The ubiquitin ligase RNF5 determines acute myeloid leukemia growth and susceptibility to histone deacetylase inhibitors

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Acute myeloid leukemia (AML) remains incurable, largely due to its resistance to conventional treatments. Here, we find that increased abundance of the ubiquitin ligase RNF5 contributes to AML development and survival. High RNF5 expression in AML patient specimens correlates with poor prognosis. RNF5 inhibition decreases AML cell growth in culture, in patient-derived xenograft (PDX) samples and in vivo, and delays development of MLL-AF9-driven leukemogenesis in mice, prolonging their survival. RNF5 inhibition causes transcriptional changes that overlap with those seen upon histone deacetylase (HDAC) inhibition. RNF5 induces the formation of K29 ubiquitin chains on the histone-binding protein RBBP4, promoting its recruitment to and subsequent epigenetic regulation of genes involved in AML maintenance. Correspondingly, RNF5 or RBBP4 knockdown enhances AML cell sensitivity to HDAC inhibitors. Notably, low expression of both RNF5 and HDAC coincides with a favorable prognosis. Our studies identify an ERAD-independent role for RNF5, demonstrating that its control of RBBP4 constitutes an epigenetic pathway that drives AML, and highlight RNF5/RBBP4 as markers useful to stratify patients for treatment with HDAC inhibitors.
AML is a heterogeneous hematological cancer characterized by the accumulation of somatic mutations in immature myeloid progenitor cells. Such mutations alter the self-renewal, proliferation, and differentiation capabilities of these progenitor cells\(^1^,\)\(^2\). The prognosis of AML patients is strongly influenced by the type of chromosomal or genetic alterations and by changes in gene expression\(^1^,\)\(^3\). Although numerous mutations and chromosomal aberrations that drive AML development have been identified\(^4^,\)\(^5\), the molecular components and epigenetic modulators that contribute to AML etiology and pathophysiology are not well defined. Approximately one-third of AML patients fail to achieve complete remission in response to chemotherapy, and 40–70% of those who do enter remission relapse within 5 years. Thus, there is an urgent need to better understand the molecular mechanisms underlying AML development and progression to facilitate the development of more effective therapies.

RING finger protein 5 (RNF5) is an ER-associated E3 ubiquitin ligase and component of the UBC6e-p97 complex, which is implicated in ER-associated degradation (ERAD)\(^6^,\)\(^7\), a pathway involved in maintaining protein homeostasis. RNF5 recognized misfolded proteins and promotes their ubiquitination and proteasome-dependent degradation\(^8^,\)\(^9\). RNF5 expression is relatively increased in several cancers, including breast cancer, hepatocellular carcinoma, and AML\(^10\). RNF5 regulates glutamine metabolism by promoting degradation of misfolded glutamine carrier proteins, a function important in the cancer cell response to ER stress-inducing chemotherapies such as paclitaxel\(^11\). RNF5 promotes degradation of the protease ATG4B, which limits basal amounts of autophagy\(^9\). RNF5 also limits intestinal inflammation by controlling S100A8 protein stability\(^12\). Given the important pathophysiological roles of RNF5, the observation that it is upregulated in AML cells and patient samples prompted us to investigate the possible contribution of RNF5 to the development and progression of this disease.

Histone modification by acetylation contributes to the dynamic regulation of chromatin structure and affects gene expression programs. Histone acetylation status is associated with transcriptional regulation of leukemic fusion proteins, such as AML1-ETO, PML-RARa, and MLL-CBP\(^11^,\)\(^12\). Correspondingly, histone deacetylases (HDACs) are implicated in the etiology and progression of leukemia\(^13\), and HDAC inhibitors can block growth or promote differentiation or apoptosis of leukemia cells\(^14\). The retinoblastoma binding protein 4 (RBBP4) is a component of multi-protein complexes that function in nucelesome assembly and histone modifications, modulate gene transcription, and regulate the cell cycle and proliferation\(^15\). Such complexes include the nucleosome remodeling and deacetylase (NuRD) complex, polycomb repressor complex 2 (PRC2), and switch independent 3 A (SIN3A)\(^15^,\)\(^16\). Overexpression of RBBP4 or HDAC1 correlates with clinicopathologic characteristics and poor prognosis in breast cancer\(^17\), and RBBP4 expression positively correlates with hepatitis metastasis and poor prognosis in colon cancer patients\(^18\). RBBP4 is also implicated in the regulation of DNA repair genes, and its suppression in glioblastoma enhances tumor sensitivity to temozolomide chemotherapy\(^19\). However, the function of RBBP4 in AML has not been studied.

Here, we identified a central role for the RNF5-RBBP4 axis in AML maintenance and responsiveness to HDAC inhibitors. Our data suggest that targeting RNF5 and HDAC pathways represents a therapeutic modality for AML and that RNF5 or RBBP4 abundance could serve as a prognostic marker and means to stratify patients for treatment with HDAC inhibitors.

### Results

Increased abundance of RNF5 in AML patient samples correlates with poor prognosis. Analysis of RNA-seq datasets for various cancer cells in the Cancer Cell Line Encyclopedia (CCLE) database identified a higher copy number and levels of RNF5 transcripts in AML, chronic myeloid leukemia (CML), and T-cell acute lymphoblastic leukemia (T-ALL) relative to other tumor types (Fig. 1a and Supplementary Fig. 1a). Higher levels of RNF5 protein were confirmed in AML and CML cell lines compared with other cancer lines (Supplementary Fig. 1b). To assess the clinical relevance of RNF5 expression in AML, we analyzed levels of RNF5 mRNA and protein in peripheral blood mononuclear cells (PBMCs) from independent cohorts of AML patients. Similar to results in AML lines, the average abundance of RNF5 protein was significantly higher in PBMCs from AML patients relative to control samples (CD34\(^+\) and PBMCs) (Fig. 1b, c, Supplementary Fig. 1c, d). The patient cohort included an equal number of females (24; median age = 59) and males (24; median age = 64.5; Supplementary Table 1). Given that RNF5 is a ubiquitin ligase, its transcript levels are not as reflective of activity as are protein levels, as self-degradation or other post-translational modifications can alter RNF5 subcellular localization, availability and/or activity. Indeed, analysis of patient cohorts revealed a significant increase in RNF5 protein but not transcript levels in patients as compared to healthy subjects (Fig. 1b, c and Supplementary Fig. 1c). Stratification of the 50 patients into two groups based on the top high (n = 8, 15%) and low (n = 42, 85%) RNF5 protein levels revealed that high abundance coincided with poor overall survival (P = 0.05, Fig. 1d). Notably, this difference was not due to blast counts, as they did not differ significantly among patients showing high or low RNF5 levels (Supplementary Fig. 1f, g). Independent analysis of AML patients (n = 154) based on The Cancer Genome Atlas (TCGA) dataset confirmed a significant positive correlation between high RNF5 expression (10%) and poor survival (P = 0.009, Fig. 1e). Notably, there was no pattern of RNF5 abundance that either positively or negatively correlated with the presence of FLT3 or NPM1 mutations (Supplementary Fig. 1h, i), suggesting that the significance of RNF5 activity to AML may not depend on any specific oncogenic driver(s) or activation of particular signaling pathways.

Assessment of an independent AML patient cohort (from the Rambam Health Campus Center, Haifa, Israel which included multiple samples obtained from 5 females with a median age of 62 and 6 males with a median age of 63.5, as detailed in Supplementary Table 1) confirmed higher levels of RNF5 protein in AML patient blood samples (n = 18) relative to samples taken from healthy donors (n = 5) (Fig. 1f, g). Because this cohort included samples taken from patients both prior to and following therapy, we were able to compare RNF5 abundance before and after therapy and at remission or relapse stages. Notably, RNF5 abundance markedly decreased following chemotherapy and during remission (n = 8) (Fig. 1h, i and Supplementary Fig. 1j). Conversely, RNF5 levels at diagnosis were similar to those seen in patients that either relapsed or were refractory to treatment (n = 5) (Fig. 1h, j). These results suggest that RNF5 levels in AML blasts may serve as a prognostic marker for AML.
showed reduced colony formation in soft agar relative to controls (Fig. 2d) and increased abundance of proteins associated with apoptosis, as reflected by levels of cleaved forms of caspase-3 (Fig. 2e) and poly ADP ribose polymerase (PARP) (Supplementary Fig. 2d). Effects of RNF5-KD on U937 and MOLM-13 cells were confirmed in two additional AML cell lines HL-60 and THP-1 (Supplementary Fig. 2e–h). Importantly, re-expression of RNF5 WT, but not the catalytically inactive RING mutant (RNF5 RM), restored cell proliferation (Fig. 2f, g), confirming the specificity of these phenotypes and suggesting that RNF5 catalytic activity is required for AML cell proliferation.

