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KLF4/MLL3 axis Drives NRBP2 Transcription to Eliminate Acute Myeloid Leukemia Cells

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Article

Keywords:

Posted Date: September 30th, 2025

DOI: https://doi.org/10.21203/rs.3.rs-7639334/v1

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Additional Declarations: There is **NO** conflict of interest to disclose.

Version of Record: A version of this preprint was published at Leukemia on September 2nd, 2026. See the published version at https://doi.org/10.1038/s41375-026-03117-8.

Abstract

Acute myeloid leukemia (AML) is a heterogeneous malignancy rooted in hematopoietic stem cell dysregulation. Despite therapeutic advances, clinical outcomes remain unsatisfactory. Here, we identify KLF4, a zinc finger transcription factor with previously reported context-dependent roles in hematologic malignancies, as a negative regulator of AML proliferation. Mechanistically, KLF4 interacts with the MLL3 histone methyltransferase complex, comprising MLL3, WDR5, RBBP5 and ASH2L, through its zinc finger (ZnF) and transrepression domain (TRD) domains, and actives transcription of the tumor suppressor gene NRBP2. Furthermore, integrated transcriptional profiling revealed TNIK as a convergent effector of the KLF4–NRBP2 tumor-suppressive circuit in acute myeloid leukemia. Pharmacological blockade of TNIK with the selective small-molecule inhibitor TNIK-IN-1 selectively impaired leukemic cell proliferation while sparing normal haematopoiesis. Consequently, our findings reveal a previously unrecognized KLF4–MLL3–NRBP2 transcriptional axis and its pharmacological re-engagement with TNIK-IN-1 establishes this axis as a directly druggable vulnerability in AML.

Introduction

Acute myeloid leukemia (AML) is an aggressive hematologic malignancy characterized by clonal expansion of dysregulated myeloid progenitors, resulting in disruption of normal hematopoiesis. Although conventional chemotherapy induces complete remission (CR) in most AML patients, relapse rates remain high, yielding a 5-year overall survival (OS) of approximately 30%[1]. Despite recent approvals of novel agents, AML remains a persistent therapeutic challenge[1, 2]. Transcription factors (TFs) orchestrate hematopoiesis and hematological malignancies binding directly to regulatory elements to elegantly regulate gene expression by forming transcription complexes (TCs) in a context-dependent manner. Despite their pivotal role, studies of TFs in acute myeloid leukemia (AML) remain further exploration.

Kruppel-like factor 4 (KLF4), a member of the belongs to TFs contains 3 consecutive C2H2 finger structures at its C-terminal, which can bind to specific methylated DNA sequences[3]. KLF4 has dual functions as a tumor suppressor or an oncogene in solid tumors[4] and a contradictory role in hematological malignant disease[5, 6]. Such as, KLF4 is elevated in Burkitt lymphoma. Also, low KLF4 expression could predict a favorable overall survival[6]. However, in acute lymphoblastic leukemia (ALL), low expression of KLF4 accelerates the NOTCH1-induced T-ALL through repressing MAP2K7 kinase[5]. KLF4 overexpression enhances chemosensitivity to conventional chemotherapeutics in ALL cells[7]. In AML, KLF4 is reactivated via PPARγ signaling as a viable therapeutic strategy in AML patients[8]. Also, KLF4 is upregulated by RUNX1 through p57 in t(8;21) leukemia (AML)[9]. Furthermore, our previous work performed that KLF4 and SOX2 play a dominant role in eliminating leukemia cells while sparing hematopoietic stem and progenitor cells (HSPCs)[10]. Based on these, the function of KLF4 and transcriptional regulation mechanism in molecular still deserves exploring.

Myeloid/lymphoid or mixed lineage leukemia 3 (MLL3), also known as lysine methyltransferase 2C (KMT2C), is a member of the MLL family of H3K4 histone methyltransferases. It primarily regulates distal enhancers and, to varying degrees, depends on core complex subunits including tryptophan-aspartate repeat protein-5 (WDR5), retinoblastoma-binding protein-5 (RBBP5), and absent small homeotic-2-like (ASH2L) for enzymatic activity[11]. Also, exome sequencing identified a recurrent MLL3 germ-line mutation in AML patients[12]. However, the MLL3/WDR5/RBBP5/ASH2L complex was whether required for KLF4 in AML regulation still largely unknown.

