Targeting the RNA m6A reader YTHDF2 selectively compromises cancer stem cells in acute myeloid leukemia

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Targeting the RNA m\(^6\)A Reader YTHDF2 Selectively Compromises Cancer Stem Cells in Acute Myeloid Leukemia

Graphical Abstract

Highlights
- YTHDF2 is highly expressed across human AML and is essential for leukemia initiation
- YTHDF2 shortens the half-life of m\(^6\)A-modified transcripts in AML
- Loss of YTHDF2 expands HSCs but does not derail hematopoiesis
- YTHDF2 protects AML cells from apoptosis by downregulating TNFR2

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In Brief
Elimination of cancer stem cells in acute myeloid leukemia (AML) while preserving hematopoiesis is a challenge in leukemia treatment. Paris et al. demonstrate that inactivation of the RNA m\(^6\)A reader YTHDF2 increases the half-life of m\(^6\)A-modified transcripts and specifically compromises cancer stem cells, highlighting YTHDF2 as an essential regulator of AML.
Targeting the RNA m\(6\)A Reader YTHDF2 Selectively Compromises Cancer Stem Cells in Acute Myeloid Leukemia

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SUMMARY

Acute myeloid leukemia (AML) is an aggressive clonal disorder of hematopoietic stem cells (HSCs) and primitive progenitors that blocks their myeloid differentiation, generating self-renewing leukemic stem cells (LSCs). Here, we show that the mRNA m\(6\)A reader YTHDF2 is overexpressed in a broad spectrum of human AML and is required for disease initiation as well as propagation in mouse and human AML. YTHDF2 decreases the half-life of diverse m\(6\)A transcripts that contribute to the overall integrity of LSC function, including the tumor necrosis factor receptor Tnfrsf2, whose upregulation in Ythdf2-deficient LSCs primes cells for apoptosis. Intriguingly, YTHDF2 is not essential for normal HSC function, with YTHDF2 deficiency actually enhancing HSC activity. Thus, we identify YTHDF2 as a unique therapeutic target whose inhibition selectively targets LSCs while promoting HSC expansion.

INTRODUCTION

Hematopoiesis critically depends on hematopoietic stem cells (HSCs), which possess unique self-renewal capacity and multineage differentiation potential, replenishing all blood lineages (Orkin and Zon, 2008). Acute myeloid leukemia (AML) is an aggressive clonal disorder of hematopoietic stem and progenitor cells (HSPCs) in which the acquisition of mutations by HSPCs results in a block in their myeloid differentiation and the generation of self-renewing leukemic stem cells (LSCs) (Döhner et al., 2015). LSCs initiate and propagate the disease, and given that they are treatment resistant, they often fuel disease relapses. Therefore, identification of specific therapeutic targets for LSC elimination is an unmet clinical need.

Emerging evidence indicates an involvement of mRNA N6-methyladenosine (m\(6\)A) modification, the most abundant internal mRNA modification (Desrosiers et al., 1974; Perry and Kelley, 1974), in hematopoietic specification, differentiation, and pathogenesis of AML (Barbieri et al., 2017; Li et al., 2017; Vu et al., 2017; Weng et al., 2018; Zhang et al., 2017). The m\(6\)A modification is deposited by the m\(6\)A methyltransferase complex (m\(6\)A writer) composed of a METTL3 and METTL14 heterodimeric enzymatic core and their regulator, WTAP (Bokar et al., 1997; Liu et al., 2014; Ping et al., 2014; Tuck, 1992; Wang et al., 2014b), and reversed by m\(6\)A demethylases (FTO and AlkBH5; Jia et al., 2011; Zheng et al., 2013) referred to as m\(6\)A erasers. Recent studies revealed the requirement for METTL3, METTL14, and FTO in leukemic transformation and established the importance of m\(6\)A modification in AML (Barbieri et al., 2017; Li et al., 2017; Vu et al., 2017; Weng et al., 2018). However, while m\(6\)A modification regulates mRNA processing, translation, and degradation (Fu et al., 2014), the functional contributions of these m\(6\)A-dependent processes to leukemic transformation have not been explored.

The outcome of RNA m\(6\)A modification is executed by the YTH (YT521-B homology) domain proteins (known as readers), including nuclear YTHDC1 (Xiao et al., 2016a; Xu et al., 2014) and cytoplasmic YTHDF1–YTHDF3 and YTHDC2 (Shi et al., 2017; Tanabe et al., 2016; Wang et al., 2014a, 2015). Nuclear YTHDC1 regulates mRNA splicing and nuclear export (Xiao
et al., 2016a). While YTHDF1 and YTHDF3 binding to m⁶A enhances mRNA translation (Shi et al., 2017; Wang et al., 2015), YTHDF2 recognizes m⁶A mRNA within the GACU/A consensus to mediate degradation of m⁶A transcripts (Du et al., 2016; Wang et al., 2015). Although previous studies perturbing the whole m⁶A pathway have established its significance in AML pathogenesis (Barbier et al., 2017; Li et al., 2017; Vu et al., 2017; Weng et al., 2018), the functions of specific m⁶A readers in leukemia remain unexplored. However, recent studies implicated Ythdf2 in the regulation of HSC homeostasis and hematopoietic regeneration (Li et al., 2018; Wang et al., 2018). Here, we reveal that targeting YTHDF2 extends the half-life of m⁶A-modified transcripts to selectively compromise AML initiation and propagation without derailing normal hematopoiesis.

