Functional screen of MSI2 interactors identifies an essential role for SYNCRIp in myeloid leukemia stem cells

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The identity of the RNA-binding proteins (RBPs) that govern cancer stem cells remains poorly characterized. The MSI2 RBP is a central regulator of translation of cancer stem cells programs. Through proteomic analysis of the MSI2-interacting RBP network and functional shRNA screening, we identified 24 genes required for in vivo leukemia. Syncrip was the most differentially required gene between normal and myeloid leukemia cells. Syncrip depletion increased apoptosis and differentiation while delaying leukemogenesis. Gene expression profiling of Syncrip-depleted cells demonstrated a loss of the MLL and HOXA9 leukemia stem cell program. Syncrip and MSi2 interact indirectly through shared mRNA targets. Syncrip maintains HOXA9 translation, and MSi2 or HOX9 overexpression rescued the effects of Syncrip depletion. Altogether, our data identify Syncrip as a new RBP that controls the myeloid leukemia stem cell program. We propose that targeting these RBP complexes might provide a novel therapeutic strategy in leukemia.

Acute myeloid leukemia (AML) is a genetically complex and heterogeneous set of diseases characterized by a diverse set of mutations3. Despite an increased understanding of the molecular basis of AML pathogenesis, overall survival of adult patients with AML has only improved modestly in the past 30 years2. Leukemia stem cells (LSCs) are a subpopulation characterized by a self-renewal capacity and an ability to recapitulate the phenotypic heterogeneity of the disease3,4. While somatic alterations in genetic and epigenetic mechanisms in leukemogenesis are intensively studied, how post-transcriptional and translational regulation of mRNA/protein expression influences leukemia progression and LSC function remains poorly defined. Post-transcriptional regulation provides the abundance and diversity of the proteome that can contribute to cell fate decisions. RBPs are the central arbiters of this complex regulatory process. Recently, RBPs have emerged as an important class of gene expression regulators in cancer and hematological malignancies5,6. Mutations in genes that encode proteins involved in RNA processing and metabolism—such as DKC1 (ref. 8), RPS19 (ref. 9), and splicing factors10,11—have been shown to...
RESULTS

Pool in vivo shRNA screening of the MSII interactome identified novel regulators of leukemia

To understand which RBPs are required for the survival of myeloid leukemia cells, we conducted an in vivo pooled shRNA screen in MLL-AF9-driven leukemia cells enriched for LSCs. The MLL (mixed-lineage leukemia) gene has been shown to be involved in chromosomal translocations in over 70% of childhood leukemias and 5–10% of leukemias in adults.17

The t(9;11) MLL-AF9 translocation is the most common translocation in AML. In mice, expression of the fusion protein MLL-AF9 in granulocyte–monocyte progenitor cells (GMPs) results in an established, robust, and short-latency leukemia model, where LSCs can be enriched after serial transplantation.18,19 Using the same leukemia model, we previously found that MSII function is required for self-renewal of LSCs.15 Thus, to establish a relevant interacting riboproteomic network, we used MSII as a founding factor and performed mass spectrometry analysis of FLAG-MSII-immunoprecipitated complexes in the human leukemia cell line K562 (Supplementary Fig. 1a). A group of 234 proteins of multiple RBP classes was identified in association with MSII (Fig. 1a and Supplementary Table 1). Functional gene ontology (GO) term analysis linked these 234 proteins to RNA-binding functions, including poly(A) binding and helicase activity (Supplementary Fig. 1b). To obtain a comprehensive assessment of MSII functional networks, we also used data generated from our MSII-associated genomic studies to prioritize genes in different candidate pools for functional screening. Fifty-one genes with differential expression were found in MSII-depleted chronic myeloid leukemia (CML) or AML cell lines, MSII-overexpressing Lin− ‘Scal’− ‘Kit’− (LSK) cells12, and MSII-knockout LSK cells13 were prioritized on the basis of their associations with hematopoietic/leukemic gene sets (Supplementary Table 2), and gene set enrichment analysis leading-edge genes were included in pool 1 (Supplementary Tables 3 and 4). Genes identified as MSII1-binding mRNA targets (Supplementary Table 5) were ranked and evaluated for their relevance to MSII and the hematopoietic system, and 19 genes were included in pool 2 (Supplementary Table 6). Similarly, 58 genes that were discovered from MSII protein–protein interactions and had relevance to hematopoietic/leukemic gene sets were selected for pool 3 (Supplementary Table 7).

In total, we curated a list of 128 genes (Supplementary Table 8) and obtained 5–7 shRNAs targeting each gene transcript. Using a pooled library of titrated shRNA lentiviruses, we transduced sorted LSC-enriched populations (a tertiary transplant of c-Kit−-enriched MLL-AF9-dsRed leukemia) and subsequently transplanted them into sublethally irradiated recipient mice (Fig. 1b). We allowed cells to engraft and then quantified the relative representation of each shRNA in leukemia cells from the bone marrow and spleen at day 0 and day 16 after transplantation (Supplementary Table 9). We ultimately recovered a pool of shRNAs with greater than 20-fold depletion, indicating strong selection against their expression during leukemia progression (Fig. 1c and Supplementary Fig. 1c–f). We prioritized the top 24 hits, each of which had at least five shRNAs that gave 20-fold depletion in both the bone marrow and spleen (Fig. 1d,c and Supplementary Table 10). GO analysis of the top genes showed significant enrichment for RBPs. The majority of the hits (20/24) were in the MSII protein–protein interaction group (pool 3), suggesting that these complexes are important for disease progression (Fig. 1f,g). Among the top 24 scored genes, we selected 7 genes (of which 4 encode RBPs) for in vitro validation, including Syncrip, Caprin, Dyrk2, Hnrrpr, Cct3, Mybpb1, and Hnrrpa3 (Fig. 1h and Supplementary Fig. 1g). For all of the genes tested, we confirmed knockdown and observed a reduction in colony formation (Fig. 1i and Supplementary Fig. 1h). Additionally, MLL-AF9 leukemia cells were generally more sensitive to shRNA depletion than normal cells (c-Kit−-enriched cells from bone marrow), except for Dyrk2, which was equally depleted in both cell types (Fig. 1i). These data suggest that a dysregulated RBP network is differentially required for leukemia cell survival in comparison to normal cells.