NRBP2 (nuclear receptor-binding protein 2), a pseudokinase of approximately 500 amino acids, lacks 7 of 15 conserved residues in its kinase domain[13]. While catalytically inactive, pseudokinases function as critical regulators in signaling pathways[14]. NRBP2 plays an significant role in solid tumor. In $CD133^{+}$ hepatoma carcinoma cells, downregulation of NRBP2 modulates stemness-associated genes and enhances chemosensitivity[15]. In thyroid cancer, overexpression of NRBP2 suppresses tumor growth, reduces pro-angiogenic markers in the tumor microenvironment (TME), and inhibits angiogenesis[16]. These findings establish NRBP2 as a tumor suppressor that constrains proliferation, regulates stemness, and increases drug sensitivity—supporting its therapeutic relevance. However, the function of NRBP2 function in AML remains unexplored.

AML represents a biologically and genetically heterogeneous hematologic malignancy. While intensive chemotherapy has historically constituted the therapeutic cornerstone, molecular stratification of AML heterogeneity has enabled refined prognostic classification and development of mutation-specific targeted agents[17–26]. Despite targeted advanced drug, the 5-year overall survival of AML patients is still frustrated. Therefore, developing more effective AML therapies remains a critical unmet need, necessitating deeper mechanistic insights into AML pathogenesis and identification of novel molecular targets.

In this study, we identify KLF4 as a direct interactor of the MLL3 histone methyltransferase complex, which activates transcription of the tumor suppressor NRBP2, a previously uncharacterized factor in AML. Specificity, we found that the small molecule compound TNIK-IN-1 enhances the KLF4-NRBP2 axis, thereby attenuating leukemic cell growth and promoting apoptosis. These findings define a KLF4–MLL3–NRBP2 transcriptional circuit with potential therapeutic relevance and provide mechanistic insight into transcriptional regulation in AML.

Materials and Methods

Antibodies and reagents

The information was showed in Supplemental Table 1.

Mice and Human Samples

C57BL/6J mice (8–10 weeks old) were housed under specific pathogen-free conditions in individually ventilated cages at the State Key Laboratory of Experimental Hematology (SKLEH), Tianjin, China. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of SKLEH and the Institute of Hematology. BM samples from AML patients and healthy donors were obtained from the Institute of Hematology & Blood Diseases Hospital, Tianjin, China. The use of human samples was approved by the Institutional Review Boards (IRBs) of SKLEH and the Institute of Hematology & Blood Diseases Hospital.

Leukemia model and transplantation and human mononuclear cells (MNC) enrichment were performed in Supplemental Data.

Cell Culture

THP-1, HEL and Molm-13 cells lines were obtained from SKLEH's experimental pathology cell bank. Kasumi and HL60 cells were purchased from American Type Culture Collection. All cell lines were cultured in RPMI1640 with 10% FBS. MA9 cells were cultured in IMDM with 15% FBS, 10 ng/mL mIL-6, 10ng/mL mIL-3 and 50 ng/mL mSCF. c-kit + cells were cultured in serum-free expansion medium (SFEM) with 100 ng/mL mTPO and 100 ng/mL mSCF.

Functional and molecular assays

We performed Colony forming assays, plasmids virus transfection, luciferase reporter assay, Immunofluorescence, cell proliferation, cell viability, cell apoptosis assay, qRT-PCR, Western blotting Chromatin Immunoprecipitation Sequencing (ChIP-seq), CUT&Tag and ATAC-seq. Experimental details are provided in the Supplemental Data.

Statistical Analysis

Data from biological triplicate experiments are presented, and data represent means ± SD. An unpaired two-tailed Student's t test was used to compare two groups of data. ANOVA with Bonferroni's correction was used to compare multiple groups of data. Survival curves analysis was using log-rank test. A P value < 0.05 was considered statistically significant. Statistical significance was defined as $p < 0.05$. All analyses and visualization were performed with GraphPad Prism (v10.0.0).