**RESULTS**

**Ythdf2 Is Essential for LSC Development and AML Initiation**

We found that YTHDF2 was expressed significantly higher across AML samples with diverse cytogenetic abnormalities compared to non-leukemic controls (Figure 1A), and YTHDF2 protein was highly expressed in primary AML samples (Figure 1B). We next compared YTHDF2 expression in datasets from AML cells with LSC activity and AML cells without LSC activity validated by xenotransplantation (Ng et al., 2016) and found that YTHDF2 expression correlated with LSC activity (Figure 1C). Given that the majority of CD34⁺ and a minority of CD34⁺ fractions have LSC activity (Eppert et al., 2011; Sarry et al., 2011), we also compared YTHDF2 expression between these fractions and found that YTHDF2 was expressed at higher levels in CD34⁺ fractions (Figure S1A). To investigate the requirement for YTHDF2 in leukemogenesis, we employed conditional genetics and a mouse AML model in which Meis1 and Hoxa9, oncogenes frequently overexpressed in human AML (Drabkin et al., 2002; Lawrence et al., 1999), drive leukemogenesis. In this model (Figure 1D), HSPCs are transduced with retroviruses co-expressing Meis1 and Hoxa9 and serially replated, generating preleukemic cells, which upon transplantation to recipient mice generate self-renewing LSCs, causing AML (Guitart et al., 2017; Kroon et al., 1998; Vukovic et al., 2015). We utilized the conditional and reporter Yhdf2 fl/fl mouse allele in which exon 2 of Yhdf2 was flanked by loxp sites and GFP was inserted after the start codon of Yhdf2 in exon 1, generating a fully functional GFP-YTHDF2 fusion protein (Ivanova et al., 2017). We combined the Yhdf2 allele with Vav-iCre (de Boer et al., 2003) to generate Ythdf2CKO, Vav-iCre (Ythdf2CKO) mice in which Yhdf2 is specifically deleted in the hematopoietic system shortly after the emergence of HSCs (Figures 1E and 1F). Ythdf2CKO and control Ythdf2fl/fl (Yhdf2CKO) mice showed normal Mendelian distribution (Yhdf2fl/fl x Ythdf2fl/fl, Vav-iCre matings resulted in 65 Ythdf2CKO and 47 Ythdf2CKO mice at weaning; p = 0.28) and had comparable survival. We transduced Ythdf2CKO and Ythdf2CKO HSPCs with Meis1-Hoxa9 retroviruses and found that while Ythdf2CKO cells produced significantly lower colony numbers upon serial replating (Figure 1G), they had unaffected expression of c-Kit, CD11b, and Gr-1 (Figure 1H). Notably, Yhdf2-deficient preleukemic cells generated AML with substantially longer latency compared to control cells (Figures 1I and 1J). The loss of YTHDF2 expression was confirmed in Ythdf2CKO cells isolated from moribund recipient mice (Figure 1K). To enumerate LSCs in the leukemic recipients of Meis1-Hoxa9-transduced Ythdf2CKO and Ythdf2CKO cells, we performed a limiting dilution assay with donor-derived CD45.2⁺ bone marrow (BM) cells isolated from primary recipients. We found that LSC frequency in recipients of Ythdf2CKO cells was significantly decreased (Figure 1L). Therefore, Yhdf2 is required for LSC development and AML initiation.

To test whether Yhdf2 is required for leukemic transformation driven by other oncogenes, we used PML-RARA, which causes acute promyelocytic leukemia, and MOZ-TIF2, which is associated with AML with inv(8)(p11q13). Serial replating assays revealed that Ythdf2CKO c-Kit⁺ cells transduced with either PML-RARA or MOZ-TIF2 retroviruses failed to efficiently generate colonies (Figure 1M). Thus, Ythdf2 is essential for leukemic transformation driven also by other oncogenes.

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**Figure 1. YTHDF2 Is Upregulated in Different AML Subtypes and Is Essential for AML Development**

(A) YTHDF2 gene expression in control (CTL) and different cytogenetic subgroups of human AML bone marrow samples. Violin plots show the distribution of log₂ expression values. Horizontal line in the boxplots indicates median. CNG, cytologically normal with good prognosis; CNI, cytologically normal with intermediate prognosis; CAO, cytologically abnormal not otherwise specified.

(B) Western blot of YTHDF2 in normal human CD34⁺ cells and AML samples (karyotype details are shown in STAR Methods) (left). -Histone 3 (H3) was used as a loading control. Quantification of YTHDF2 normalized to H3 expression is presented (right).

(C) YTHDF2 gene expression in primitive AML cell compartments with (LSC⁺) and without (LSC⁻) leukemic engraftment potential.

(D) Control Yhdf2fl/fl (Yhdf2CKO) and Yhdf2fl/fl, Vav-iCre (Ythdf2CKO) fetal liver (FL) c-Kit⁺ cells were co-transduced with Meis1 and Hoxa9 retroviruses and serially replated. c-Kit⁺ preleukemic cells were transplanted into recipient mice (n = 12–14). (E) A representative histogram showing GFP-YTHDF2 protein expression in Ythdf2CKO FL LSK cells and the lack of GFP-YTHDF2 expression in Yhdf2CKO FL LSK cells.

(F) Percentage of GFP-positive cells in the 14.5 days post coitum (dpc) FL LSK cell population from FLs of Ythdf2CKO and Yhdf2CKO embryos (n = 5).

(G) CFC counts at each replating (n = 3).

(H) Percentage of CD11b⁺Gr-1⁻, CD11b⁺Gr-1⁺, and c-Kit⁺ cells in the preleukemic cell compartment (n = 4–5).

(I) Percentage of CD45.2⁺ leukemic cells in the PB of recipient mice (n = 12–14 per genotype).