Syncrip is required for survival of leukemia cells

Of these in vitro–validated genes, Syncrip demonstrated the most differential effect (tenfold) in colony-forming ability between leukemia cells and normal c-Kit−-enriched cells. Thus, we focused our investigation on Syncrip (sytaptotagmin–binding, cytoplasmic RNA-interacting protein; also known as NSAP1 or hnRNPQ1 in humans), which has three tandem RNA-recognition motifs and is implicated in regulation of RNA processing, transcript turnover, and protein translation.20–23 Several studies suggest that Syncrip may have a role in neuronal morphogenesis.24,25 Yet, the role for Syncrip in cancer or leukemia has not been studied. To further evaluate the effects of Syncrip depletion, we first confirmed that shRNAs specific for Syncrip resulted in the reduction of Syncrip levels by immunoblot in MLL-AF9-transformed leukemia cells (Fig. 2a and Supplementary Fig. 2a). Syncrip depletion in leukemia cells resulted in rapid increase in transformed leukemia cells (Fig. 2b). Syncrip depletion in leukemia cells resulted in rapid increase in myeloid differentiation as demonstrated by increased Gr-1 and Mac-1, F480, and CD115 surface staining (Fig. 2b,c and Supplementary Fig. 2b,c) and cellular morphology (Fig. 2d) at day 4 after transduction. Significant change in c-Kit level was observed for Syncrip-depleted cells with one shRNA (shRNA 2) but not the other shRNA (shRNA 1) (Supplementary Fig. 2d). Syncrip knockdown also resulted in apoptosis of leukemia cells at 5 d after transduction (Fig. 2e and Supplementary Fig. 2e,f), suggesting that differentiating cells subsequently underwent apoptosis. Of note, Syncrip function was not restricted to MLL-AF9-driven leukemia, as we observed a similar reduction in the colony formation of AML-ETO9a-driven leukemia cells depleted for Syncrip (Supplementary Fig. 2g,h). We further performed shRNA-mediated depletion in MLL-AF9 leukemia cells and found a requirement for leukemia in vivo (Fig. 2f), as diseased mice with Syncrip-shRNA-expressing leukemia cells selected for attenuated Syncrip knockdown (Supplementary Fig. 2i,j).

To rule out the potential for off-target effects from shRNA-mediated knockdown and test an additional leukemia line, we developed Syncrip guide RNAs (gRNAs) for CRISPR–Cas9-mediated deletion. RN2 mouse myeloid leukemia cells (MLL-AF9, NRASG12D, and expressing rTAT-RN2 cells; ref. 26) were transduced with vectors expressing inducible (tetO) Cas9 and gRNAs specific for Syncrip or an empty vector (Cas9-EV) and sorted for GFP positivity after induction by doxycycline.
MSI2 direct protein interactors

(a) MSII direct protein interactors

(b) Summary of the pooled shRNA screening strategy from primary leukemia cells. (c) Waterfall plot depicting normalized depletion levels of all shRNAs in bone marrow (BM). Control shRNAs and shRNAs targeting selected candidate genes are highlighted. (d) Venn diagram showing the score of 24 hits in bone marrow and spleen samples. (e) Heat map depicting normalized depletion levels of all shRNAs targeting the top 24 genes scored both in bone marrow and spleen (SP). (f) Pie chart showing the scoring percentage of each screening pool in comparison to the predicted score based on pool representation. (g) GO analysis of the top 24 genes scored in the in vivo screen. (h) The log2 fold depletion in the bone marrow and spleen of all the shRNAs against seven candidate genes in the pooled shRNA screen. Error bars, s.e.m. **P < 0.01, two-tailed t test. (i) Colony formation was impaired in knockdown leukemia cells. The number of colonies formed was normalized to that of control MLL-AF9 leukemia cells or control normal c-Kit+ cells. n = 4 independent experiments; error bars, s.e.m. P values were calculated by two-tailed t test.

Consistent with an on-target effect of CRISPR–Cas9-mediated deletion of *Syncrip*, ectopic expression of human *SYNCRIP* could rescue the reduction in colony formation and reverse the increased differentiation resulting from *SYNCRIP* deletion (Fig. 2k and Supplementary Fig. 2p.q). Therefore, our data strongly suggest that *SYNCRIP* is required for leukemic cell growth, cell survival, and maintenance of the undifferentiated state.

CRISPR–Cas9-mediated deletion of *SYNCRIP* impairs leukemogenesis but not normal hematopoiesis