Results

KLF4 interacts with MLL3 complex in AML cells

Despite transformative advances in leukemia therapy, selectively eliminating leukemic cells remains a persistent challenge. Building on our previous findings that two member of Yamanaka factors (OCT3/4, SOX2, KLF4 and c-MYC), KLF4 and SOX2 promote selective eradication of leukemia cells while sparing

HSPCs[10]. To further elucidate the mechanistic of KLF4 in AML, we established a doxycycline (DOX) inducible KLF4-overexpression MLL-AF9 (MA9) mouse model (Fig. 1A). KLF4 overexpression markedly reduced colony forming capacity in MA9 cells (Fig. 1B), an effect recapitulated in THP-1 cells (Fig. S1A-C). These data supported that KLF4 exerts growth-suppressive effects in AML cells, prompting us to define its downstream molecular mechanisms.

To uncover KLF4-associated protein networks, we performed affinity purification coupled with mass spectrometry (AP-MS) using DOX induced Flag-tagged KLF4 expressed THP1 cells (Supplemental Table 2). Proteomic profiling identified interactions between KLF4 and the MLL3 (KMT2C) histone methyltransferase complex, including WDR5, RBBP5, ASH2L (Fig. 1C-D). In addition, we use PPI interaction prediction to confirm the MLL3 (KMT2C) complex including WDR5, RBBP5 and ASH2L (Fig. 1E) (https://cn.string-db.org/). To further identifying the potential interaction between KLF4 and MLL3 complex, we conducted co-immunoprecipitation (co-IP) assays. From IP with Flag, KLF4 or MLL3 antibodies and IB with indicate antibodies, the results confirmed reciprocal binding between KLF4 and each MLL3 complex component in THP-1 cells (Fig. 1F-G). The immunofluorescence (IF) assays also demonstrated a significantly co-localization in nucleus frequency between KLF4 and MLL3, WDR5, RBBP5, ASH2L in THP-1 cells (Fig. 1H). Also, the expression of KLF4 showed a moderate positive correlation with MLL3 complex members (Fig. S1D) (http://gepia.cancer-pku.cn/), which indicates a potential functional consistency within KLF4 and MLL3 complex. Together, these findings reveal a previously unrecognized interaction between KLF4 and the MLL3 complex, suggesting a functional epigenetic axis in AMLs.

TRD and ZnF domain of KLF4 mediates association of KLF4 and MLL3 complex

Protein-protein interactions between transcription factors and epigenetic modifiers are often mediated by specific modular domains. Defining these interfaces is essential for understanding transcriptional regulatory logic and for future therapeutic disruption. Furthermore, KLF4 belongs to the zinc finger family of transcription factors[27] and we next defined the mechanistic basis for synergistic transcriptional regulation.

To test this idea, we generated 12 (T1-T12) truncation mutants of KLF4 based on its major domains (transactivation domain, TAD; transrepression domain, TRD; zinc finger, ZnF) to clarify the structural basis underlying the interaction between KLF4 and the MLL3 complex [27, 28] (Fig. 2A). 12 Flag-tagged truncation mutants were directionally cloned into a DOX-inducible lentiviral vectors overexpressed KLF4, subsequent lentiviral transduction to yield stable expressed THP1 cell lines, enabling precise interrogation of each variant's functional impact. Western-blot proved that KLF4 truncations successfully constructed (Fig. 2B). Subsequently, we preformed co-IP assays through IP with Flag antibody and IB with indicated antibodies to identifying the interactions between KLF4 domains and MLL3 complex. Co-IP results revealed that the T3, T6, and T11 truncations robustly interact with three proteins of MLL3

complex, RBBP5, ASH2L, and WDR5. While RBBP5 and ASH2L associate with most truncations from T7 to T12, and WDR5 additionally binds the T10 truncation (Fig. 2B, C). These results suggest that the linker region (amino acids 120–184), TRD, and ZnF domain of KLF4 mediate interactions with RBBP5 and ASH2L, while the TRD and ZnF domains are critical for binding to WDR5.