(J) Survival curve of recipients transplanted with preleukemic cells (n = 12–14).

(K) Percentage of GFP-positive cells in the CD45.2⁺ cell population from moribund recipients of Ythdf2CKO and Yhdf2CKO (n = 5–6).

(L) Limiting dilution assay (LDA). Secondary recipients (n = 5–8) were transplanted with indicated doses of CD45.2⁺ BM cells from primary recipients.

(M) Ythdf2CKO and Yhdf2CKO FL c-Kit⁺ cells were transplanted with MOZ-TIF2 or PML-RARA retroviruses and serially replated. CFC counts at each replating are shown (n = 3).

Data represent mean ± SEM; *p < 0.05; **p < 0.01; ****p < 0.0001.
A

CD45.2* FL

Mels1/Hoxa9

Ythdf2* (Ythdf2^+/+)

Ythdf2* (Ythdf2^+/-)

Ythdf2* (Ythdf2^-/-)

Meis1/avr activation

c-Ki67* cells

primary recipients

LSCs

secondary recipients

B

% of GFP cells in CD45.2* cell compartment

% of CD45.2* leukemic cells in secondary recipients

D

Surviving animals (%)

Days after transplantation

E

% of GFP cells in CD45.2* cell compartment

after 2^* transplantation

F

YTHDF2 mRNA

YTHDF2

H3

G

Cell number (X 10^6 cells/ml)

0 24 48 72 96

H

Apoptotic cells (%)

I

% of cells

CD11b^- CD14^+

CD11b^- CD14^+

CD11b^+ CD14^+

CD11b^+ CD14^+

J

Human CD45.2* CD33* cells (%)

BM Liver Spleen PB

K

Surviving animals (%)

Days after transplantation

L

Colony number (x 10^6)

AML1 AML2 AML3

M

Primary AML cells

(legend on next page)
**Ythdf2 Is Critical for AML Propagation**

We next asked whether acute deletion of Ythdf2 from established LSCs using Mx1-Cre impacts LSC maintenance and leukemia propagation. We generated experimental Ythdf2^{fl/fl}; Mx1-Cre (Ythdf2^{CKO}, Mx1-Cre) and control Ythdf2^{fl/fl} (Ythdf2^{CTRL}) mice, transduced HSPCs with Meis1-Hoxa9 retroviruses, and transplanted them into lethally irradiated primary recipients (Figure 2A). Upon leukemia development, c-Kit+ cells (a population enriched for LSCs; Somervaille and Cleary, 2006) were isolated, and given the leakiness of Mx1-Cre upon transplantation (Velasco-Hernandez et al., 2016), the population was further sorted for GFP+ cells to enrich for those expressing YTHDF2 (Figure 2B). While Ythdf2^{CTRL} c-Kit+GFP+ cells showed significant engraftment and caused aggressive AML in secondary recipients (Figures 2C and 2D), Ythdf2^{CKO} c-Kit+GFP+ cells lost YTHDF2 expression (Figure 2E) due to spontaneous Mx1-Cre activation (even without the administration of polyinosinic-polycytidylic acid [pIpC]) and failed to efficiently engraft and propagate the disease (Figures 2C and 2D). Therefore, YTHDF2 is critical for LSC maintenance.

**Targeting YTHDF2 Disables Human AML Cells**

To investigate the requirement for YTHDF2 in human leukemic cells, we knocked down the expression of YTHDF2 in human AML THP-1 cells harboring MLL-AF9 translocation using two independent short hairpins targeting YTHDF2. YTHDF2 knockdown (Figure 2F) inhibited their proliferative capacity (Figure 2G) and increased their apoptosis (Figure 2H) but had no impact on their myeloid differentiation (Figure 2I). This finding was corroborated in NMO-1 AML cells harboring MLL-AF9 translocation (Figures S1B and S1C). THP-1 cells with YTHDF2 knockdown had compromised capacity to engraft AML (Figure 2J) and displayed impaired ability to cause fatal AML (Figure 2K). Finally, we performed knockdown experiments in independent human primary AML samples and found that YTHDF2 depletion significantly decreased the clonogenic potential of AML cells in colony-forming cell (CFC) assays (Figures 2L and 2M). Thus, YTHDF2 is necessary for human AML cell survival and leukemic cell engraftment.

**Ythdf2 Deletion Does Not Derail Normal Hematopoiesis**

We next investigated whether Ythdf2 deletion has any detrimental effects on HSC functions and multilineage hematopoiesis. To determine the YTHDF2 expression at different levels of the hematopoietic differentiation hierarchy, we employed Ythdf2^{CTRL} mice harboring the GFP-YTHDF2 fusion protein (Ivanova et al., 2017). All hematopoietic cells in adult BM expressed GFP-YTHDF2 (Figures 3A and S2A). YTHDF2 was highly expressed in Lin- Sca-1- c-Kit+ (LSK) stem and/or progenitor cells, HSCs, multipotent progenitors (MPPs), primitive hematopoietic progenitors (HPC-1 and HPC-2 populations), and myeloid progenitors, and its expression was decreased in differentiated Lin+ cells (Figures 3A and S2A).

Peripheral blood (PB) analyses of Ythdf2^{CKO} mice revealed modest decreases in numbers of white blood cells (WBCs), red blood cells (RBCs), B cells, and CD8+ T cells and elevated platelet levels (Figure 3B). Apart from a decrease in CD8+ T cells, Ythdf2^{CKO} mice had essentially normal numbers of differentiated cells in their spleens (Figure S2B). We found unaffected numbers of granulocyte/macrophage progenitors (GMPs), increased numbers of pre-megakaryocyte/erythroid progenitors (pre-MegEs) and megakaryocyte progenitors (MkPs) and an imbalance between pre-colony forming unit-erythroid (pre-CFU-E) and colony forming unit-erythroid (CFU-E) (Figure S2C). CFC assays showed normal differentiation potential of Ythdf2^{CKO} BM cells (Figure 3C). Thus, YTHDF2 is not critical for steady-state hematopoiesis.