To further assess the function of *SYNCRIP* in normal and malignant hematopoiesis in vivo, we developed mice deficient for *SYNCRIP* using a CRISPR–Cas9 approach (co-injection of gRNAs and Cas9 mRNA into the pronuclei of mouse zygotes). We then collected fetal liver cells from developed embryos in pseudo-mothers at embryonic day (E) 14 and determined fetal liver genotypes by PCR (Fig. S3, a, Supplementary Fig. 3a, and Supplementary Table S11). We performed analysis on confirmed wild-type and CRISPR–Cas9-mediated knockout samples and found equivalent frequencies of phenotypic hematopoietic
stem cells (HSCs) in wild-type versus knockout animals (Fig. 3c and Supplementary Fig. 3b) and a modest increase in granulocyte–macrophage colony-forming units (CFU-GM) in the knockout mice (Supplementary Fig. 3c). We then performed analysis of SYNCRIP function during normal hematopoiesis in vivo by monitoring the engraftment efficiency of wild-type and knockout transplanted cells.
in recipient mice. We confirmed reduction of SYNCRIP protein expression in engrafted bone marrow cells from knockout as compared to wild-type recipients (Fig. 3c). We observed no defect in engraftment of the knockout cells in primary transplant mice (Fig. 3f). To determine whether SYNCRIP is required for development of leukemia in vivo, we then isolated LSK cells from the bone marrow of Syncrip wild-type and knockout primary transplanted mice, transduced them with MLL-AF9-GFP-expressing viruses, and injected GFP+ MLL-AF9-transformed cells into recipient mice (Fig. 3g). We also performed secondary bone marrow transplantation of Syncrip wild-type and knockout bone marrow cells into lethally irradiated mice and found a mild reduction in engraftment (Fig. 3h,i). On the other hand, MLL-AF9-transformed LSK cells derived from knockout recipients showed a delay in leukemogenesis in vivo when compared to cells from wild-type recipients (Fig. 3j).

However, we found that expression of SYNCRIP was maintained when the animals died of leukemia (Fig. 3k and Supplementary Fig. 3d). These data imply that the CRISPR-driven knockout fetal liver samples were mosaic for wild-type Syncrip and Syncrip knockout, resulting in outgrowth of the residual leukemia cells with wild-type SYNCRIP. Overall, these data indicate at requirement for SYNCRIP for the development of leukemia in a genetic mouse model.

**SYNCRIP is highly expressed in and essential for human leukemia cells**

To define the role of SYNCRIP in human leukemia, we surveyed a previously published expression data set and found that SYNCRIP expression was elevated in patients with AML who had diverse genetic alterations in comparison to normal hematopoietic stem and
Figure 4  SYNCRIP is highly expressed in human AML cells, and SYNCRIP depletion results in inhibition of cell growth and apoptosis in human AML cells. (a) SYNCRIP is upregulated in samples from patients with AML. The graph shows the log_2-transformed expression of SYNCRIP from transcriptional profiling of bone marrow cells from patients with various subtypes of AML and of normal hematopoietic stem and progenitor cells (HSPCs) from healthy donors. AML, n = 142; AML inv(16)/t(16;16), n = 27; AML t(11q23)/MLL, n = 38; AML ETO, n = 39; APL, n = 37; HSC, n = 8; HPC, n = 4; Prog, n = 9; B cells, n = 5; Mono, n = 14; T cells CD4/CD8, n = 10. Error bars, s.e.m. ****P < 0.0001, two-tailed t-test. (Hemaexplorer data of SYNCRIP probe 209024_s_at from the U133 Plus 2.0 array.) (b) SYNCRIP is highly expressed in multiple human AML cell lines. Immunoblot of various myeloid leukemia cell lines in comparison to cord blood–derived CD34+ cells. (c) Primary AML samples expressing SYNCRIP. Actin serves as a loading control. (d–g) Cell proliferation in the indicated cell lines after transduction with lentivirus expressing control or SYNCRIP-specific shRNAs: scrambled control (black) and two shRNAs (shRNA 1 and 2; blue). n = 3 independent experiments per cell line; error bars, s.e.m. ***P < 0.001, two-tailed t-test. (h) Annexin-V staining assessed by flow cytometry 24 h after puromycin selection. n = 3 independent experiments per cell line; error bars, s.e.m. *P < 0.05, **P < 0.001, two-tailed t-test.

SYNCRIP and MSI2 co-regulate LSC gene expression programs
To better understand the molecular function of SYNCRIP in leukemia, we performed RNA sequencing on MLL-AF9 leukemia cells transduced with shRNAs against Syncrip 4d after transduction. The transcriptional profile of cells transduced with Syncrip shRNA was significantly altered, and the results obtained from two independent shRNAs were highly correlated (Supplementary Fig. 5a).

We found 282 genes that were differentially expressed, where 57 were downregulated (Syncrip was ranked as the ninth most downregulated gene) and 225 were upregulated (log_2 (fold change) > 1.5, false discovery rate (FDR) < 0.01; Fig. 5a and Supplementary Table 12). We then functionally annotated our RNA sequencing analysis by performing GSEA on all curated gene sets in the Molecular Signatures Database (MSigDB; 3,256 gene sets) in combination with an additional set of relevant gene sets (92 gene sets from our experimentally derived or published hematopoietic self-renewal and differentiation signatures) using the ranked list of differentially expressed genes in Syncrip shRNA samples (control/Syncrip shRNA; Supplementary Table 13). Genes upregulated after SYNCRIP depletion were enriched in 236 gene sets and downregulated genes were enriched in 172 gene sets (Supplementary Tables 14–16). A set of genes downregulated in HSCs (CD133+ versus CD133−)28, an LSC-related gene signature associated with good prognosis in AML31, and the myeloid development program were significantly enriched for upregulated genes in SYNCRIP-depleted cells (Fig. 5b–d and Supplementary Table 16). Moreover, we found that MLL-AF9 direct targets were enriched for genes downregulated after SYNCRIP depletion (Fig. 5e,f). Consistent with the MLL program being reversed upon Syncrip knockdown, genes negatively regulated by HOX-A9 and MEIS1 were enriched for genes suppressed by SYNCRIP (Fig. 5g). Overall, these data suggest that SYNCRIP depletion results in loss of the HSC/LSC program and the MLL-AF9 gene expression program. On the basis of previous studies and our data, we hypothesized that SYNCRIP and MSI2 might co-regulate the LSC/MLL epigenetic program. Consistent with this observation, genes downregulated after SYNCRIP depletion were...
Figure 5 SYNCRIP regulates the myeloid LSC gene expression program. (a) Gene expression heat map of the top 191 upregulated and downregulated genes from RNA sequencing analysis of MLL-AF9 leukemia cells transduced with control shRNA and shRNAs against SYNCRIP. n = 3 biological replicates. (b–i) GSEA analysis showing the gene expression signature for genes enriched in HSCs (b) and LSCs (c) and genes upregulated in the myeloid development program (d) enriched in Syncrip-knockdown cells and MLL-AF9 direct target genes (e,f) and HOXA9–MEIS1 target genes (g) downregulated in Syncrip-knockdown cells. (h.i) Enrichment of MSII target genes among SYNCRIP-regulated genes.