To verify changes seen upon KD, we used CRISPR-Cas9 gene-editing technology to deplete RNF5 in MOLM-13 cells stably expressing Cas9 using RNF5-targeting guide RNAs (sgRNAs).
Relative to control cells transduced with Renilla luciferase-targeting sgRNAs, cells transduced with RNF5-targeting sgRNAs showed impaired growth based on CellTiter-Glo luminescence assay (Fig. 2h, i).

To further assess RNF5 function in AML, we monitored the viability of xenografted patient-derived AML cells (PDX, AML-660) transduced with shRN5 or control constructs. As expected, using two independent shRNF5s (albeit limited KD efficiency), we observed decreased viability of xenografted RNF5-KD cells relative to controls (Fig. 2j-i). These findings confirm our observations in AML cell lines and support the notion that RNF5 downregulation impairs the proliferation of AML blasts.

RNF5 inhibition enhances ER stress-induced apoptosis of AML cells. As RNF5 functions as part of ERAD and the ER stress response, we asked whether changing RNF5 abundance would alter the ER stress response in AML cells. To do so, we exposed RNF5-KD or control MOLM-13 cells to thapsigargin or tunicamycin to inhibit the ER Ca\(^{2+}\)-ATPase (SERCA) or protein glycosylation, respectively, as a mean to induce ER stress. Thapsigargin treatment of MOLM-13 RNF5-KD cells increased apoptotic markers to levels higher than those seen in control cells (Fig. 3a, b). Thapsigargin treatment also decreased the viability of MOLM-13 RNF5-KD cells to a greater extent than seen in control MOLM-13 WT RNF5 cells (Fig. 3c). Tunicamycin treatment also decreased the viability of RNF5-KD HL-60 cells compared to tunicamycin-treated control HL-60 cells (Supplementary Fig. 3a). Consistent with a function in ER stress, RNF5-KD increased levels of transcripts encoding key UPR components, including CHOP, ATF3, and sXBP1, in thapsigargin-treated MOLM-13 (Fig. 3d) and HL-60 cells (Supplementary Fig. 3b), relative to mock-transduced controls.

Given the link between ER stress and protosomal degradation, we assessed potential synergy between RNF5 KD and protosomal inhibition. Indeed, RNF5-KD MOLM-13 cells treated with the proteasome inhibitor bortezomib (BTZ) showed increased levels of apoptotic markers such as cleaved forms of caspase-3 and PARP (Fig. 3e) and decreased viability (Fig. 3f) relative to control-treated cells. Using annexin V and propidium iodide staining, which monitor the degree of programmed cell death, we showed that RNF5-KD also enhanced apoptosis of BTZ-treated HL-60 cells (Fig. 3f), decreasing the BTZ IC\(_{50}\) from 9.6 nM in controls to 5.4 nM in RNF5-KD cells (Fig. 3g). These data suggest that RNF5 plays a role in the response of AML cells to proteotoxic stress.

RNF5 loss delays leukemia establishment and progression. We next asked whether RNF5 activity modulates leukemia growth in vivo. To do so, we used a human AML xenograft model in which luciferase-expressing U937 cells (U937-pGFL) were transduced with doxycycline-inducible shRNF5 or control shRNA before being injected intravenously into NOD/SCID mice (Supplementary Fig. 4a). Following leukemia establishment, as confirmed by bioluminescence, mice were fed a doxycycline-containing diet and monitored for disease progression and overall survival. Interestingly, animals injected with RNF5-KD cells exhibited a markedly decreased leukemia burden and prolonged survival relative to control mice (Fig. 4a, b and Supplementary Fig. 4b). RT-PCR analysis of splenocytes isolated from mice transplanted with RNF5-KD cells confirmed expression of shRNF5 (Supplementary Fig. 4c). Western blot analysis of splenocyte lysates revealed more abundant expression of the cell cycle regulatory protein p27 in shRNF5 relative to control cells (Supplementary Fig. 4d), consistent with our in vitro data and with the delayed leukemia progression observed following RNF5 KD (Fig. 4a, b). Collectively, these data indicate that RNF5 is required for AML cell proliferation in vivo.

We also asked whether RNF5 functions in AML initiation using the MLL-AF9 model for in vitro and in vivo studies. For in vitro analysis, we purified hematopoietic stem and progenitor (Lin-depleted) cells (HSPCs) from the bone marrow of Rnf5\(^{-/-}\), which exhibit normal development and hematopoiesis, and wild-type (WT) C57/BL6 mice. HSPCs from these mice were retrovirally transduced with a bicistronic construct harboring MLL-AF9 linked to a green fluorescent protein (GFP) marker. When we assessed colony-forming capacity (CFC), we found that compared to WT GFP-MLL-AF9 cells, Rnf5\(^{-/-}\) GFP-MLL-AF9 cells exhibited markedly reduced CFC in methylcellulose after 7, 14, and 21 days in culture and observed a striking reduction in the number of blast-like colonies (Fig. 4d, e). These phenotypes are consistent with apparent terminal differentiation of Rnf5\(^{-/-}\) cells, as reflected by a greater cytoplasm/nucleus ratio and more vacuolated cytoplasm (Fig. 4e, f).

To assess leukemogenesis in vivo, we injected sublethally-irradiated WT C57/BL6 recipient mice with GFP-MLL-AF9–transduced Rnf5\(^{WT}\) or Rnf5\(^{-/-}\) cells and monitored cell engraftment by flow cytometry for GFP-positive (GFP\(^{+}\)) cells in peripheral blood (Fig. 4c). Analysis on days 15 and 28 post-injection identified fewer GFP\(^{+}\) cells in mice injected with GFP-MLL-AF9 Rnf5\(^{-/-}\) cells than in mice injected with GFP-MLL-AF9 Rnf5\(^{WT}\) cells, indicating a delay in leukemia development (Fig. 4g). Moreover, mice harboring GFP-MLL-AF9 Rnf5\(^{-/-}\) cells exhibited prolonged survival relative to mice injected with GFP-MLL-AF9 Rnf5\(^{WT}\) cells (Fig. 4h). Collectively, these data show that RNF5 loss decreases the colony-forming capacity of MLL-AF9–transformed pre-leukemic cells in vitro and delays leukemia progression in vivo.
RNF5 activity modulates transcription in AML cells. To identify pathways modulated by RNF5 activity in AML cells, we first monitored transcriptional changes in MOLM-13, U937, and HL-60 AML lines expressing either RNF5-KD or control constructs. RNA sequencing (RNA-seq) analysis identified a total of 237, 814, and 1380 dysregulated genes in MOLM-13, U937, and HL-60, respectively, following RNF5 KD relative to control (RNF5-WT) cells (Fig. 5a, b and Supplementary Data file 1). Ingenuity Pathway Analysis identified selective enrichment of genes implicated in myeloid cell function such as NF-κB signaling, IL-8 signaling, reactive oxygen species, and several pathways related to cell migration such as Rho GTPase and Tec kinase signaling (Supplementary Fig. 5a). Expression of a total of 59 genes (35 up- and 24 downregulated) were significantly altered by RN5F KD in all
three AML lines (Fig. 5a, b). Among upregulated genes were CDKN1A and CDKN2D, which encode cell cycle inhibitors; LIMK1, which encodes a kinase functioning in regulation of the actin cytoskeleton; ANXA1, which encodes a calcium-binding protein functioning in metabolism, EGFR signaling, and cell death programs; and NCF1, which encodes a subunit of NADPH oxidase (Fig. 5c and Supplementary Fig. 5b). Downregulated genes included antiapoptotic BCL2A1, and SAP18, which encodes a histone deacetylase complex subunit functioning in transcriptional repression (Supplementary Fig. 5b). Moreover, such changes were consistent with phenotypic changes seen in RNF5-KD AML cell lines, such as reduced proliferation and increased apoptosis. Interestingly, analysis of the Library of Integrated Network-Based Cellular Signatures (LINCS) drug screening database identified a notable overlap between transcriptomic changes induced by the HDAC1 inhibitor mocetinostat in various cancer cells and those seen in shRNF5-expressing MOLM-13 and HL-60 cells (Fig. 5d). Five and one out of the top ten transcriptional changes identified in LINCS following HDAC inhibition overlapped with those seen following RNF5 KD in MOLM-13 and HL-60 cells, respectively (Fig. 5d, Supplementary Fig. 5c and Supplementary Data file 2). Among commonly affected pathways were activation of GP6 and Rho GTPase signaling and repression of the nucleotide excision repair (NER) pathway (Fig. 5e). These observations suggest that RNF5 may regulate HDAC activity in AML cells.