**Ythdf2 Loss Results in HSC Expansion**

We next investigated the impact of Ythdf2 deletion on stem and progenitor cells. Adult Ythdf2^{CKO} mice displayed expansion of LSK cells, HSCs, and HPC-1 and HPC-2 progenitor cells compared to Ythdf2^{CTRL} mice (Figures 3D and 3E). We also inducibly ablated Ythdf2 using Mx1-Cre, which upon pIpC injection acutely deletes Ythdf2 in Ythdf2^{CKO} adult mice (Figure 3F). Acute Ythdf2 deletion (Figure 3G) had no impact on mouse survival (data not shown) or multilineage hematopoiesis (Figures 3H and 3I; Table S1) and resulted in increased numbers of LSK cells, but not myeloid progenitor cells (Figure 3J). Thus,
hematopoiesis-specific Ythdf2 ablation during development or acute deletion in adult mice leads to an expansion of the primitive cell compartment at the top of the hematopoietic hierarchy and does not derails normal hematopoiesis.

To reveal the repopulation capacity of Ythdf2-deficient HSCs, we competitively transplanted HSCs from Ythdf2CKO and Ythdf2WT mice into lethally irradiated recipients. HSCs of both genotypes gave equal overall long-term reconstitution (Figure 3K). However, while Ythdf2CKO HSCs had enhanced myeloid lineage reconstitution capacity, they had normal B cell and compromised T cell reconstitution potentials (Figure 3K). Strikingly, Ythdf2CKO HSCs displayed significantly increased capacity to contribute to the BM HSC and progenitor cell compartments and differentiated cell compartments (Figures 3L and 3M). The analyses of donor-derived compartment of the recipients revealed increased frequencies of Ythdf2CKO LSK, HPC-1, and HPC-2 cells (Figure S2D). The myeloid bias of Ythdf2-deficient HSCs and its connection to a shift in balance among the HSCs, MPP, and HPC populations upon Ythdf2 deletion merit future investigation. Therefore, targeting Ythdf2 promotes stem or primitive progenitor cell expansion and enhances their reconstitution and myeloid differentiation potentials.

YTHDF2 Decreases m6A RNA Stability in AML

We next sought to understand the mechanism by which YTHDF2 loss impedes LSC function. YTHDF2 is known to promote transcript decay through deadenylation (Du et al., 2016; Wang et al., 2014a). Indeed, the loss of YTHDF2 resulted in deregulated gene expression with 754 upregulated and 528 downregulated genes (p < 0.05) in Ythdf2CKO compared to Ythdf2WT preleukemic cells (Figure 4A). Gene Ontology analysis of deregulated genes in preleukemic cells revealed generic metabolic processes in the upregulated genes and immune response processes in the downregulated genes (Figure S3A). To understand which of the deregulated transcripts could be direct targets of YTHDF2, we determined transcriptome-wide mRNA m6A in Ythdf2WT and Ythdf2CKO preleukemic cells. This revealed the expected m6A consensus motif as well as distribution of m6A within the transcriptome and enrichment around the stop codons within transcripts in both genotypes (Figures S3B–S3D). Furthermore, Ythdf2 deficiency did not alter any of these parameters (Figures S3B–S3D). YTHDF2 loss is expected to result in the upregulation of direct target transcripts; indeed, we observed an enrichment for m6A occupancy in the significantly upregulated genes (p < 0.05; 754 genes) in Ythdf2CKO preleukemic cells compared to the corresponding unchanged or downregulated gene sets (Figure 4B). Reciprocally, we analyzed the transcriptome based on RNA m6A modification and found that transcripts that contain m6A show increased expression in Ythdf2CKO preleukemic cells (Figures 4C and 4D). To understand if these observations are extended to the AML in vivo, we isolated LSCs from mice with AML derived from Ythdf2WT and Ythdf2CKO preleukemic cells and performed gene expression analysis (Figure S3E). The relationship between m6A occupancy and increased transcript dosage was also observed in Ythdf2CKO LSCs (Figures S3F–S3H). The upregulation of m6A-containing transcripts in the absence of YTHDF2 may arise from an increase in their half-life. We therefore measured mRNA half-life transcriptome-wide in preleukemic cells using thiol(SH)-linked alkylation for the metabolic sequencing of RNA (SLAM-seq; Herzog et al., 2017), which revealed an overall modest increase in mRNA half-life in Ythdf2CKO cells (Figure 4E). Interestingly, m6A-containing transcripts displayed overall shorter half-lives than non-m6A transcripts in Ythdf2WT cells (Figure 4F). YTHDF2 deficiency did not alter the translational efficiency of either m6A or non-m6A-containing transcripts (Figure 4I). These data indicate that m6A-directed YTHDF2-mediated mRNA decay contributes to the regulation of the leukemic transcriptome.