SYNCRIP and MSII interact through shared mRNA targets

To further probe the interaction between SYNCRIP and MSII, we performed reciprocal immunoprecipitations in MSII-overexpressing K562 cells to confirm SYNCRIP as an interacting partner of MSII, based on our mass spectrometry data. We detected the interaction by reciprocal immunoprecipitation using antibodies against either MSII or SYNCRIP. Interestingly, we found that the interaction was RNA dependent, as treatment of the lysate with RNase diminished the interaction of the two proteins (Fig. 6a). A similar interaction was observed in MOLM13, a myeloid leukemia cell line carrying MLL-AF9 fusion protein (Supplementary Fig. 6a). To investigate whether SYNCRIP and MSII share the previously validated targets of MSII in MLL-AF9-driven leukemia15, we performed RNA immunoprecipitation and found that SYNCRIP could also bind Myc, Hoxa9, and Iκzf2 mRNA (Fig. 6b).

SYNCRIP regulates HOXA9 expression post-transcriptionally

Next, we sought to determine whether SYNCRIP regulates expression of these candidate genes. Targeted depletion of SYNCRIP with CRISPR–Cas9 or shRNA in multiple myeloid leukemia cells (RN2 cells, mouse dsRed MLL-AF9 cells, and MOLM13 cells) significantly reduced HOXA9 (Fig. 6c,d and Supplementary Fig. 6b–c). A decrease in MYC and IKZF2 was observed with CRISPR–Cas9 depletion of SYNCRIP in RN2 cells (Fig. 6c), while reduction in MYC was observed at 4 d after transduction in comparison to 3 d after transduction in both dsRed MLL-AF9 cells and MOLM13 cells (Fig. 6d and Supplementary Fig. 6b,c). While these changes occurred at the protein level, we also observed variable reductions in the mRNA levels of Hoxa9, Myc, and Iκzf2 (Supplementary Fig. 6d,e,h–j). As previously described, MSII depletion reduced HOXA9 and MYC protein expression and downregulated Hoxa9, Myc, and Iκzf2 mRNA levels at 4 d after transduction, similar to the phenotype observed in SYNCRIP-knockdown cells (Supplementary Fig. 6f,g).

To further understand the mechanism for SYNCRIP regulation of HOXA9 expression, we examined the effects of SYNCRIP depletion on total RNA but found no change in SYNCRIP-depleted cells in comparison to control cells (Supplementary Fig. 6k). Additionally, reduced HOXA9, MYC, and IKZF2 protein levels in SYNCRIP-depleted cells were not due to an effect on mRNA stability, as the mRNA levels of these genes were equivalent after the addition of actinomycin D to block transcription (Supplementary Fig. 6l,m). Moreover, measurement of newly synthesized proteins based on AHA incorporation showed a significant decrease in AHA-labeled HOXA9 protein in SYNCRIP-depleted cells. Despite its short half-life, MYC labeling at day 3 remained unchanged (Fig. 6e), and global peptide synthesis was modestly increased as determined by quantification of total OP-Puro incorporation (Fig. 6f,g). These data suggest that SYNCRIP in part controls the translation of specific targets, including HOXA9.

HOXA9 is a functional target of SYNCRIP in leukemia cells

In support of SYNCRIP and MSII co-regulating the MLL-associated transcriptional program, MSII overexpression could rescue the reduced colony formation and reversed the reduction of HOXA9 after
shRNA-mediated Syncrip depletion (Fig. 7a and Supplementary Fig. 7a,b). Verifying the functional relationship between Syncrip and HOXA9, retroviral HOXA9 overexpression partially reversed the reduction in colony formation of dsRed MLL-AF9 cells after Syncrip depletion (Fig. 7b,c and Supplementary Fig. 7c,d). Similarly, overexpression of HOXA9 also rescued the cell growth in Syncrip-knockdown MOLM13 cells (Fig. 7d,e). In contrast, forced MYC expression failed to rescue the effects of Syncrip depletion (Supplementary Fig. 7e–h). Interestingly, MYC protein levels were also reduced after Syncrip depletion but remained higher than the controls after shRNA depletion. These data suggest that Syncrip regulates the translation of the MLL and LSC program in part through its control of HOXA9 expression.

To directly demonstrate the relevance of Syncrip function in human leukemia, we knocked down Syncrip expression in primary leukemia cells derived from a patient with AML (Supplementary Table 17) by transducing cells with control shRNA or Syncrip-targeting shRNA. We monitored engraftment of the cells in vivo after transplantation of sorted GFP+ transduced cells into recipient mice. Depletion of Syncrip protein expression resulted in a marked reduction in engraftment of human CD45*GFP+ double-positive leukemia cells at week 10 (for shRNA 1) and week 16 (for both shRNAs 1 and 2) (Fig. 7f). Notably, HOXA9 expression in the primary AML cells correlated with the extent of Syncrip shRNAs depletion (Fig. 7g). Altogether, these data indicate that Syncrip regulates myeloid leukemia cell proliferation at least in part through regulating the expression of HOXA9, a critical target in leukemia (Fig. 7h).