RNF5 interacts with and ubiquitinates the retinoblastoma binding protein 4. We hypothesized that RNF5 elicits transcriptional changes through intermediate regulatory components(s). Thus, to identify RNF5-interacting proteins or substrates we performed liquid chromatography-tandem mass spectrometry (LC-MS/MS) and compared proteins immunoprecipitated from lysates of MOLM-13 cells expressing inducible Flag-tagged RNF5 versus those expressing empty vector. Among 65 RNF5-interacting proteins identified were previously reported substrates, such as 26S proteasome components, VCP and S100A8/9,10, as well as proteins implicated in AML development, such as DHX1524 and gelsolin25,26 (Supplementary Data file 3). Among the more abundant RNF5-bound proteins were components of ERAD, translation initiation, proteolytic and mRNA catabolic processes (Fig. 5f, Supplementary Fig. 5d, e and Supplementary Data file 3).

Although none of the interacting proteins identified here were transcription factors, epigenetic modifications initiated by changes in RNF5 expression could also underlie changes in gene expression. In fact, we identified one RNF5-interacting protein as the epigenetic regulator histone-binding protein RBBP4 (Fig. 5f and Supplementary Data file 3). Analysis of transcriptome data from TCGA revealed an inverse correlation of RBBP4 expression with expression of genes upregulated in RNF5-KD cells (Fig. 5g and Supplementary Fig. 5f), suggesting that RNF5 positively controls RBBP4 transcriptional regulatory function. RBBP4 is a component of several chromatin assembly, remodeling, and nucleosome modification complexes, including PRC2 and the NuRD co-repressor complex, which contains HDAC1 and HDAC2.28 Indeed, the inverse correlation between RBBP4 expression and RNF5-upregulated genes was mirrored when we analyzed HDAC1, HDAC2, and EZH2 expression relative to RNF5-upregulated genes (Supplementary Fig. 5g–i). Increased RBBP4 expression was also correlated with malignant phenotypes of several human tumors including AML.29 Analysis of tumor data in TCGA revealed high RBBP4 expression in AML, compared with other tumor types (Supplementary Fig. 5j). Assessment of an AML patient cohort confirmed higher RBBP4 expression in samples from AML patients compared to healthy donors (Fig. 5h and Supplementary Fig. 5k). Stratification of AML patients based on RBBP4 expression indicated that high expression (the top 30%) correlated with poor overall survival (Fig. 5i).

If RNF5 positively regulates RBBP4, RBBP4 KD should promote phenotypic changes in AML cells similar to RNF5 KD. Indeed, shRNA-based RBBP4 KD in MOLM-13 and U937 cells impaired their growth (Fig. 5j and Supplementary Fig. 5l), promoted PARP cleavage indicative of apoptosis (Fig. 5k and Supplementary Fig. 5m), and induced expression of genes that were also induced by RNF5-KD (Fig. 5i). Furthermore, when we examined RBBP4 function in vivo using the U937 xenograft model, mice harboring RBBP4 KD in xenografted cells showed delayed AML development and prolonged survival relative to WT-RBBP4 controls, phenocopying changes seen upon RNF5 KD (Fig. 5m, n and Supplementary Fig. 5n, o). Notably, western blot analysis of splenocytes from mice transplanted with RBBP4 KD cells showed that these cells retained RBBP4 expression (Supplementary Fig. 5p). Since RBBP4 KD was confirmed prior to injection of these cells, it is likely that these cells emerged as escapers during in vivo selection (Supplementary Fig. 5n, o). The latter explains the shorter survival observed in mice that harbored the escapers, compared with mice that retained RBBP4 KD (Fig. 5n). Interestingly, similar to outcomes seen in RNF5-KD AML cells, RBBP4 KD blocked the growth of AML, but not CML and T-ALL, cell lines (Supplementary Fig. 6a, b), confirming a link between RNF5 and RBBP4 in the context of AML.

RNF5 is a transmembrane protein primarily associated with the ER, and its ubiquitin ligase domain is located in the cytosol.6,30 We assessed potential interaction between RNF5 and RBBP4 in the HEK293T line by coimmunoprecipitation of ectopically expressed WT RNF5, a catalytically inactive RING mutant (RNF5 RM), or a
was ubiquitinated by WT RNF5, but not by RNF5 RM or RNF5 Flag-tagged RNF5 constructs in HEK293T cells revealed that RBBP4 Co-expression of HA-tagged ubiquitin, Myc-tagged RBBP4, and expressed RNF5 was also con

Reciprocal IP using RBBP4 as bait constructs, suggesting that both the RING and transmembrane domains are dispensable for protein-protein interaction (Fig.6b). Endogenous RBBP4 coimmunoprecipitated with all RNF5 domains are dispensable for protein-protein interaction (Fig.6b).

Next, we assessed potential effect of RNF5 on RBBP4 ubiquitination. Correspondingly, RNF5-KD in HEK293T or MOLM-13 cells decreased RBBP4 ubiquitination relative to controls (Supplementary Fig. 6c, d). Notably, neither RNF5 overexpression nor RNF5 KD altered the abundance of RBBP4 protein, suggesting that RBBP4 ubiquitination by RNF5 does not occur via the formation of proteasome-targeting K48 ubiquitin chains and does not alter RBBP4 stability (Fig. 6f, g and Supplementary Fig. 6e).

Immunoprecipitation of RBBP4 and immunoblot using an antibody specific for the K63 chain topology revealed no notable differences in cells overexpressing any form of RNF5, suggesting domain-dependent) and membrane association are both required for an RNF5-mediated increase in RBBP4 ubiquitination.

C-terminal transmembrane domain deletion mutant (RNF5 ΔCT) (Fig. 6a). Endogenous RBBP4 coimmunoprecipitated with all RNF5 constructs, suggesting that both the RING and transmembrane domains are dispensable for protein-protein interaction (Fig. 6b). Reciprocal IP using RBBP4 as bait confirmed interaction with RNF5 (Fig. 6c). Interaction between endogenous RBBP4 and ectopically expressed RNF5 was also confirmed in MOLM-13 cells (Fig. 6d).

Next, we assessed potential effect of RNF5 on RBBP4 ubiquitination. Co-expression of HA-tagged ubiquitin, Myc-tagged RBBP4, and Flag-tagged RNF5 constructs in HEK293T cells revealed that RBBP4 was ubiquitinated by WT RNF5, but not by RNF5 RM or RNF5 ΔCT (Fig. 6e), indicating that ubiquitin ligase activity (RING
that RNF5 does not induce K63 ubiquitination of RBBP4 (Supplementary Fig. 6f). To assess the linkage-specific poly-ubiquitin induced by RNF5 on RBBP4, we used mutant HA-ubiquitin constructs with only one lysine available for linkage (K-only mutants: K29, K11, K27, K6, and K33) in which all lysine residues except that indicated are mutated to arginine allowing a single type homotypic chain. We then monitored changes in Myc-tagged RBBP4 ubiquitination in cells overexpressing Flag-tagged RNF5. Poly-ubiquitination of RBBP4 was enhanced by RNF5 only in the presence of K29 ubiquitin (Fig. 6h), strongly suggesting that RNF5 induces K29-topology polyubiquitination of RBBP4.
**Fig. 4 RNF5 suppression impairs leukemia establishment and progression in vivo.** a Graph depicting growth in mice of luciferase-expressing U937-pGFL cells transduced with empty vector (pLKO) or inducible shRNF5. Bioluminescence was quantified to monitor disease burden. Data are presented as the mean ± SD of 7 mice per group. $P$ values were determined using an unpaired two-tailed t-test. b Kaplan-Meier survival curves of mice injected with U937-pGFL cells expressing pLKO (n = 7 mice/group) or inducible shRNF5 (n = 7 mice/group). $P < 0.001$ by two-sided Mantel–Cox log-rank test. c Schematic representation of the experiment. Lin−Sca1+c-Kit+ (LSK) cells were purified from the bone marrow of WT or Rnf5−/− mice, transduced in vitro with a GFP-tagged MLL-AF9 fusion gene, and then either analyzed by colony-forming assays in vitro or intravenously injected into sub-lethally irradiated WT C57BL/6 mice. d Quantification of total colonies (left) or blast-like and differentiated colonies (right) of GFP-MLL-AF9-transformed WT or Rnf5−/− cells after 7, 14, or 21 days in culture. Data are presented as the mean ± SD of three independent experiments. $P$ values were determined using a paired two-tailed t-test. e Representative pictures of colonies of GFP-MLL-AF9 expression seen after treatment with HDACi in MOLM-13 cells (made RNF5 deficient using the CRISPR/Cas9 system; Supplementary Fig. 7g). These observations further support the role for RNF5 in sensitizing AML cells to HDAC inhibition.