To investigate their functional roles, we overexpressed individual KLF4 truncations and assessed their impact on AML cell proliferation. Overexpression of T3, T6, and T11 truncations recapitulated the effects of full-length KLF4, and most truncations containing the ZnF domain exhibited varying degrees of proliferation suppression in AML cells (Fig. 2D). Notably, overexpression of the isolated KLF4-ZnF domain (T9) had no effect on proliferation, whereas the presence of the TRD domain enhanced the suppressive effect of ZnF-containing truncations. Given the role of the ZnF domain in DNA binding, we propose that KLF4's anti-leukemic activity stems from its transcriptional regulatory function, with the TRD domain facilitating interactions with the MLL3 complex to support this activity (T5 vs T6). Together, the TRD and ZnF domains are essential for maintaining KLF4's primary function in AML cells.

KLF4/MLL3 axis promotes NRBP2 transcription in AML cells

To address the downstream target genes regulated by KLF4's transcriptional activity in AML, we employed the DOX inducible MA9 mouse model for overexpressing KLF4. Following disease onset, primary MA9 cells were isolated and treated with DOX to induce KLF4 overexpression *in vitro* (Fig. S2A). RNA-seq and ATAC-seq were performed at 0, 6, 12, and 24 hours post-induction to assess gene expression and chromatin accessibility (Fig. 3A-B). To further elucidate the early regulatory effects of KLF4 overexpression, ChIP-seq analysis was conducted 6 hours after DOX induction (Fig. 3A and Fig. S2B). The results revealed that KLF4 predominantly binds to promoter regions in AML cells, with RNA-seq data indicating dynamic changes in target gene expression upon KLF4 overexpression, accompanied by varying degrees of chromatin accessibility. To identify genes consistently exhibiting high expression and active chromatin states across different time points of KLF4 overexpression, we performed an integrated analysis of sequencing data (Fig. 3B), identifying 19 target genes that maintained elevated expression and accessibility (Fig. 3C).

To pick candidate target gene, we performed q-PCR to verify KLF4, NRBP2, EVPL, GRHL3, NLRC3 and KLF3, with NRBP2 emerging as a target due to the highest level in 6, 12, 24, 36h (Fig. 3D). Additionally, visual analysis using Integrative Genomics Viewer (IGV) revealed enhanced chromatin accessibility and active enhancer signatures at the NRBP2 cis-regulatory region (CRR) in overexpression KLF4 MA9 cells of ATAC-seq and ChIP-seq (Fig. 3E). Multi-modal chromatin profiling demonstrated chromatin openness gradually increased from 0h to 24h in ATAC-seq peaks and overexpression of KLF4 significantly increased binding at the NRBP2 cis-acting element, collectively indicating a transcriptionally permissive chromatin state at this locus. Cumulatively, these data demonstrate that KLF4 functions as a transcriptional regulator of NRBP2 in AML, directly modulating its expression through chromatin accessibility changes at CRR.

Based on these observations, we postulated a mechanistic cooperation between KLF4 and the MLL3 complex in orchestrating NRBP2 transcriptional regulation. To experimentally validate this regulatory paradigm, we engineered a dual-luciferase reporter system incorporating a NRBP2 promoter fragment. Overexpression KLF4 in 293T cells significantly enhanced reporter activity, whereas concomitant siRNA-MLL3 or ASH2L attenuated this activation (Fig. 3F, G). Western-blot analysis confirmed coordinated expression changes: KLF4 overexpression upregulated NRBP2 protein levels while MLL3/ASH2L knockdown reduced NRBP2 expression rather than MLL4 (Fig. 3H-I, S2E). ChIP-qPCR was performed to verify KLF4-MLL3 complex induce NRBP2 expression (Fig. 3J, K). These results indicated that KLF4 interacted with MLL3 complex and regulates NRBP2 expression to modulate AML development.