**DISCUSSION**

Our study uncovered a functionally dysregulated riboproteomic network in myeloid leukemia by performing an in vivo shRNA screen for the MS2 interactome. We identified several RBPs, including Syncrip, that were essential for the survival of myeloid leukemia cells. Other studies have used similar approaches to identify novel epigenetic regulators in leukemia, for example, BRD4 (ref. 33), SIRT-1 (ref. 34), and JMJD1c (ref. 19), or to identify unknown functions of cancer-associated genes in leukemia, for example, ITGB3 (ref. 18). We uncovered an RBP-associated network that allowed us to further explore the functional interactions between its components, in particular MS2 and Syncrip. We found that MS2 and Syncrip co-regulated the myeloid leukemia self-renewal post-transcriptional program, including many of the downstream targets of MLL-AF9, specifically, HOXA9 (Fig. 7h). RBPs act in concert to orchestrate the regulatory processes of protein expression in a manner similar to that of many well-characterized functional complexes that mediate epigenetic regulation.

Syncrip binds and regulates the expression of the mRNA targets of MS2, including **Hoxa9**,** Ikzf2**, and **Myc**. Mechanistically, we also demonstrated that Syncrip regulates synthesis of HOXA9 protein, one of the major functional downstream targets of the MLL-AF9 transcriptional program. These data indicate that Syncrip shares with MS2 a set of common target genes, which are critical for leukemia. However, it is likely that Syncrip also controls expression of genes that are not associated with MS2 or the MLL-associated network. Future studies that directly profile
SYNCRIP’s binding targets would further expand understanding of SYNCRIP’s function in leukemia.

The function of SYNCRIP in normal development and in cancer is poorly characterized. It has been mostly studied in the context of neuronal tissue and mRNA trafficking in neurons. Recently, it has been shown that SYNCRIP is involved in the riboproteomic network and in the regulation of mRNA trafficking in neurons. SYNCRIP has been found to be overexpressed in leukemia and cancer cells, which suggests that it may play a role in the development and progression of these diseases. In normal cells, SYNCRIP is expressed at low levels, while in cancer cells, it is expressed at higher levels. This suggests that SYNCRIP may be a potential therapeutic target for the treatment of cancer.

In summary, as the roles for RBPs in leukemia and cancer have become clinically relevant, these proteins represent a new class of targets for therapeutic interventions. Small-molecule inhibitors that specifically block RBP binding to RNAs or antisense oligonucleotides (ASOs) that can knock down these proteins could be potential strategies for targeting these RBP complexes. Overall, we propose that targeting the riboproteomic network in leukemia could be a new therapeutic strategy in cancer.
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COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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25. 875
ONLINE METHODS
Mass spectrometry methods and analysis (for pool 3). K562 cells expressing either MSCV-IRE-5′GFp (control) or FLAG-MSI2 were grown in RPMI medium with 10% FBS. Immunoprecipitation with anti-FLAG antibody was performed as previously described13. Immunoprecipitate from 150 million cells per experiment was run on an SDS–PAGE gel and stained with Coomassie blue. The gel was then cut into six slices for the control or eight equal slices for FLAG-MSI2, except for the slice that encompassed MSI2 (slice 6), by the Children’s Proteomics Core Facility and then run on a Thermo Fisher Orbitrap XL top 6 with data-dependent acquisition using a 60-min gradient. Control and MSI2-interacting proteins were considered to be represented if there were two or more peptides found and there was high confidence with a MASCOT score of equal to or greater than 20 or 77. Pairwise analysis was performed for two independent immunoprecipitation experiments and mass spectrometry analyses. TOPGENE was performed with 234 MSI2 direct binding targets.

Intestinal MSI1 CLIP–CHIP used for pool 2 gene prioritization. UV-crosslinked mouse intestinal cells were used for immunoprecipitation of MSI1 (ab9397, Millipore). The CLIP protocol was similar to that used in Park et al.29, with the exception that RNA was randomly primed and hybridized to the Affymetrix 1.0ST array. Fold enrichment was ranked over control IgG.

Pool description for the gene prioritization for the in vivo shRNA screen. The number of genes used in our screen was determined on the basis of our previous studies, which included approximately 100 genes22. Therefore, the genes in each pool were prioritized according to the following criteria. Pool 1 was made up of two subpools, 1a and 1b. Pool 1a was composed of differentially expressed genes prioritized on the basis of Ms2 gene expression and other hematopoietic gene sets with a matrix score of 6 or greater (Supplementary Table 3). Pool 1b was selected on the basis of genes found in the leading edge taken from the GSEA with MSI2 overexpression in LSK cells overlapped with genes upregulated after shRNA depletion in CML/AML and other hematopoietic gene sets with a matrix score of 6 or greater. (Supplementary Table 5). These genes were ranked if they were MSI2 target genes and hematopoietic-relevant genes. A combined score of 3 or more was included into the screen (Supplementary Table 6).

Pool 3 included direct protein interactors of MSI2. A matrix score of 3 or more was more on the basis of the ability of proteins with this score to interact with MSI2 and was included in an MSI2 or hematopoietic-relevant gene set (Supplementary Table 7). A list summarizing the genes included in the shRNA screen is provided in Supplementary Table 8.

Lentiviral production, infection, and in vivo shRNA screen. Lentiviruses expressing shRNAs in the pLKO.1 vector were obtained from the RNAi Consortium at the Broad Institute. Virus production and preparation of pooled and single-shRNA viruses were performed as previously described15. Approximately 200,000 cells were infected with a lentivirus expressing shRNA directed against MSI1 or control shRNA. Transduced cells were analyzed by FACS for GFP positivity. 200,000 GFP + cells and 250,000 GFP− cells were sorted for transplantation and analyzed for integration by PCR with primers specific for the detection of Synergos exons 3–4. DNA was resolved on a 1.5% agarose gel. 500,000 cells from confirmed B6 CD45.2 + wild-type and C57BL/6 mice were transduced with lentiviruses expressing a puromycin resistance gene and shRNAs against Syncrip or control shRNA. Transduced cells were selected with 2 µg/ml puromycin for 2 d. 50,000 selected cells were injected retro-orbitally into female C57BL/6 recipient mice (6–8 weeks old) that had been sublethally irradiated with 475 Gy. All animal studies were performed on animal protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the Memorial Sloan Kettering Cancer Center.