RNF5 promotes recruitment of RBBP4 to gene promoters. Because RNF5 activity does not alter RBBP4 stability, we asked whether RNF5 affects RBBP4 localization or interactions with other proteins. Subcellular fractionation in MOLM-13 cells and immunofluorescent analyses of nuclear and chromatin-bound RBBP4 did not identify changes in RBBP4 localization following RNF5 KD (Supplementary Fig. 6g and h). Next, since RBBP4 is a component of PRC2 and complexes containing HDACs, we asked whether RNF5 activity alters the formation of these complexes or their recruitment to target gene promoters. Neither overexpression nor KD of RNF5 affected RBBP4 interaction with HDAC1, HDAC2, or EZH2 (Fig. 6i, j and Supplementary Fig. 6i), suggesting that RBBP4 ubiquitination by RNF5 is not required for assembly of RBBP4-containing these complexes.

We then used chromatin immunoprecipitation (ChIP) and quantitative PCR (qPCR) to investigate RBBP4 recruitment to promoters of genes regulated by either RNF5 or RBBP4. RNF5 KD decreased RBBP4 recruitment to ANXA1, NCF1, and CDKN1A promoters (Fig. 6k). Examination of histone modifications at promoters of these genes identified that RNF5 KD increased H3K9 and H3K27 acetylation (Fig. 6l, m) and reduced H3K27 methylation (Fig. 6n), changes indicative of increased gene expression. These changes are consistent with increased expression of these genes following RNF5 KD (Fig. 5c) and suggest that RNF5 control of gene expression in AML cells is mediated by RBBP4.

RNF5 inhibition sensitizes AML cells to HDAC inhibitors. As an independent support for the function of the RNF5-RBBP4 regulatory axis in promoting AML cell growth, we screened for synergistic interactions between RNF5 and epigenetic modulators. To do so, we assessed the effect of 261 epigenetic inhibitors at two concentrations (Supplementary Table 2) on the growth of U937 cells that stably express inducible shRNF5 (Fig. 7a, b). Of epigenetic inhibitors tested, 49 decreased viability of shRNF5-expressing cells relative to control WT RNF5 AML cells (Fig. 7b). Among the 49 inhibitors were several hypomethylation agents, including several histone methyltransferases (such as G9a), histone demethylases (such as Jumonji histone demethylases), and HDAC inhibitors (such as TMC269, pimelic diphenylamide 106, and N-acetyldalinine [CI-994]). Because RBBP4 is a key component of the HDAC complex and given that RNF5 KD induces transcriptional changes comparable to HDAC1 inhibition (Fig. 5d, e), we assessed possible synergy between RNF5 inhibition and HDAC inhibitors. To do so, we selected the HDAC inhibitor CI-994, which is in clinical trials against several cancers (https://www.drugbank.ca/drugs/DB12291), for additional validation. Indeed, U937 and HL-60 cells subjected to RNF5-KD were more sensitive to CI-994 relative to control cells (Fig. 7c and Supplementary Fig. 7a), suggesting that RNF5 KD sensitizes AML cells to HDAC inhibition.

We note that the HDAC inhibitor romidepsin (also known as FK228) did not score positively in our screen. This was likely due to the relatively high concentrations tested, which were lethal to both RNF5-KD and control U937 cells. FK228 has been approved by the Food and Drug Administration (FDA) to treat peripheral T-cell lymphoma and has been investigated in preclinical studies as a potential treatment for AML. Therefore, we re-assessed FK228 at non-lethal concentrations (up to 6 nM for 24 h) using multiple AML cell lines. Notably, when combined with RNF5-KD, FK228 had an additive effect in decreasing cell viability (Fig. 7d, e and Supplementary Fig. 7b–d) and inducing apoptosis (Fig. 7f). We also confirmed the additive effect of RNF5 loss plus treatment with HDACi in MOLM-13 cells (made RNF5 deficient using the CRISPR/Cas9 system; Supplementary Fig. 7e). The additive effect on AML cell death of RNF5-KD plus FK228 treatment was lost upon RNF5 re-expression (Fig. 7g), confirming a specific role for RNF5 in sensitizing AML cells to HDAC inhibition.

Of AML cell lines tested, the MV-4-11 line showed very low levels of RNF5 protein (Supplementary Fig. 1b). MV-4-11 cells were also most sensitive to FK228 treatment (Supplementary Fig. 7g), and RNF5 KD did not increase their sensitivity (Supplementary Fig. 7g). These observations further support the importance of RNF5 abundance in the response of AML cells to HDAC inhibitors. Moreover, RBBP4 KD sensitized AML cells to FK228 treatment (Fig. 7h and Supplementary Fig. 7h), consistent with our findings that RNF5 positively regulates RBBP4. Notably, elevated H3K9 acetylation at the promoters of RNF5- or RBBP4-regulated genes, such as ANXA1 and CDKN1A, was seen following FK228 treatment and further increased upon RNF5 KD (Fig. 7i). The latter finding is consistent with increased ANXA1 and CDKN1A expression seen after treatment with FK228 alone or in combination with RNF5 KD (Fig. 7i and Supplementary Fig. 7i). Notably, RNF5 KD in the K562 (CML) line did not sensitize cells to FK228, and RNF5 KD in Jurkat cells (T-ALL) only slightly enhanced cell sensitivity to FK228 (Supplementary Fig. 7j and k).

Next, to corroborate these findings in primary AML blasts, we performed an ex-vivo analysis of AML patients’ samples (n = 4) and assessed their sensitivity to FK228 treatment. These samples were selected based on RNF5 and RBBP4 protein levels (2 high, 2 low). Interestingly, and similar to phenotypes seen in our KD experiments, samples expressing low RNF5 and RBBP4 were more sensitive (AML-075B logIC50 = −10.7 M, AML-034 logIC50 = −10.4 M) to FK228 treatment, compared with the high-expressing group (AML-013 logIC50 = −9.9 M, AML-072B logIC50 = −9.6 M) (Fig. 7k–m).
Finally, to assess the relevance of combined RNF5 loss and HDAC treatment in patients, we analyzed data from a bioinformatic pipeline that identifies clinically relevant synthetic lethal interactions. This analysis revealed a more favorable prognosis in patients with concomitant downregulation of both HDAC and RNF5 (Fig. 7n), substantiating the sensitization to HDAC in RNF5 low expressing specimens. Collectively, these findings suggest that RNF5 signaling is a critical determinant of AML cell sensitivity to HDAC inhibitors.

**Discussion**

High mortality seen in patients with AML predominantly results from failure to achieve complete remission following
chemotherapy, coupled with a high relapse rate. Here we identify an important role for the ubiquitin ligase RNF5 in AML and demonstrate how RNF5 contributes to this form of leukemia. Our studies establish a function for RNF5 beyond its previously characterized activity in ERAD and proteostasis,6,30 and reveal mechanisms underlying its regulation of gene expression programs governing AML development and response to HDAC inhibitors. The clinical relevance of RNF5 and RBBP4 to AML is supported by our studies of patient samples and genetic mouse models. In mice, RNF5 or RBBP4 depletion inhibited AML progression and prolonged mouse survival (Fig. 4). In human, analysis of AML samples from two independent clinical cohorts revealed that a high abundance of RNF5 protein, which is commonly seen in AML patient samples, correlates with poor prognosis. Those phenotypes are mediated via RNF5 interaction with the chromatin remodeling protein RBBP4, which results in its non-canonical (K29 topology) ubiquitination that promotes RBBP4 recruitment to specific gene promoters (among them, ANXA1, NCF1, and CDKN1A), and a concomitant regulation of genes implicated in AML maintenance. Our finding that RNF5 modulates RBBP4 in a way that alters the expression of AML-related genes is confirmed by our ChIP analysis showing that RNF5 promotes recruitment of RBBP4 to gene promoters. Moreover, an inverse correlation between RBBP4 expression and the expression of genes that were upregulated in RNF5 KD cells was also found in AML samples from the TCGA database (Fig. 5). Future genome-wide assessment of promoter-bound RBBP4 will likely identify additional genes whose transcription is regulated by RNF5-modified RBBP4.