Next, we sought to determine if the m6A-modified transcripts deregulated upon Ythdf2 deletion in mouse AML are relevant to human AML. We found that transcripts significantly upregulated in the Ythdf2CKO preleukemic cells are preferentially methylated in human AML cell lines (Figure 4J). To understand the molecular pathways underpinned by upregulated transcripts methylated both in mouse and human, we performed ConsensusPathDB
AB C D E HI KJ MN O P Q

(legend on next page)

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Ythdf2 Deletion Sensitizes AML Cells to TNF

Inspecting the genes that negatively correlate with YTHDF2 expression in human AML, contain m\(^6\)A in both mouse and human AML, are upregulated in Ythdf2\(^{CKO}\) LSCs, and are associated with weak LSC function, we found TNF receptor 2 (TNFR2) encoded by Tnfrsf1b gene (Figure 4L). We focused on TNFR2, as TNF signaling was also identified as a node in the CPDB network analysis (Figure 4K) and TNFR2, together with TNFR1, restricts the accumulation of leukemic cells (Höckendorf et al., 2016). Tnfrsf1b expression is significantly decreased in AML samples compared to non-leukemic controls (Figure S3K), and its expression negatively correlates with LSC activity (Figure S3L). Notably, Tnfrsf1b is highly methylated in mouse preleukemic cells and human AML cells (Figure 4M). RNA immunoprecipitation (RIP)-qPCR revealed co-precipitation of the Tnfrsf1b transcript with Ythdf2 (Figure 4N). Concurrent with the increased half-life of Tnfrsf1b transcript (Figure 4O), the surface expression of TNFR2 is upregulated on Ythdf2\(^{CKO}\) preleukemic cells (Figure 4P). We therefore tested if TNF stimulation had differential impact on Ythdf2\(^{CKO}\) and Ythdf2\(^{CTL}\) preleukemic cells. YTHDF2 loss rendered cells more sensitive to TNF-induced apoptosis (Figure 4Q). This highlights at least one molecular mechanism by which YTHDF2 loss negatively impacts AML.

**DISCUSSION**

Through the analysis of mRNA m\(^6\)A methyltransferases and demethylase, a key role for mRNA m\(^6\)A has been shown in AML pathogenesis (Barbieri et al., 2017; Li et al., 2017; Vu et al., 2017; Weng et al., 2018). The modification of mRNA with m\(^6\)A can have multiple outcomes on the respective transcript (Zhao et al., 2017), but here we demonstrate that the YTHDF2-mediated (CPDB) network analysis and found enrichment for RNA processing, mitochondrial function, ubiquitination as well as tumor necrosis factor (TNF) signaling (Figures 4K and S3). To reveal why the loss of YTHDF2 is correlated with a weak leukemogenic potential, we interrogated gene sets from human AML samples associated with different leukemogenic potential in vivo (Ng et al., 2016). The upregulated transcripts in Ythdf2\(^{CKO}\) preleukemic cells that contain m\(^6\)A in both mouse and human AML cells were divided into groups whose expression positively or negatively correlates with YTHDF2 expression in 1,732 human AML samples (Figure S3J). We found that transcripts that negatively correlate with YTHDF2 expression are highly associated with the loss of leukemogenic potential (Figure 4L). In this way, when an AML sample expresses low amounts of YTHDF2, transcripts associated with the loss of leukemogenic potential have greater expression. In contrast, transcripts whose expression correlates with that of YTHDF2 are depleted from transcripts associated with weak LSC activity (Figure S3J). Thus, YTHDF2 negatively regulates transcripts whose expression limits LSC activity.

![Figure 4. YTHDF2 Targets m\(^6\)A-Methylated Transcripts for Degradation](image-url)
component of the pathway is also critical for cancer stem cells in AML. We find that inhibition of YTHDF2 specifically compromises LSC development and propagation. Given the more severe impact of Ythd2 deletion or knockdown on established AML compared to disease development, AML propagation may be even more dependent on YTHDF2 than disease initiation. Furthermore, consistent with recent findings in mouse and human HSCs (Li et al., 2018; Wang et al., 2018), we demonstrate that targeting Ythd2 expands HSCs and enhances their myeloid reconstitution. These are unique properties of YTHDF2, which, coupled with the fact that the loss of YTHDF2 is permissive in adult mice, underscores the therapeutic potential of YTHDF2 inhibition as a strategy for AML treatment. Such an intervention would have the dual benefits of eradicating malignant LSCs while bestowing a competitive advantage to normal HSCs. Given that isolation of HSCs in sufficient quantities is a limiting factor for the usage of HSC transplantation for a variety of diseases, inhibition of YTHDF2 could be employed to expand HSCs in vitro or in vivo to circumvent this challenge. In summary, we revealed the m^6A reader YTHDF2 as a critical mediator of LSCs whose inhibition selectively compromises AML implying its future applications in treatment of this hematologic malignancy.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.stem.2019.03.021.

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**AUTHOR CONTRIBUTIONS**


**DECLARATION OF INTERESTS**

K.R.K. and D.O. are inventors on patent number GB 1805287.8.

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**REFERENCES**


### STAR★METHODS

#### KEY RESOURCES TABLE

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**Bacterial and Virus Strains**

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents may be directed to, and will be fulfilled by the Lead Contact, Kamil Kranc (kamil.kranc@qmul.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
All experiments on animals were performed under UK Home Office authorisation. All mice were on the C57BL/6 genetic background. Ythdf2fl/fl mice were described previously (Ivanova et al., 2017). Vav-iCre (de Boer et al., 2003), Mx1-Cre (Kühn et al., 1995), and NOD scid gamma mice were purchased from the Jackson Laboratory. All transgenic and knockout mice were CD45.2+. Congenic recipient mice were CD45.1+/CD45.2+.