However, based on our results performed that KLF4 interacts with MLL3 complex through TRD and ZnF domain, and this raises another question whether KLF4 through TRD + ZnF domains to regulate NRBP2. To verify this, we overexpressed full-length KLF4, T2 (without TRD + ZnF) and T3 (with TRD + ZnF) truncations in THP-1 cells, respectively. Analysis of RNA and protein levels revealed that NRBP2 was specifically upregulated in cells overexpressing full-length KLF4 and the T3 truncation, but not T2 truncation (Fig. 3L, S2F, G). Furthermore, we performed CUT&Tag-seq analysis using MLL3, ASH2L or H3K4me1 antibody within Full-length, T2, T3 or T6 truncation overexpressed THP1 cells. Analysis revealed that overexpression of full-length KLF4 and its T3 and T6 truncations enhanced the recruitment of MLL3 complex components to the CRR of NRBP2, accompanied by increased histone modifications at this locus (Fig. 3M, S2H). These findings indicate that the TRD and ZnF domains mediate KLF4's interaction with the MLL3 complex to regulate NRBP2 transcription (Fig. S2I).

In summary, KLF4 overexpression in AML enhances the recruitment of the MLL3 complex to the CRR of the target gene NRBP2, with KLF4's transcriptional regulation of NRBP2 being dependent on its TRD and ZnF domains.

KLF4 suppresses AML cell proliferation through NRBP2

NRBP2 has been reported to be overexpressed increasing the chemosensitivity of hepatocellular carcinoma cells[15]. However, studies in hematologic malignancies are still limited. Given that such characteristics of NRBP2 are similar to those of KLF4, we further studied NRBP2 function in AML cells. Therefore, we overexpressed NRBP2 (OE NRBP2) in THP-1 cells and observed a dramatic reduction of cell proliferation (Fig. 4A), cell viability (Fig. 4B), and an increase of apoptosis (Fig. 4C), which recapitulated the phenotypes resulting from KLF4 (Fig. S1A-C). To further clarify the effect of NRBP2 overexpression on the gene expression of AML cells, we collected MA9 cells with NRBP2 overexpression for RNA-seq detection. GO analysis revealed that the caspase-mediated cleavage of cytoskeletal proteins pathway was significantly enriched in cells with NRBP2 overexpression (Fig. 4D). As a result, we examined apoptosis-related gene expression and found that Caspase-7 was increased in OE NRBP2 cells (Fig. 4E). In addition, DOX induced OE NRBP2 MA9 mouse model was performed that survival was prolonged (Fig. 4F).

Based on these, we examined whether NRBP2 decreased could rescue any of the phenotypes of KLF4 overexpression AML cells by NRBP2 knockdown in THP-1 cells (Fig. 4G). Forced decreased NRBP2 partially yet significantly restored KLF4 overexpression-induced AML cell apoptosis (Fig. 4H). Moreover, overexpressed KLF4 and NRBP2 could enhanced the phenotype of proliferation by colonies (Fig. 4I-J). These results demonstrate that NRBP2 overexpression significantly promotes apoptosis and inhibits proliferation in AML cells, and that KLF4's suppression of AML cell proliferation is mediated through NRBP2.

KLF4/NRBP2 axis downregulates TNIK and TNIK inhibitor suppresses AML progression

Our previous results indicated that both KLF4 and NRBP2 overexpression could inhibit the proliferation and apoptosis in AML. To further elucidate the mechanisms by which KLF4/NRBP2 regulate AML cells, we reanalyzed RNA-seq data from KLF4 and NRBP2 overexpression experiments. As a result, we overlapped the downregulated genes which overexpressed KLF4 and NRBP2 in RNA-seq (|log fold change| >0.5) and 110 candidate genes were found (Fig. 5A, Supplemental Table 3).

To systematically evaluate the therapeutic efficacy of targeting KLF4/NRBP2 in AML, we assessed whether small-molecule inhibition suppresses AML expansion. In overlapped 110 genes, we found that three candidate genes including ABCA1, TNIK and AASS have small molecular inhibitors (Fig. 5A). To find out the specific target, RT-PCR was performed in THP-1 cells that overexpressed KLF4 or NRBP2 respectively. TNIK, TRAF2 and NCK-interacting kinase[29], a therapeutic target in lung squamous cell[30], was significantly decreased in mRNA level among three candidate genes (Fig. 5B-C), similarly, its protein level was also decreased in 24h (Fig. 5D). Based on these, we further investigated whether TNIK inhibitors (TNIK-IN-1) exert their effects on AML through the KLF4/NRBP2 axis.