Independent support for a function for RNF5 in recruiting RBBP4 to transcriptional regulatory complexes comes from our finding that RNF5/RBBP4 abundance governs the sensitivity of AML cells to HDAC inhibitors. Correspondingly, transcriptional changes induced by RNF5 KD overlapped with those seen following treatment with HDAC1 inhibitors. Furthermore, AML primary blasts expressing low RNF5/RBBP4 levels were more sensitive to FK228 compared to high-expressing blasts. Along these lines, synthetic lethal analysis identified a favorable prognosis in a cohort of AML patients with low expression of both HDAC and RNF5 (Fig. 7). These findings suggest that RNF5 or RBBP4 abundance may serve as useful markers for the stratification of AML patients for treatment with HDAC inhibitors.

Notably, although RNF5 is expressed at high levels in AML, CML, and T-ALL cell lines, it is critical for cell survival only in AML cells. In fact, the CCLE database reveals that CML and T-ALL lines express on average higher levels of RNF5 than do AML lines. Nonetheless, K-562 (CML) and Jurkat (T-ALL) lines subjected to RNF5 KD do not exhibit growth inhibition or undergo cell death, while similarly treated AML lines do. Likewise, inhibition of RBBP4 does not impact CML or T-ALL cell but rather inhibits AML cell growth in a manner similar to that seen after RNF5 inhibition. Along these lines, RNF5 regulation and function are likely to be cell type and tissue-dependent. Our earlier studies in breast cancer and melanoma further support such context-dependent functions: RNF5 promotes melanoma growth via changes in immune and intestinal epithelial cells, while inhibits breast cancer growth through the tumor-intrinsic expression of glutamine carrier proteins.35,38

It is important to note that relatively high RNF5 expression in AML cell lines is likely due to a high copy number, as shown by analysis of copy number alterations in various cancer cells. The analysis of the TCGA database reveals increases in RNF5 mRNA levels in 3% of patients. Our patient cohorts revealed a significant increase in RNF5 abundance but not transcription levels (Fig. 1b, c and Supplementary Fig. 1c). We therefore believe that RNF5 overexpression (at the protein level) is attributable to relative increases in RNF5 protein stability, as supported by our assessments of two independent AML cohorts (Fig. 1). As a ubiquitin ligase, RNF5 activity is regulated primarily by post-translational modifications affecting its stability, rather than transcription. However, that these increases may be linked to a pre-existing mutation that could increase RNF5 abundance in AML patient PMBCs or to micro-vesicle-based cell–cell communication cannot be ruled out.

Given that RNF5 protein is ER-anchored, its interaction with a chromatin regulatory protein such as RBBP4 is unanticipated. Among that possibilities that may explain such interaction are: (i) The interaction may occur as the RBBP4 gene is translated, prior to nuclear translocation, a mechanism reported for other ubiquitin ligases,35, (ii) An as yet unidentified post-translational modification may promote nuclear localization of RNF5. For example, the possibility that RNF5 undergoes sumoylation should be considered given the high probability predicted using GPS-SUMO tool.49, Finally, (iii) RBBP4/RNF5 interaction may occur at specific phases of the cell cycle, for example, at the entry to mitosis, when the nuclear envelop breaks down and nuclear contents are released to the cytoplasm. Future studies are needed to further examine these possibilities.
In summary, using genetic mouse models and clinical data, our findings establish a central role for the RNF5-RBBP4 axis in AML maintenance and responsiveness to HDAC inhibitors. Our identification of crosstalk between ubiquitination and epigenetic regulation offers a new paradigm for ERAD-independent RNF5 function in controlling RBBP4 activity and subsequent transcriptional networks implicated in AML. Our findings also demonstrate the ability of HDAC inhibitors to treat AML,
particularl AM expressing low levels of RNF5, and provides a method to stratify AML patients for treatment with HDAC inhibitors.

Methods

Animal studies. All animal experiments were approved by the Sanford Burnham Prebys Medical Discovery Institute’s Institutional Animal Care and Use Committee (approval AUP 16-028). Animal care followed institutional guidelines. Rnf5−/− mice were generated on a C57BL/6 background as described23. C57BL/6 WT mice and U937-pGFL cells (2 × 104 per mouse) were injected intravenously. Leukemia burden was serially assessed using noninvasive bioluminescence imaging by injecting mice intraperitoneally (i.p.) with 150 μg/kg D-Luciferin (PerkinElmer, Waltham, MA, USA) and imaging them on an IVIS Spectrum (PerkinElmer). For in vivo RNF5 KD experiments, at disease onset (day 15, as measured by bioluminescent imaging), mice were sedated upon signs of morbidity resulting from leukemic engraftment (>10% weight loss, lethargy, and ruffling fur).

Cell culture. Human HEK293T and A375 cells were obtained from the American Type Culture Collection (ATCC), U937 and K562 cells were kindly provided by Prof. Tsila Zuckerman, and M-4-11, GRANTA-THP-1, and MEC-1 cells were from Dr. Netanel Horowitz. MOLT-13 cells were kindly provided by Dr. Ani Deshpande (SBP Discovery Institute, Houston, TX, USA). THP-1, K562, Jurkat, and RPMI-8226 cultured in RPMI medium; HL-60, M-4-11, K-562, MEC-1, HAP-1, and KG-1a cells were cultured in IMDM; and GRANTA, A375, and HEK293T cells were cultured in DMEM. All media were supplemented with 10% fetal bovine serum (FBS), 1% l-glutamine, penicillin (83 U/mL), and streptomycin (83 μg/mL) (Gibco). Cells were regularly checked for mycoplasma contamination using a luminescence-based kit (Lonza).

Primary AML MDS. AML patient samples were obtained from Scripps MD Anderson, La Jolla, CA (IRB-approved protocol 13-6180), and written informed consent was obtained from each participant. Samples were also obtained from the Rambam Health Campus Center, Haifa, Israel (IRB-approved protocol 0372-17). Fresh blood samples were obtained by a peripheral blood draw, PICC line, or central catheter. Fiblagrumobilized peripheral blood cells were collected from healthy donors and cryopreserved in DMSO. PBMCs were isolated by centrifugation through ficoll-paque™ plus (17-1440-02, GE Healthcare). Residual red blood cells were removed using RBC Lysis Buffer for humans (Alfa Aesar, cat. # J62990) according to the manufacturer’s instructions. The final PBMC pellets were resuspended in Bambanker serum-free freezing medium (Wako Pure Chemical Industries, Ltd) and stored under liquid N2. Patients’ information is provided in Supplementary Table 1.

Mll-AF9 patient-derived xenograft (PDX) samples (from the Jerubens Lab, Munich, Germany) were cultured in IMDM medium with 20% B2 (Stemcell Technologies), human cytokines, and StemRegenin 1 (SRI) and UM171, as described34. Cells were transduced with empty vector or different shRNF5 constructs as described below (see transfections and transsection section) and plated in 100 μL per well of a complete medium in 96-well plates. Growth was monitored every 24 h using the CellTiter Glo reagent.

Animals were injected i.p. with DMSO at 10 mM and serially diluted (1/3, x13 concentrations) in a Labcety Echo Low Dead Volume (LDV) plate. 25 μLs of drugs at 1000x concentration were spotted in quadruplicate in 384-well plates (Greiner #781098) using a Labcety Ech 550 acoustic dispenser, and patient AML cells (described above) were seeded (2.5 x cells/well in 25 μLs) on 3 plates with a Multidrop Combi Reagent Dispenser (Thermo). After 2 days, cell viability was assessed by adding 10 μLs/well of CellTiter-Glo reagent (Promega #G7572) using a Multidrop Combi, and luminescence was read on an EnVision plate reader (Perkin Elmer). Raw data were processed in Microsoft Excel, with cell viability values normalized to percentages relative to vehicle (0.1% DMSO) controls. Data were graphed and subjected to statistical analyses using GraphPad Prism software (v9.1.1).