Human tissue & ethical approvals
Use of human tissue was in compliance with the ethical and legal framework of the United Kingdom’s Human Tissue Act, 2004. Primary human AML samples were from Manchester Cancer Research Centre’s Tissue Biobank (instituted with approval of the South Manchester Research Ethics Committee). Their use was authorized following ethical review by the Tissue Biobank’s scientific sub-committee, and with the informed consent of the donor. Normal CD34+ HSPCs surplus to requirements were from patients undergoing autologous transplantation for lymphoma or myeloma. Their use was authorized by the Salford and Trafford Research Ethics Committee and, for samples collected since 2006, following the written informed consent of donors.

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**METHOD DETAILS**

**Flow cytometry**

All BM and FL cells were prepared and analyzed as described previously (Guitart et al., 2017; Guitart et al., 2013; Kranc et al., 2009; Mortensen et al., 2011; Vukovic et al., 2016). BM cells were isolated by crushing tibias and femurs using a pestle and mortar. FL cells were prepared by mashing the tissue and passing through a 70 μm strainer. Single cell suspensions from BM, FL or PB were incubated with Fc block and then stained with antibodies. For HSC and progenitor cell analyses, following incubation with Fc block, unfractionated BM cells were stained with lineage markers containing biotin-conjugated anti-CD4, anti-CD5, anti-CD8a, anti-CD11b, anti-B220, anti-Gr-1 and anti-Ter119 antibodies together with APC-Cy7-conjugated anti-c-Kit, Pacific Blue-conjugated anti-Sca-1, PE-conjugated anti-CD48 and PE-Cy7-conjugated anti-CD150 antibodies. Biotin-conjugated antibodies were then stained with PerCP-conjugated streptavidin. For analyses of differentiated cells, following incubation with Fc block, spleen or BM cell suspensions were stained with PerCP-conjugated anti-B220 and APC-Cy7-conjugated anti-CD19 antibodies for B cells; Pacific Blue-conjugated anti-CD11b and PE-Cy7-conjugated anti-Gr-1 for myeloid cells; APC-conjugated anti-CD8 antibodies and PE-conjugated anti-CD4 antibodies for T cells.

To distinguish CD45.2+ donor derived cells in PB or BM of transplanted mice, BV711-conjugated anti-CD45.1 and Pacific Blue-conjugated anti-CD45.2 antibodies were used. For HSC and progenitor staining in transplanted mice, APC-conjugated anti-c-Kit, and APC-Cy7-conjugated anti-Sca-1 were used; the remainder of the staining was as described above. For analyses of differentiated cells in BM of transplanted mice, myeloid cells were stained with PE-conjugated anti-CD11b, PE-Cy7-conjugated anti-Gr-1 and APC-conjugated anti-Ter119 for erythroid cells. Lymphoid cells were stained separately, as described above. PB of transplanted mice was stained with BV711-conjugated anti-CD45.1, Pacific Blue-conjugated anti-CD45.2, APC-conjugated anti-CD4 and anti-CD8a, PE-Cy7-conjugated anti-Gr-1, APC-conjugated anti-CD11b, and APC-Cy7-conjugated anti-CD19. TO-PRO-3 or DAPI were used for dead cell exclusion.

Flow cytometry analyses were performed using a LSRFortessa (BD). Cell sorting was performed on a FACSAria Fusion (BD).

**Colony forming cells (CFC) assays**

CFC assays were carried out using MethoCultTM M3434 (STEMCELL Technologies) methylcellulose medium. Two technical replicates were used per each biological replicate in each experiment. Colonies were tallied at day 10. Human primary AML samples were enumerated after 7 days of culture in semisolid medium in the presence of recombinant IL-6, G-CSF and TPO (20 ng/ml) using puromycin as the selectable marker.

**Leukemic transformation**

c-Kit+ cells were prepared from FLs of 14.5 dpc embryos using c-Kit (CD117) enrichment with MACS columns (Miltenyi Biotec). 200,000 c-Kit+ cells were co-transduced with MSCV-Meis1a-puro and MSCV-Hoxa9-neo retroviruses. Transduced cells were subjected to three rounds of CFC assays in MethoCultTM M3231 (STEMCELL Technologies) supplemented with 20 ng/ml SCF, 10 ng/ml IL-3, 10 ng/ml IL-6 and 10 ng/ml GM-SF. Colonies were counted 5 days after plating, and 2,000 cells were re-plated.

**Syngeneic transplantation assays**

CD45.1+/CD45.2+ recipient mice were lethally irradiated using a split dose of 11 Gy (two doses of 5.5 Gy administered at least 4 hours apart) at an average rate of 0.58 Gy/min using a Cesium 137 GammaCell 40 irradiator.

For primary transplantations 200 LSKCD48-CD150+ HSCs (per recipient) sorted from BM of the donor mice were mixed with 200,000 support CD45.1+ BM cells and transferred into lethally irradiated CD45.1+/CD45.2+ recipients. For secondary transplantations 2,000-3,000 CD45.2+ LSK cells sorted from BM of primary recipients were mixed with 200,000 support CD45.1+ wild-type BM cells and re-transplanted. All recipient mice were culled and analyzed 16-20 weeks post-transplantation.

For transplantations of leukemic cells, 50,000-100,000 Meis1/Hoxa9-transduced c-Kit+ cells were transplanted into lethally irradiated CD45.1+/CD45.2+ recipient mice (together with 200,000 unfractionated support CD45.1+ wild-type BM cells). For secondary transplantation, 10,000 CD45.2+c-Kit+ cells sorted from BM of primary recipients were transplanted into lethally irradiated secondary CD45.1+/CD45.2+ recipient mice (together with 200,000 unfractionated support CD45.1+ wild-type BM cells).

