to the GRCh39 genome concatenated with *EIF6* and *MLL-AF9-GFP* transgenes. All downstream analyses were implemented using R (v4.3.1) and the Seurat package (v4.3.0)³⁵ unless otherwise stated. Quantification and removal of ambient mRNA was performed using SoupX (v1.6.2).³⁶ To remove low-quality transcriptomes, cells with lower than 2,500 genes and lower than 5,000 or greater than 40,000 unique molecular identifiers were filtered out. Moreover, cells with lower than 15 or greater than 36% ribosomal protein RNA, lower than 1 or greater than 4% of mitochondrial RNA, and greater than 0.001% hemoglobin RNA were removed. In total, 18,426 cells passed quality control, with an average of 4,733 genes and 21,700 unique molecular identifiers detected per cell. Data normalization was performed using the LogNormalize function. The FindVariableFeatures function was performed using the 4,000 most variable features, and these were used as input for the RunPCA function. The first 20 principal components were used as input for the RunUMAP function.

The FindClusters function was performed using a resolution parameter of 0.5. Cluster marker gene identification and differential gene expression analysis were performed using MAST.⁴⁰ The AddModuleScore function was used to calculate the LSC, ribosome biogenesis and p53 cell scores, and the respective signature genes are listed in [Table S6](#). Gene ontology analyses were performed using clusterProfiler (v4.8.1).³⁷

QUANTIFICATION AND STATISTICAL ANALYSIS

The data analysis was performed using Prism 9 and 10 (Graphpad) and Microsoft Excel (Microsoft) except for mass spectrometry and scRNA-seq data for which detailed analyses are described in their respective sections. Data are presented as mean $\pm$ standard deviation unless stated otherwise. The exact value of n and what it represents as well as the specific statistical test used for each experiment are described in figure legends. To test statistical significance between two groups, a two-tailed Student's *t*-test was used except for [Figure 2E](#) where paired sample *t* test was applied. When multiple groups were compared, statistical significance was evaluated using one-way ANOVA with post hoc Tukey correction. Chi-square test was used to evaluate statistical difference in [Figure 6D](#). Log rank (Mantel Cox) test was used to determine statistical significance in survival analyses. No blinding was used and no statistical method was used to predetermine sample size.