Plasmas and constructs. Plasmids expressing Flag-RNF5-WT, Flag-RNF5-RM, and Flag-RNF5-ΔCT were described previously35. To generate doxycycline-inducible RNF5-WT, RNF5-RM, and RNF5-ΔCT overexpression vectors, coding sequences were amplified by PCR from pcDNA3.1-RNF5-WT, pcDNA3.1-RNF5-RM, and pcDNA3.1-RNF5-ΔCT, respectively, and the product was inserted into EcoRI-linearized pLVX TetOne-puro plasmid (Clontech) using the NEBuilder HiFi Assembly Kit (New England BioLabs). Expression vectors encoding Myc-RBBP4 (#9532, Cell Signaling Technology, 1:1000), mouse anti-UBiquitin (#3939, Cell Signaling Technology, 1:1000), rabbit anti-Phospho-p21 (Thr14, #2947, Cell Signaling Technology, 1:1000), rabbit anti-p21 (#9215, Cell Signaling Technology, 1:1000), and mouse anti-UBiquitin (#3939, Cell Signaling Technology, 1:1000), rabbit anti-K6-3 linkage Specific Polyubiquitin (#5621, Cell Signaling Technology, 1:1000), rabbit anti-Actin (#4970, Cell Signaling Technology, 1:1000), mouse anti-HSP90 (sc-13119, Santa Cruz Biotechnology, 1:1000), and mouse anti-CALRE4 (sc-66837, Santa Cruz Biotechnology, 1:1000). HRP-conjugated secondary antibodies were from Jackson ImmunoResearch (goat-anti-mouse-HRP (AB_233804) and goat-anti-rabbit-HRP (AB_233798) and diluted 1:10000.

Romipidine and N-acetyladrenaline were purchased from Cayman Chemicals. Thapsigargin and tunicamycin were purchased from Sigma-Aldrich. MG132 was obtained from Selleckchem. Puromycin was purchased from Merck. Annexin V-APC and propidium iodide were from BioLegend.

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Gene silencing. Lentiviral plKO1 vectors expressing RNF5 or RBP4-specific shRNAs were obtained from the La Jolla Institute for Immunology RNAi Center (La Jolla, CA, USA). Sequences were: shRNF5 #1 (TRCN0000004785) GAGTTGTCGACGTTTCAGTATGGAAGCT, shRNF5 #2 (TRCN0000004786) GAGCTGTGCGTCAGTATGGAAGCT, shRNF5 #3 GAGGTGATGTTGAGAGAA, and inducible shRNF5, which has the same sequence as shRNF5#1. Sequences for RBP4-specific shRNAs were: shRBP4#1 (TRCN0000026103) GCTTTCTTTCATCCTTATA, and

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Transfections and transduction. Transient transfections were carried out using CalFectin (SignaGen) according to the manufacturer’s recommendations. Lentiviral particles were prepared using standard protocols. In brief, HEK293T cells were transfected with relevant vectors and the second-generation packaging plasmids ΔR8.2 and Vsv-G (Addgene). Virus-containing supernatants were collected 48 h later and then added in the presence of Polybrene to AML cells pre-seeded at ~5 × 10^5/well in 24-well plates (Sigma-Aldrich). After 8 h, cells were transferred to 10 cm culture dishes for an additional 24 h prior to experiments.
Western blotting. Cells were washed twice with cold PBS and lyzed by addition of Tris-buffered saline (TBS)-lysate buffer (TBS [50 mM Tris-HCl pH 7.5, 150 mM NaCl], 0.5% Nonidet P-40, 1× protease inhibitor cocktail [Merck], and 1× phosphatase inhibitor cocktail II [Sigma]) followed by incubation on ice for 20 min. Blood cells from healthy control subjects and AML patients were lysed using hot lysis buffer (100 mM Tris-HCl pH 7.5, 5 mM sodium dodecyl sulfate [SDS]) followed by incubation 5 min at 95 °C and sonication. Some samples were subjected to fractionation using a subcellular protein fractionation kit (Thermo Scientific Pierce), as indicated. Samples were resolved on SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated for 1 h at room temperature with blocking solution (0.1% Tween 20/5% non-fat milk in TBS) and then overnight at 4 °C with primary antibodies. Membranes were washed with TBS and incubated for 1 h at room temperature with appropriate secondary antibodies (Jackson Immunoresearch). Finally, proteins were visualized using a chemiluminescence method (Image-Quant LAS400, GE Healthcare, or ChemiDoc MP imaging system, BioResearch). Finally, proteins were visualized using a chemiluminescence method. Raw data were analyzed using MaxQuant (v1.5.5.0) with default settings. Protein efficiency was measured in preliminary experiments, and reporter potential contaminants. Cytoscape (version 2.0)242 repository were deposited to filter potential contaminants. Cytoscape (version 3.8.1.3)45 was used to generate the RNFS interaction network and pathway enrichment analysis. Raw MS data were deposited in the MassIVE repository under the accession code MSV000083160.

Immunofluorescence microscopy. Cells were plated on coverslips on glass slides using a StatSpin cytofuge and fixed with 4% paraformaldehyde for 20 min at room temperature. Slides were then rinsed three times in PBS, and cells were permeabilized in 0.2% Triton X-100 in PBS for 5 min and blocked with 0.2% TX-100/100% FBS in PBS for 30 min. Primary antibodies were diluted in staining buffer (0.2% Triton X-100/2% FBS in PBS) and added to cells, and the slides were then incubated overnight at 4 °C in a humidified chamber. Slides were then washed three times in staining buffer, and secondary antibodies (Life Technologies) were diluted in staining buffer and added to slides for 1 h at room temperature in a humidified chamber shielded from light. Finally, slides were washed three times in staining buffer and mounted with Fluoroshield Mounting Medium containing 4′, 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Cells were analyzed using a fluorescence microscope (DMi8; Leica) with a 60× oil immersion objective. Images were processed using the 3D deconvolution tool from LASX software (Leica), and the same parameters were used to analyze all images.

Cell viability assay. Cell viability and growth were assayed using the CellTiter Glo kit (Promega) according to the manufacturer’s recommendations. Cell lines were plated in white 96-well clear-bottomed plates (Corning) at a density of 7 × 10³ cells/well, and growth was monitored every 24 h using CellTiter Glo reagent. Viability was quantified by measuring luminescence intensity with an Infinite 200 Pro reader (Tecan).

Cell cycle analysis. The distribution of cells in each phase of the cell cycle was analyzed by propidium iodide staining (Merck). Briefly, 1 × 10⁶ cells were washed twice with cold PBS and fixed in 70% ethanol in PBS at 4 °C overnight. Cells were washed, pelleted by centrifugation, and treated with RNase A (100 µg/mL) and propidium iodide (40 µg/mL) at room temperature for 30 min. Cell cycle distribution was assessed by flow cytometry (BD LSRFortessa™, BD Biosciences), and data were analyzed using FlowJo software.

Annexin V and propidium iodide staining. Cells were collected in FACS tubes, washed twice with ice-cold PBS, and resuspended in 100 µL PBS. Annexin V-APC (1.4 µg/mL) was added for 15 min at room temperature in the dark. Then, cells were washed in PBS and resuspended in 200 µL PBS, and then propidium iodide (50 µg/mL) was added. Finally, samples were analyzed by flow cytometry (BD LSRFortessa™, BD Biosciences). The gating strategy is provided in Supplementary Fig. 8c.

Colonoy-forming assays. For the soft agar assay, a base layer was formed by mixing 1.5% soft agar (low-melting-point agarose, Bio-Rad) and culture medium at a 1:1 ratio and placing the mixture in 6-well plates. Cells were resuspended in a medium containing 0.3% soft agar and added to the base layer at 1 × 10³ (MOLM-13) or 5 × 10² (U937) cells/well. Agar was solidified by incubation at 4 °C for 10 min before incubation at 37 °C. Plates were incubated at 37 °C in a humidified atmosphere for 18–28 days. Cells were then fixed overnight with 4% paraformaldehyde, washed with PBS, and stained with 0.05% crystal violet (Merck) for 20 min at room temperature, and washed again with PBS. Plates were photographed and colonies were counted on the captured images.