CRISPR–Cas9 approach to create Syncrip–knockout mice. The CRISPR gRNAs used to delete exons 3 and 4 of the Syncrip gene were designed using the approach of Romanienko et al.41. The sequence for the 5′ gRNA was 5′-GTACCTGATTACCCAAATGC-3′, and the sequence for the 3′ gRNA was 5′-CAATTGGAGATTGCACGAC-3′. Both were produced in vitro transcription using the pU6T7 promoter in the hybrid plasmid described. To initiate cleavage of the target locus in mice, gRNA C67 and gRNA C69 in conjunction with Cas9 mRNA were co-injected into the pronuclei of mouse zygotes at a concentration of 50 ng/µl each, using conventional techniques43. Deleted samples were assayed using PCR primers located outside of the gRNA cleavage sites (outside of Syncrip exons 3–4), thereby revealing the size of the deletion on the basis of the nucleotide length of the amplicon obtained (~900 bp for wild-type versus ~300 bp for the deletion).

Isolation of fetal liver cells. Fetal liver cells were isolated and placed in single-cell suspension according to standard protocols42. ~200,000 fetal liver cells after red blood cell lysis were used for DNA extraction with HotSHOT genomic DNA preparation methods. 2 µl of supernatant containing DNA was used for PCR reactions with primers specific for the detection of Syncrip exons 3–4. DNA was resolved on a 1.5% agarose gel. 500,000 cells from confirmed B6 CD45.2+ wild-type and Syncrip–knockout fetal livers were retro-orbitally injected into lethally irradiated B6 CD45.1+ Pep Boy, a C57BL/6 congenic strain carrying the differential Ptprca pan-leukocyte marker CD45.1, or Ly5.1+ recipient mice. For secondary transplantation, 1 million bone marrow cells were retro-orbitally injected into lethally irradiated CD45.1+ recipient mice.

Generation of MLL-AF9 primary leukemia and transplantation. Recipient mice at 6–10 weeks of age previously transplanted with cells from wild-type (WT) or CRISPR-KO fetal livers (KO) were used. Bone marrow cells were isolated and subsequently enriched for c-Ki-r+ cells. c-Ki-r+–enriched cells were stained with a lineage antibody cocktail (antibodies to CD3, CD4, CD8, Gr-1, B220, CD19, and Ter119 conjugated with PeCy5) and with Sca1–Pac Blue, CD34–FITC, SLAM–APC, CD48–PE, and c-Ki–APC-Cy7 antibodies. LSK cells were sorted using a BD FACSAria instrument. Sorted cells were grown overnight in SEM medium with 10 ng/ml IL-3, 10 ng/ml IL-6, 50 ng/ml SCF, 10 ng/ml thrombopoietin (TPO), and 20 ng/ml FL/T3. Cells were transduced twice with supernatant containing retroviruses encoding MLL-AF9 and GFP (a gift from S. Armstrong, Memorial Sloan Kettering Cancer Center) on retrovirus-coated 96-well flat-bottom plates. The cells were expanded for 1 week in GFM3434 methylcellulose (Stemcell Technologies). MLL-AF9–transformed cells were sorted for GFP positivity. 200,000 GFP+ cells and 250,000 helper cells were injected retro-orbitally into each lethally irradiated 6– to 8-week-old C57BL/6 mouse.

 Colony-forming assays. 10,000 cells were plated on MethoCult GFM3434 (Stemcell Technologies). Colonies were scored every 5 d for leukemia cells and every 7 d for normal c-Ki-r–enriched bone marrow cells.

In vivo transplantation of leukemia cells. MLL-AF9 tertiary mouse leukemia cells were transduced with lentiviruses expressing a puromycin resistance gene and shRNAs against Syncrip or a control shRNA. Transduced cells were selected with 2 µg/ml puromycin for 2 d. 50,000 selected cells were injected retro-orbitally into female C57BL/6 recipient mice (6–8 weeks old) that had been sublethally irradiated with 475 Gy. All animal studies were performed on animal protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the Memorial Sloan Kettering Cancer Center.

Tibia and femurs, pelvic bones, and arm bones from leukemic or wild-type MLL-AF9–transduced B6 CD45.1+ Pep Boy, a C57BL/6 congenic strain carrying the differential Ptprca pan-leukocyte marker CD45.1, or Ly5.1+ recipient mice. For secondary transplantation, 1 million bone marrow cells were retro-orbitally injected into lethally irradiated CD45.1+ recipient mice.

Isolation, infection, and selection of mouse leukemia and normal cells. Tibia and femurs, pelvic bones, and arm bones from leukemic or wild-type C57BL/6 mice (6–8 weeks old) were collected, crushed, filtered and subjected to red blood cell lysis (Qagen). To isolate c-Ki– cells, bone marrow cells were incubated with anti-CD117 Microbeads (Miltenyi Biotec), according to the manufacturer’s instructions, and then subjected to positive selection using an autoMACS Pro Separator. Cells were spininfected in RPMI medium with 10% FBS and cytokines: SCF (10 ng/ml), IL-3 (10 ng/ml), IL-6 (10 ng/ml), and GM-CSF (10 ng/ml). 48 h after transduction, cells were treated with 2 µg/ml puromycin. Two days after the start of puromycin selection, cells were collected for further analysis.