Subsequently, we test different drug concentration from 0-100μM in THP-1 cells firstly (Fig. 5E, S3A), and choose 0, 1, 5, 10μM for further investigation, finally. Moreover, the results shown that TNIK-IN-1 effectively decreased TNIK protein levels in THP-1 cells (Fig. 5F). Furthermore, to extend the universality in AML, different AML cell lines and primary mouse cells were cultured in DMSO with or without TNIK-IN-1 for 5–6 days, after which cell proliferation and apoptosis were evaluated (Fig. 5G). The results indicated that TNIK-IN-1 treatment significantly decreased THP1, MOLM 13, HEL, HL60, and Kasumi cell proliferation and increased apoptosis relative to the DMSO control (Fig. 5G, S3B-E). Also, the TNIK protein level was inhibited (Fig. 5F, S3F). To further investigate the effects of TNIK inhibitors on primary mouse cells, we conducted *in vitro* drug response assays using MA9 and c-Kit$^+$ cells. The results demonstrated that both high and low concentrations of TNIK-IN-1 significantly suppressed MA9 cell proliferation and promoted apoptosis, whereas a low concentration (1μM) of the TNIK-IN-1 had minimal impact on c-Kit cells (Fig. 5H-I). To assess the therapeutic efficacy of TNIK-IN-1 *in vivo*, we employed an MA9 leukemia transplantation model in C57BL/6J mice. Starting at disease onset as confirmed by peripheral blood (PB) analysis (approximately day 14 post-transplantation), mice were treated with TNIK-IN-1 by intraperitoneal injection (IP) (Fig. 5J). To minimize potential pharmacological toxicity from

prolonged exposure, treatment was administered in two cycles separated by a one-week interval, with three consecutive injections per cycle at 24-hour intervals. Survival analysis revealed that TNIK-IN-1 significantly prolonged lifespan compared to vehicle controls (Fig. 5K), highlighting the therapeutic potential of TNIK inhibitor TNIK-IN-1 in AML.

TNIK inhibition by TNIK-IN-1 suppresses human leukemia cell proliferation

To evaluate the therapeutic potential of the small-molecule TNIK inhibitor TNIK-IN-1 in AML of human, we collected bone marrow (BM) samples from healthy individuals and AML patients, isolated mononuclear cells (MNCs), and performed *in vitro* experiments to assess the effects of TNIK-IN-1 on cell proliferation (Fig. 6A). Our results showed that both high and low concentrations of TNIK-IN-1 significantly inhibited proliferation of MNCs from AML patients, whereas a low concentration (1 μM) had minimal impact on the proliferation of MNCs from healthy individuals (Fig. 6B-C). To determine whether the effects of TNIK-IN-1 on MNCs were mediated by suppression of TNIK protein levels, we conducted WB analysis on protein samples (Fig. 6D). The results revealed that TNIK-IN-1 markedly reduced TNIK expression in MNCs from both healthy individuals and AML patients, indicating that TNIK-IN-1 inhibits AML cell proliferation by suppressing TNIK expression. Collectively, these findings underscore the clinical potential of TNIK-IN-1 and provide a novel therapeutic strategy for AML treatment.