For methylcellulose assay, WT or Rfp⁻/⁻ Lin- Scal⁺ Cell Kit cells transformed with GFP-MLL-AF9 were resuspended in methylcellulose M3234 (Stem Cell Technologies) supplemented with 6 ng/mL IL-3, 10 ng/mL IL-6, and 20 ng/mL stem cell factor (PeproTech). Cells were then added to 35 mm dishes at 10³ cells/well and incubated for 6–7 days. Colonies were classified as compact and hypercellular (blast-like) or small and diffuse (differentiated). Virtually all colonies fell into one of these two categories.

RT-qPCR analysis. RNA was extracted using a GenElute Mammalian Total RNA Purification Kit (Sigma) according to standard protocols. RNA concentration was measured using a NanoDrop spectrophotometer (ThermoFisher). CDNA was synthesized from aliquots of 1 µg total RNA using a qScript CDNA Synthesis Kit (Quanta). Quantitative PCR was performed with SYBR Green I dye master mix (Roche) and a CFX connect Real-Time PCR System (Bio-Rad). Primer sequences are listed in Supplementary Table 3. Primer efficiency was measured in preliminary experiments, and amplification specificity was confirmed by dissociation curve analysis.
Gene targeting using CRISPR/Cas9. RNF5 sgRNAs were cloned into the pKLV2-U6gRNA-(BbsI)-PGKpuro2A5BF-P lentiviral expression vector and transduced into C2C12 myoblasts. All gRNAs were used at the gRNA expression vector as previously described. Briefly, HEK293T cells were co-transfected with pKLV2-U6gRNA-(BbsI)-PGKpuro2A5BF-P and ecotopic packaging plasmids using CalFectin transfection reagent (SigmaGen). Virus-containing supernatants were collected 48 h later. MOLM-13 cells were infected by the addition of supernatants for 4 h. Cells were then selected with puromycin (0.5 μg/mL) for 48 h and viability was measured. The RNF5-targeting sgRNA sequences were: sgRNF5 #3 F-GCACTCTGATACCCGGCGAAAA and R-TTCCCGCCGGGTAACAGGGC and sgRNF5 #4 F-GTGGGCGGGGTTCTAGCTG and R-CACCTGTACCCCGGCGGA.

RNA-seq analysis. PolyA RNA was isolated using the NBE/NexT PolyA (mRNA Magnetic Beads Kit, Madison, WI), and magnetic-attached libraries were constructed using the NEB/NexT Ultra High Yield RNA Library Prep Kit for Illumina (NER, Ipswich, MA). Libraries were pooled and single end-sequenced (1 × 75) on the Illumina NexSeq 500 using the High output V2 kit (Illumina, San Diego, CA). Quality control was performed using Fastqc (v0.11.5, Andrews S, 2010), reads were trimmed for adapters, low quality 5 bases, and the minimum length of 20 using CUTADAPT (v1.1). The number of reads per sample and the number of aligned reads suggested that read quality and number were good and that the data were valid for analysis. High-quality data were then mapped to a human reference genome (hg19) using the STAR mapping algorithm (version 2.5.2a). Feature Counts implemented in Subread (v1.5.30) were used to count the sequencing reads from mapped BAM files. Analyses of differentially expressed genes were subsequently performed using a negative binomial test method (edgeR, v3.34.0) implemented using SARTools R Package (v1.2.0). A list of the differentially expressed genes was exported into an excel file (Supplementary Data file 1), and pathway analysis was performed by uploading the lists of differentially expressed genes to www.genomecommons.org (known as “Bing Pathway”). The list was uploaded into the following formula: 
\[ \text{log}_2(\text{fold-change}) > 0.4 \text{ and } P < 0.05 \]
Pathway list and analysis were performed by uploading the lists of differentially expressed genes to IPA (http://www.ingenuity.com) using the following criteria: | log2(FC) | > 1 and | p-value | < 0.05.

Chromatin immunoprecipitation (ChIP). ChIP analysis was performed using a ChIP Assay Kit (Millipore) following the manufacturer’s instructions. Briefly, 1 × 10^6 cells were used for each reaction. Cells were fixed in 1% formaldehyde at 37 °C for 10 min, and nuclei were isolated with nuclear lysis buffer (Millipore) supplemented with a protease inhibitor cocktail (Millipore). Chromatin DNA was sonicated and sheared to a length between 200 bp and 1000 bp. Sheared chromatin was immunoprecipitated at 4 °C overnight with anti-H3K9ac (9649, Cell Signaling Technology), or anti-H3K27ac (ab5394, Abcam), anti-CCCP27 (9733, Cell Signaling Technology), or anti-RBBP4 (HRP) (Millipore, 07-2808). IgG was used as a negative control and anti-RNA polII (Millipore) served as a positive control antibody. Protein A/G beads-chromatin complexes were washed with low salt buffer, high salt buffer, LiCl buffer, and then TE buffer to eliminate nonspecific binding. Protein/DNA complexes were reverse cross-linked, and DNA was purified using SureClean (s) (Promega, Madison, WI). Enrichment of the indicated DNA regions were calculated using the Percent Input Method according to the manufacturer’s instructions and are presented as % input.

Small molecule epigenetic regulator screen. Aliquots of compounds (10 mM in DMSO) from a library of 261 epigenetic regulators were dispensed at final concentrations of 0.5 μM or 5 μM in the wells of a Greiner (Monroe, NC, Cat #781080) 384-well TC-treated black plate using a Lababyte Echo 555 acoustic pipette (Labyte, San Jose, CA). U937 cells expressing an inducible shRNF5 vector were induced with doxycycline for 72 h and dispensed into the prepared plates at a density of 5 × 10^4/well. CM518-1 cells were selected with puromycin (1 μg/mL) for 48 h, then transfected with the gRNA expression vector as previously described. For the analysis, the intensity of induced shRNF5-expressing cells was divided by the intensity of uninduced cells. Ratios were log2 transformed and thresholds were calculated based on the distribution of the log2 ratios. The upper threshold was calculated as the Q3 + 1.5x(Q3 - Q1), where Q1 is the first quartile and Q3 is the third quartile and Q1 is the interquartile. The lower threshold was calculated as the Q1 - 1.5x(Q3 - Q1), where Q1 is the first quartile. Ratios outside these thresholds were considered outliers from the global ratio distribution and thus were potential candidates for having a differential effect on RNF5-KD or control cells.

MLL-AF9-mediated transformation of bone marrow cells and generation of MLL-AF9-leukemic mice. HEK293T cells were co-transfected with Murine Stem Cell Virus (MSCV)-based MLL-AF9 IRES-GFP22 and ectopic packaging plasmids. Viral supernatants were collected 48 h later and added to Lin- Sca-1^c Kit^c cells isolated from the bone marrow of WT or Rnf5^−/− C57BL/6 mice. Transduced cells were maintained in DMEM supplemented with 15% FBS, 6 μg/mL IL-3, 10 ng/mL IL-6, and 20 ng/mL stem cell factor, and transformed cells were selected by sorting for GFP^+ cells. To generate “primary AML mice,” GFP^− MLL-AF9^− transduced cells were resuspended in PBS at 1 × 10^6 cells/200 μL and injected intravenously into sublethally irradiated (650 Rad) 6- to 8-week-old C57BL/6 female mice.

Statistical analysis. Differences between two groups were assessed using two-tailed unpaired or paired t-tests or Wilcoxon rank-sum test, and differences between group means were evaluated using t-test or ANOVA. Two-way ANOVA with Tukey’s multiple comparison test was used to evaluate experiments involving multiple groups. Survival was analyzed by the Kaplan–Meier method and evaluated with a log-rank test. All data were analyzed using GraphPad Prism version 8 or 9 (GraphPad, La Jolla, CA, USA) and expressed as means ± SD or SEM. P < 0.05 was considered significant. NS stands for not statistically significant.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The RAW MS data have been deposited in the MassIVE repository under accession code MSV00083160 (https://massive.ucsd.edu/ProteoSAFe/dataset.jsp?task=321ee7f174ba890da284d56603). RNA-Seq RAW data in FASTQ format from RNF5 knockdown experiments have been deposited in the NCBI Gene Expression Omnibus (accession code GSE155929). The raw data of TCGA database are available through GDC Data Portal (https://portal.gdc.cancer.gov/). The raw data of LINCS are available through NIH LINCS (https://lincsproject.org/). All other data are available within the article and its Supplementary Information. Source data are provided with this paper.