**Xenotransplantation experiments**

THP-1 cells transduced with CTL or KD lentiviruses were tail vein injected into non-irradiated 12 week-old female non-obese diabetic (NOD)/LtSz-severe combined immune-deficiency (SCID) IL-2Rγcnull (NSG) mice (1x10^6 cells per 200 μL per mouse). Mice were killed one month after transplantation. For survival curve analyses, 10,000 or 1,000 cells per NSG mouse were injected. To assess human AML burden, cells were stained with anti-human PE-conjugated anti-CD45 and APC-conjugated anti-CD33.

**plpC administration**

Mice were injected intraperitoneally every other day with 300 μg plpC (GE Healthcare) for a total of 6 doses, as previously described (Guitart et al., 2017; Guitart et al., 2013; Kranc et al., 2009).
shRNA-mediated YTHDF2 knockdown

THP-1 cells were transduced with lentiviruses expressing shRNAs (shRNA KD1, 5'-TACTGATTAAGTCGAGTAA-3' [TRCN000254410, Sigma-Aldrich]; shRNA KD2, 5'-CCTTCCATATACTATAAC-3' [TRCN0000254336, Sigma-Aldrich]; and shRNA CTL, 5'-TCTCTGAGCTGTCACGT-3'; GE Healthcare). Selection of efficiently transduced cells was achieved by treatment with puromycin (2 μg/mL final concentration).

Cell proliferation, cell death and cell differentiation analyses

Lentivirus-transduced THP-1 were seeded at 15x10^4/mL after puromycin selection. Viable cells were counted by trypan blue exclusion at the indicated time points. To analyze cells undergoing apoptosis, cells were suspended in binding buffer containing Annexin V-PE and DAPI. To assess myeloid differentiation, cells were stained with PE-conjugated anti-CD14 and APC-conjugated anti-CD11b antibodies.

Primary human AML patient derived samples

For western blotting shown in Figure 1B, the following samples were used: 70 (karyotype 46,XY.del(7)(q22q32)[20]), 104 (karyotype 46,XX,t(15;17)(p11.2;q21)[7]/46,sl,-6,add(16)(q12),+mar3[46,XX][3]), 163 (karyotype 45,X,Y,t(8;21)(p22;q22)[8]/46,XY[2]), 191 (karyotype 46,XX [20]), 205 (karyotype 46,XX,t(1;22)(p21;p11.2),ins(10;11)(p12;q23q14)[10] nb variant of t(10;11) MLL-MLLT10 fusion), 539 (karyotype 46,XY [20]), 685 (karyotype 46,XX,t(6;9)).

For CFC assays shown in Figures 2L and 2M, the following samples were used: 160 (AML1) (karyotype 46,XX,t(9;11)(p22;q23),der(21;22)[q10;q10],+der(21;22)[cp10]; MLL-MLLT3 rearrangement; clonal evolution with add(Xp); add(4q); add(7q); +21 at relapse), 292 (AML2) (karyotype 46,XX,t(15;17); PML-RARA rearrangement [no cyto report available]), 251 (AML3) (karyotype 46,XY,t(1;22)(p21;p11.2),ins(10;11)(p12;q23q14)[10] nb variant of t(10;11) MLL-MLLT10 fusion), 539 (karyotype 46,XY[20]), 685 (karyotype 46,XX,t(6;9)).

Western blotting

Proteins extracted from CTL, KD1 and KD2 THP-1 cells were subjected to SDS–PAGE (Bolt 4%–12% Bis-Tris Plus Gel, ThermoFisher Scientific) and probed with anti-YTHDF2 (1:5000, ON at 4°C, GE Healthcare life Sciences) and anti-CD11b antibodies.

Affymetrix

RNA extraction from Meis1/Hoxa9-transduced c-Kit+ cells was performed using TRIzol (Thermo Fisher Scientific). Total RNA was used to synthesize Biotinylated cDNA with the Ambion WT Expression kit (Ambion, 4491974). cDNA was fragmented and labeled with the Affymetrix, WT Terminal and Control Kits (Affymetrix, 901524) and then hybridized for 16 hours at 45°C on a GeneChip Mouse Gene 2.0 ST Array. The chip was later washed and stained with the Affymetrix Fluidics Station 450. Data were processed and analyzed using the Bioconductor Limma Package (Ritchie et al., 2015). Samples were normalized using the rma function and differential expression was assessed using linear modeling. Log2-fold-changes and moderated t-statistics were calculated using the contrasts.fit function. To determine the gene ontology (GO) enrichment of differentially expressed genes, we used the topGO R package. Fisher’s exact test was used to assess enrichment for the biological process ontology.

Analyses of YTHDF2 expression in human AML samples

To generate Figure 1A the following publicly available datasets were used: GSE10358, GSE52891, GSE61804, GSE68833, GSE12417, GSE13159, GSE15061, GSE15434, GSE16015, GSE19577, and GSE22845 (Bachas et al., 2015; Haferlach et al., 2009, 2010; Klein et al., 2009; Metzeler et al., 2008; Metzeler et al., 2015; Mills et al., 2009; Pigazzi et al., 2011; Taskesen et al., 2011; Tommasson et al., 2008). Exclusion criteria included datasets with less than 20 samples, samples with undefined tissue of origin, cell type and karyotype, in addition to RAEB samples. Only BM samples, with a total of 1732 samples were retained for further analysis. The Simpleafy package from Bioconductor was used to extract quality measurement of microarrays (Gentlemman et al., 2004; Wilson and Miller, 2005). RNA degradation was assessed based on 3' to 5' ratio of GAPDH and ACTNB genes. Samples with NUSE < 1.05 and relative log expression (RLE) < 0.15 were excluded from further analysis (McCall et al., 2011). The retained samples were assessed for their homogeneity using the Bioconductor arrayQualityMetrics package (Kaufmann et al., 2009). Low quality RNA and outlier samples were excluded, while high quality samples retained after quality control were background corrected and normalized using RMAexpress software (http://rnaexpress.bmbolstad.com/). Pairwise comparisons between each karyotype and control were performed using Student’s t test.