Syncrip–knockout mice. Syncrip knockout mice were backcrossed to B6 mice to remove the floxed alleles. Syncrip flox/flox mice were crossed with LSK cells were sorted using a BD FACSAria instrument. Sorted cells were grown overnight in SEM medium with 10 ng/ml IL-3, 10 ng/ml IL-6, 50 ng/ml SCF, 10 ng/ml thrombopoietin (TPO), and 20 ng/ml FL/T3. Cells were transduced twice with supernatant containing retroviruses encoding MLL-AF9 and GFP (a gift from S. Armstrong, Memorial Sloan Kettering Cancer Center) on retrovirus-coated 96-well flat-bottom plates. The cells were expanded for 1 week in GFM3434 methylcellulose (Stemcell Technologies). MLL-AF9–transformed cells were sorted for GFP positivity. 200,000 GFP+ cells and 250,000 helper cells were injected retro-orbitally into each lethally irradiated 6– to 8-week-old C57BL/6 mouse.
Proliferation assays for human leukemia cells. Human leukemia cells were infected with viruses encoding scrambled shRNA or shRNAs against SYNCRIP by spinfection of the cells grown in RPMI medium with 10% FBS together with viral supernatant. After 48 h of infection, cells were treated with 3 μg/ml puromycin. Two days after the start of puromycin selection, cells were plated at 250,000 cells/ml for proliferation assays. Cells were counted everyday using the MUSE cell analyzer (Millipore) after plating. Cell growth was calculated by normalizing the number of cells to the number of cells at plating. All cell lines were purchased from ATCC, authenticated by Genetica, and tested negative for mycoplasma contamination.

Intracellular staining and flow cytometry. For intracellular staining, cells were fixed with 1.5% paraformaldehyde at room temperature for 15 min and permeabilized with ice-cold methanol. Cells were washed three times with PBS and incubated with anti-SYNCRIP antibody (MAB11004, Millipore; 1:200 dilution) in 2% FBS in PBS for 1 h at room temperature. Cells were then washed twice with PBS and incubated with secondary antibody conjugated with Alexa Fluor 647 (Molecular Probes) for 30 min at room temperature. Cells were washed with PBS and resuspended before analysis using a BD Fortessa instrument.

Cells were stained using Mac1-PE, Gr1-APC, F480-PE-Cy7, CD115-APC, and c-Kit-APC-Cy7 antibodies and analyzed on a BD FACSM LS Fortessa instrument to assess the differentiation status of wild-type and knockdown leukemia cells. For stem and progenitor cell analysis of fetal liver hematopoietic cells, 1 million cells were stained with a stem/progenitor cell antibody panel including a lineage antibody cocktail (antibodies to CD3, CD4, CD8, Gr-1, B220, CD19, and Ter119 conjugated with PerCy5) and Sca-1–Pac Blue, c-Kit-APC-Cy7, CD34–FITC, CD16/CD32-PE-Cy7, CD48-PE, and SLAM-CD150-APC antibodies. For analysis of engraftment in recipient mice, 1 million bone marrow cells were stained with the stem/progenitor cell antibody panel and CD45.1-PE–Texas Red and CD45.2-A700 antibodies.

To measure apoptosis, cells were washed with PBS and incubated with anti-Annexin-V-PE (BD Biosciences) in Annexin-V binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 4 mM KCl, 0.75 mM MgCl2, 1 mM CaCl2) in a reaction volume of 100 μl for 15 min. DAPI was added before analysis using a BD Fortessa instrument.

O-Propargyl-puromycin flow analysis. Cells were plated at a density of 100,000 cells/ml and treated with 50 μM O-propargyl-puromycin (OP-Puro; NU-931-05, Jena Bioscience). Control cells were treated with 150 μg/ml cycloheximide for 15 min. Cells were washed twice before collection and subjected to processing using the Click-it Flow Cytometry Assay kit (C10418, Invitrogen) following the manufacturer’s instructions. Labeled cells were analyzed using a BD Fortessa instrument.

Immunoprecipitation and immunoblot analysis. K562 cells were collected by centrifugation at 277g for 5 min at 4 °C, washed twice with PBS, and then resuspended thoroughly at 2 × 106 cells/ml in 1× RIPA buffer (BP-115, Boston Bioproducts) with freshly added DTT (1 mM) and proteinase inhibitor cocktails. The supernatant was collected after the mixture was spun at 20,817g for 30 min at 4 °C. For each immunoprecipitation assay, 250 μl of cell extract was mixed with 750 μl of 1× RIPA buffer containing 2 μg of anti-SYNCRIP antibody (Millipore, MAB11004) or 2 μg of anti-MSI2 antibody (Abcam, ab 76148) and 50 μl of agarse beads. For RNA-independent assays, lysates were treated with RNase A (1 μg/ml) for 30 min at 37 °C before the coimmunoprecipitation reactions. After rotating at 4 °C overnight, beads were washed five times with 1× RIPA buffer and boiled with 1× Laemmli protein running buffer.

For immunoblot analysis, cells were collected and washed twice with cold PBS before collection. Approximately 250,000 cells were resuspended and lysed in 40 μl of 1× Laemmli protein running buffer and boiled for 5 min. Whole-cell lysates were separated by 4–15% SDS–PAGE and transferred to nitrocellulose membrane. Membranes were blocked for SYNCRIP (MAB11004 or 05-1517, Millipore; 1:1,000 dilution), IKZF2 (sc-9864, Santa Cruz Biotechnology; 1:1,000 dilution), HOXA9 (07-178, Millipore and ab140631, Abcam; 1:1,000 dilution), MYC (5605, Cell Signaling Technology; 1:1,000 dilution), MSII2 (ab76148, Abcam; 1:1,000 dilution), and actin (A3854, Sigma-Aldrich; 1:10,000 dilution).