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References.

1. Cancer Genome Atlas Research, N. et al. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. N. Engl. J. Med. 368, 2039–2074 (2013).

2. Mrozek, K., Heerema, N. A. & Bloomfield, C. D. Cytogenetics in acute leukemia. Blood Rev. 18, 115–136 (2004).

3. Shen, Y. et al. Gene mutation patterns and their prognostic impact in a cohort of 1185 patients with acute myeloid leukemia. Blood 118, 5593–5603 (2011).

4. Dohner, H. et al. Immunophenotypic and molecular analysis of adult acute myeloid leukemia: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood 115, 473–474 (2010).

5. Schrapon, A., Elmore, C., Gruenwald, P., Heimpel, A. & Schrapon, A. Epigenetic and chromatin modifications in myeloid-associated degradation by RNF5-dependent ubiquitination of JNK-associated membrane protein (JAMP). J. Biol. Chem. 284, 12099–12109 (2009).

6. Younger, J. M. et al. Sequential quality-control checkpoints triage misfolded cystic fibrosis transmembrane conductance regulator. Cell 126, 571–582 (2006).

7. Bromberg, K. D. et al. Increased expression of the E3 ubiquitin ligase RNF5 is associated with decreased survival in breast cancer. Cancer Res 67, 8172–8177 (2007).

8. Leon, Y. J. et al. Regulation of glutamine carrier proteins by RNF5 determines breast cancer response to ER stress-inducing chemotherapies. Cancer Cell 27, 354–369 (2015).

9. Tcherpakov, M. et al. Regulation of ATG4B stability by RNF5 limits basal levels of autophagy and influences susceptibility to bacterial infection. PLoS Genet 8, e1003007 (2012).

10. Fujita, Y. et al. Regulation of S100A8 stability by RNF5 in intestinal epithelial cells determines intestinal inflammation and severity of colitis. Cell Rep. 24, 3296–3311 e3296 (2018).

11. Garcia-Manero, G. et al. Phase 1 study of the oral isotype specific histone deacetylase inhibitor MGC0103 in leukemia. Blood 112, 981–989 (2008).

12. Masetti, R., Serravalle, S., Biagi, C. & Pession, A. The role of HDACs inhibitors in leukemia. Leukemia 25, 12109 (2011).

13. Dohner, H. et al. Cytogenetics and therapeutic paradigms. Blood 118, 2059 (2011).

14. Bhalla, K. N. Epigenetic and chromatin modifications in hematologic malignancies. J. Clin. Oncol. 23, 3971–3993 (2005).
Millard, C. J. et al. The structure of the core NuRD repression complex provides insights into its interaction with chromatin. Elife 5, e13941 (2016).

Zhang, Y., Iatrou, R., Erdjument-Bromage, H., Tempst, P. & Reinberg, D. Histone deactetylases and SAP18, a novel polypeptide, are components of a human Sin3 complex. Cell 89, 357–364 (1997).

Guo, Q. et al. Expression of HDAC1 and RBBP4 correlate with clinicopathologic characteristics and prognosis in breast cancer. Int J. Clin. Exp. Pathol. 13, 563–569 (2020).

Li, Y. D., Lv, Z., Xie, H. Y. & Zheng, S. S. Retinoblastoma binding protein 4 up-regulation is correlated with hepatic metastasis and poor prognosis in colon cancer patients. Hepatobiliary Pancreat. Dis. Int. 18, 446–451 (2019).

Kitagane, G. J. et al. Retinoblastoma Binding Protein 4 Modulates Taxololamide Sensitivity in Glioblastoma by Regulating DNA Repair Proteins. Cell Rep. 14, 2587–2598 (2016).

Heckl, B. C. et al. Frequent and reliable engraftment of certain adult primary acute lymphoblastic leukemias in mice. Leuk. Lymphoma Mar. 60, 848–851 (2019).

Breckenridge, D. G., Germain, M., Mathai, J. P., Nguyen, M. & Shore, G. C. Regulation of apoptosis by endoplasmic reticulum pathways. Oncogene 22, 8608–8618 (2003).

Krivosiv, A. V. et al. Transformation from committed progenitor to leukemia stem cell initiated by MLL-AF9. Nature 442, 818–822 (2006).

Delanuay, A. et al. The ER-bound RING finger protein 5 (RNF5/RMA1) causes degenerative myopathy in transgenic mice and is deregulated in inclusion bodies of AD. Proc. Natl Acad. Sci. 105, 23073–23078 (2008).

Pan, L. et al. DHX15 is associated with poor prognosis in acute myeloid leukemia (AML) and regulates cell apoptosis via the NF-kB signaling pathway. Oncotarget 8, 89643–89654 (2017).

Tabe, Y. et al. Novel role of HDAC inhibitors in AML/EoT0 AML cells activation of apoptosis and phagocytosis through induction of annexin A1. Cell Death Differ 14, 1443–1456 (2007).

Watek, M. et al. Hypogolsolinemia in patients diagnosed with acute myeloid leukemia at initial stage of sepsis. Med. Sci. Monit. 25, 1452–1458 (2019).

Kuzmichew, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P. & Reinberg, D. Histone methyltransferase activity associated with a human multiprotein complex containing the enhancer of zeste protein. Genes Dev. 16, 2893–2905 (2002).

Allen, H. F., Wade, P. A. & Kutateladze, T. G. The NuRD architecture. Cell Mol. Life Sci. 70, 3513–3524 (2013).

Casas, S. et al. Changes in apoptosis-related pathways in acute myeloid leukemia. Cancer Genet. Cytogenet 146, 89–101 (2003).

Morito, D. et al. Gp78 cooperates with RMA1 in endoplasmic reticulum-associated degradation of CPTIRdha/508. Mol. Biol. Cell 19, 1328–1336 (2008).

Xu, C. et al. Binding of different histone marks differentially regulates the activity and specificity of polycrom repressive complex 2 (PRC2). Proc. Natl Acad. Sci. USA 107, 19266–19271 (2010).

Liu, Z. et al. Structural basis of plant homeodomain finger 6 (PHF6) recognition by the retinoblastoma binding protein 4 (RBBP4) component of the nucleosome remodeling and deacteylase (NuRD) complex. J. Biol. Chem. 290, 6630–6638 (2015).

Duque-Afonso, J. et al. The HDAC class I-specific inhibitor entinostat (MS-275) effectively relieves epigenetic silencing of the LAT2 gene mediated by AML1/EoT0. Oncogene 30, 3062–3072 (2011).

Clarke, K. et al. The histone deacteylase inhibitor Romidepsin induces as a cascade of differential gene expression and altered histone H3K9 marks in myeloid leukemia cells. Oncotarget 10, 3437–3441 (2019).

Argnani, L., Broccoli, A. & Zinzani, P. L. Cutaneous T-cell lymphomas: Focusing on novel agents in relapsed and refractory disease. Cancer Treat. Rev. 61, 61–69 (2017).

Byrd, C. J. et al. A phase 1 and pharmacodynamic study of depsipeptide (FK228) in chronic lymphocytic leukemia and acute myeloid leukemia. Blood 105, 959–967 (2005).

Lee, J. et al. Harnessing synthetic lethality to predict the response to cancer treatment. Nat. Commun. 9, 2546 (2018).

Li, Y. et al. Gut microbiota dependent anti-tumor immunity restricts melanoma growth in Rnf5(−/−) mice. Nat. Commun. 10, 1492 (2019).

Foot, N., Henschel, T. & Kumar, S. Ubiquitination and the regulation of membrane proteins. Physiol. Rev. 97, 253–281 (2017).

Zhao, Q. et al. GPS-SUMO: a tool for the prediction of sumoylation sites and SUMO-interaction motifs. Nucleic Acids Res 42, W325–W330 (2014).

Pabst, C. et al. Identification of small molecules that support human leukemia stem cell activity ex vivo. Nat. Methods 11, 436–442 (2014).

Mellacherusu, D. et al. The CRA-Pome: a contaminant repository for affinity purification-mass spectrometry data. Nat. Methods 10, 730–736 (2013).

Braten, O. et al. Numerous proteins with unique characteristics are degraded by the 26S proteasome following monoubiquitination. Nat. Protoc. Acad. Sci. USA 113, E4639–E4647 (2016).