m^6^A meRIP-Seq

m^6^A meRIP-Seq library preparation was performed as previously described (Lin et al., 2016) from Ythdf2^CTL pre-leukemic cells. Three biological replicates for each condition were used. Reads were aligned to the mouse or human reference genome using HISAT2 (Kim
et al., 2015) and peaks were called using MACS2 (Zhang et al., 2008). To analyze the distribution of peaks along the transcripts, bedgraph files were converted to bigWig format and used as input for the computeMatrix function of the deepTools package (Ramírez et al., 2014). Motif enrichment was done using HOMER selecting a motif length of 6 nucleotides. Background regions were generated by shuffling peaks along the transcriptome using the shuffleBed tool from the BEDTools suite (Quinlan and Hall, 2010). Network analysis was performed using the ConsensusPathDB (CPDB) software (Kamburov et al., 2013). For gene set enrichment analysis (GSEA), the GSE76008 dataset (Ng et al., 2016) was used to rank genes according to the engraftment potential of pre-leukemic cells. The GVIZ bioconductor package was used for peak visualization (Hahne and Ivanek, 2016).

Correlation with YTHDF2 was measured to determine robust YTHDF2 targets after the knockout (Månsson et al., 2004). Briefly, Pearson correlation between YTHDF2 and the identified YTHDF2 targets was calculated using the 1732 AML samples previously described. Correlation significance was measured using parametric test with length (genes)-2 degrees of freedom (cor.test function, stats package, R project, http://www.R-project.org/), and adjusted for multiple comparisons using Benjamini & Hochberg method (Benjamini and Hochberg, 1995). Genes with negative coefficients and adjusted p value < 0.05 were considered strong targets of YTHDF2.

SLAM-seq

SLAM-seq libraries were prepared using the Lexogen catabolic kit (cat. no. 062.24) and the Lexogen QuantSeq 3’ mRNA-Seq Library Prep Kit FWD for Illumina (cat. no. 015.24) in both cases following manufacturers’ instructions. S4U was used at 2.9 µM, as determined by the cell viability titration assay. Medium with 4SU was used for pre-leukemic cells labeling for 12 hours and was later replaced with 4SU-free medium (time 0). Cells were collected immediately after medium change and at 1, 3, and 9 hours. Libraries were sequenced using an Illumina HiSeq platform in a 50 bp single-end mode. Biological triplicates for both Ythdf2CTL and Ythdf2CKO pre-leukemic cells were used to generate the different libraries sets. SLAM-seq libraries were analyzed as previously described (Herzog et al., 2017). Briefly, T to C conversion rates were obtained using the SlamDunk pipeline. Conversion rates across different time points were normalized to time 0 for each gene and were used to fit a first order decay reaction with the R stats package nls function.

RIBO-seq

RIBO-seq libraries were prepared as previously described (Reid et al., 2015). Briefly, pre-leukemic cells were lysed with CaCl₂ 4 mM, MgCl₂ 10 mM, K-HEPES pH 7.2 25 mM, KOAc 200 mM and NP-40 1%. The lysate was cleared from cell debris, diluted 1:1 in water, and digested with MNase 10 µg/ml for 30 minutes at 37°C. Digested RNA was extracted with QIAzol and later treated with PNK (NEB) for 30 minutes at 37°C. To isolate ribosome-protected mRNA fragments (RPFs), the PNK-treated RNA was resolved on a 15% Novex TBE-Urea Gel (EC6885BOX), and RPFs 25 to 40 nucleotides long were excised and purified. Libraries were then prepared using the NEBNext Multiplex Small RNA Library Prep Set for Illumina following manufacturer’s instructions. For input controls, total RNA was extracted from the pre-leukemic cell lysates before MNase digestion using QIAzol. Samples were then depleted of ribosomal RNA using the Epicenter Ribo-zero kit (cat. no. MRZH116), and libraries were generated using the SENSE Total RNA-Seq Library Prep Kit (cat no. 009.08) following manufacturer’s instructions. Libraries were sequenced with the Illumina HiSeq platform in a 50 bp single-end mode. Biological triplicates were used to generate libraries for both Ythdf2CTL and Ythdf2CKO pre-leukemic cells. For the RIBO-seq analysis, we used Kallisto (Bray et al., 2016) to obtain read counts per gene for the RPF and mRNA libraries. Read counts were then used to calculate the differential translational efficiency between Ythdf2CTL and Ythdf2CKO pre-leukemic cells with Xtail (Xiao et al., 2016b). To estimate the relative translational efficiency for genes in each condition, we compared RPF and mRNA read counts using DESeq2 (Love et al., 2014).

DATA AND SOFTWARE AVAILABILITY

Accession

Affymetrix, m⁵A meRIP-Seq, RIBO-seq and SLAM-seq datasets were deposited in ArrayExpress under the following accession numbers: E-MTAB-6783, E-MTAB-7782, E-MTAB-6791, E-MTAB-7783, E-MTAB-7785 and E-MTAB-7784. Data from NOMO-1 and MA9.3ITD human cell lines were obtained from previously published work (Su et al., 2018) through the following accession number: GSE87190.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, Inc.). P values were calculated using a two-tailed Mann–Whitney U test unless stated otherwise. Kaplan–Meier survival curve statistics were determined using the Log-rank (Mantel Cox) test.