RNA immunoprecipitation. 30 × 106 RN2 leukemia cells were used for RNA immunoprecipitation using the Magna RIP RNA-binding protein immunoprecipitation kit (03-115, Millipore). First, cells were washed with cold PBS and then lysed. Five micromolars of anti-mouse or anti-SYNCRIP antibody (18E4, Millipore) incubated with magnetic beads was used to immunoprecipitate MSI2 and SYNCRIP. After the immunoprecipitated complexes were washed, they were treated with proteinase K. RNA extraction was performed by the phenol–chloroform method, and purified RNA was converted to cDNA using the Verso cDNA kit (Thermo Scientific). qPCR was used to validate target mRNAs bound by MSI2 and SYNCRIP.

mRNA stability analysis. Control and SYNCRIP-depleted cells were treated with 5 μg/ml actinomycin D and collected at indicated time points. Total RNA was isolated using the RNeasy RNA extraction kit. 200 ng of RNA was used for reverse transcription and quantitative real-time PCR for Syncrip, Hoxa9, Ikarf2, Myc, and Actb. Actb served as a control housekeeping gene. Relative mRNA levels were normalized to levels at the starting point of treatment.

Metabolic labeling and capture of newly synthesized protein. Newly synthesized proteins were labeled using the Click-it Protein Labeling kit (Invitrogen). For this, 48 h after infection with corresponding shRNA-expressing plasmids, 1 × 107 selected MOELM13 cells were cultured at 1 × 106 cells/ml in fresh medium for 14 h. After one wash with PBS, cells were resuspended in methionine-free RPMI 1640 medium (Gibco) supplemented with 10% dialyzed FBS (Gibco) for 30 min, at which point the methionine analog L-azidohomoalanine (AHA) was added (50 μM; 14 h) to allow incorporation of AHA into nascent proteins. Cells were collected and lysed in 50 mM Tris-HCl, pH 8.0, and 1% SDS with protease and phosphatase inhibitor mixes (cOmplete and PhosSTOP, Roche). 150 μg of total protein (up to 50 μl of lysate) was used in the cross-linking of AHA-labeled nascent proteins to alkyne-derivated bifidol in Click-it Protein Reaction Buffer (Invitrogen) according to the manufacturer’s instructions. The resulting precipitated total protein pellet was resolubilized in 100 μl of 1% SDS in PBS with protease inhibitors by pipetting, vortexing, and incubating at 70 °C for 10 min. The SDS was then quenched by the addition of 100 μl of 6% NP-40 in PBS with protease inhibitors. After centrifugation at 15,000g for 5 min at room temperature to remove any insoluble particles, biotin-cross-linked nascent proteins were captured overnight with streptavidin-coated Dynabeads (M-280, Invitrogen) and eluted from the beads by boiling the samples for 5 min in 2% SDS loading buffer for Western Blot. Previously, beads were thoroughly washed first with PBS containing 0.1% BSA and 2% NP-40 and finally with PBS. The whole volume of AHA-labeled, biotin-cross-linked, streptavidin–precipitated protein was separated by SDS–PAGE together with lysate depleted of nascent protein after streptavidin incubation and input lysates.

RNA purification and quantitative real-time PCR. Total RNA was isolated using TRizol and the Qiagen RNeasy Plus mini kit. cDNA was generated from RNA using iScript cDNA Synthesis (Bio-Rad, 1708891) with random hexamers according to the manufacturer’s instructions. Real-time PCR reactions were performed using an ABI 7500 sequence detection system. qPCR for actin was performed to normalize for cDNA loading. Relative quantification of the genes was performed using the 2−ΔΔCt method as described by the manufacturer.

RNA sequencing. Total RNA was isolated from nine individually transduced and processed tertiary transplant MLL-AF9 mouse leukemia cells (n = 3 for each group, including shRNA against luciferase and two shRNAs against SYNCRIP) using TRizol and the Qiagen RNeasy Plus mini kit. RNA was denatured, and the first strand of cDNA was synthesized using oligo(dT)–primer–containing Illumina-compatible linker sequence. After removal of RNA, the second cDNA strand was synthesized with a random decamer containing another Illumina-compatible linker sequence. Illumina-compatible annealing sequences and external barcodes were introduced during amplification of the libraries.

Differential expression and pathway analyses. Quality control for raw reads was performed using FastQC (v0.11.2) to make sure there were no major flaws in sequencing. Reads were then mapped to the mm10 genome using STAR (v2.3.0c, r291) with default parameters. The mapped reads were counted
using htseq-count (v0.6.0; parameter --t exon) and gene models from Ensembl (Mus_musculus.GRCm38.75.gtf). Differential expression analysis was performed using DESeq2 (v1.2.10; default parameters).

**Statistical analysis.** Student’s t test was used for significance testing in the bar graphs, except where stated otherwise. A two-sample equal-variance model with normal distribution was used. The investigators were not blinded to the sample groups for all experiments. P values less than 0.05 were considered to be significant. Graphs and error bars reflect means + s.e.m., except where stated otherwise.

For animal studies, survival probabilities were estimated using the Kaplan–Meier method and compared with the log-rank test. Ten female mice per group were chosen to have an estimated 80% power in detecting a greater than 1.50 s.d. difference in means at a significance level of $\alpha = 0.05$ using a two-sided test. All animals were randomly assigned to the experimental groups.

All statistical analyses were carried out using GraphPad Prism 4.0 and the R statistical environment.

**Data availability.** We used gene expression data sets previously published from Hemaexplorer[27]. RNA sequencing data in this study have been deposited into the Gene Expression Omnibus (GEO) under accession GSE74178. Mass spectrometry data are available from [http://doi.org/10.5281/zenodo.375632](http://doi.org/10.5281/zenodo.375632).

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