Discussion

Previous reported that the function of KLF4 expression in solid tumor and leukemia with dual roles. Interestingly, KLF4 preformed two functions in pancreatic, which promoted early wound and inhibited advanced progression[31–33]. In our study, we deeply investigated the mechanism of KLF4 in AML, leading following vital findings. First, we verified overexpressed KLF4 inhibit MA9 and THP-1 cell proliferation. Second, KLF4 interacted with MLL3 complex, including WDR5, RBBP5 and ASH2L, through its TRD and ZnF domain and inhibits AML proliferation. Third, as a member of TFs associated with DNA binding[3], we integrated KLF4 ChIP-seq, ATCC-seq and RNA-seq data from KLF4 overexpressing MA9 cells and identified NRBP2 as a key target regulating survival, proliferation and apoptosis in AML. Fourth, by integrating RNA-seq data from THP-1 cells with overexpression of KLF4 or NRBP2, we found that TNIK was downregulated in KLF4 or NRBP2 overexpressed cells and identified the TNIK inhibitor TNIK-IN-1 may exert therapeutic benefits in AML patients at appropriate concentrations. These findings establish the KLF4/MLL3/NRBP2 axis as a novel therapeutic target for AML, with TNIK-IN-1 as a promising candidate for clinical application.

The discovery of KLF4 interacts with MLL3/WDR5/RBBP5/ASH2L expanding the transcription factor protein interaction of AML progression in hematologic malignancies. This observation aligns with our previous study[10], Yamanaka factors especially KLF4 as a significant determinant which could eliminate leukemia and AML requires deeper mechanism of KLF4. Notably, knockdown MLL3 promotes follicular lymphoma in mice[34] and MLL3/MLL4 complex is required for HOXA9/MEIS1 in leukemia[35], emerging

here as a critical dependency in AML. Furthermore, MLL3 exists as a multi-subunit complex comprising components such as WDR5, RBBP5, and ASH2L[36], which bridges the functions of multiple subunits and mediates the regulatory effects of transcription factors. Additionally, our results strongly indicate that KLF4 interacts with the MLL3 complex through its TRD and ZnF domains, with this interaction exhibiting affinity both in molecular pathways and in AML-related functions. These results were familiar with evidence that zinc finger domain of KLF4 governs its interaction with ERRα and ZnF-deletion mutants abolish this interaction in cardiac dysfunction[27], suggesting that the molecular domain of transcription factor could serve as therapeutic targeting in the future.

The mechanistic interplay between KLF4/MLL3 complex and identified downstream functional important gene NRBP2, unveils a previously unrecognized epitranscriptomic circuit that operates independently of previously KLF4 axis[9]. Our mechanistic studies establish NRBP2 as a critical executor of KLF4-dependent transcriptional programs. Knockdown ASH2L and MLL3 inhibit NRBP2 expression and emphasizing the importance of ASH2L/MLL3 mediates the regulatory function of NRBP2 within the MLL3 complex in AML biology. Moreover, this finding was similar to recent study that ASH2L is critical for MLL3 binding in cancer[37]. Although ASH2L interacted with MLL4[38], knockdown MLL4 had no effect on NRBP2. These results further indicate that ASH2L is significant for MLL3 interactive with KLF4 to regulate NRBP2. CUT&Tag profiles in ASH2L and MLL3 overexpressed KLF4 demonstrated with TRD + ZnF domain led to a significantly increased KLF4 occupancy around the CRRs. These results reveal a epitranscriptomic mechanism—whereby KLF4 interacted with MLL3/ASH2L through TRD + ZnF domain, and external promote NRBP2 expression. Functionally, NRBP2 emerges here governing AML cell proliferation arrest and apoptotic induction. Moreover, NRBP2 supports its clinical utility as a potential biomarker for risk stratification and therapeutic targeting. As a result, these point to a previously underappreciated layer of KLF4/NRBP2 axis in AML.

This epitranscriptomic circuit state also creates a therapeutic window wherein co-upregulating the KLF4/NRBP2 axis. Therefore, we explore that overexpression KLF4/NRBP2 inhibits Traf2- and Nck-interacting kinase (TNIK), as a serine/threonine kinase through RNA-seq. Although, previous study indicated that TNIK promoted colorectal and ovarian cancers through Wnt/β-catenin signaling pathway[39], it has not been reported in AML. Our result identified TNIK inhibitor (TNIK-IN-1) as a novel target to block proliferation establishing a key role of TNIK in AML. Critically, our data demonstrate that TNIK-IN-1 was more sensitive in AML cells compared with healthy donor normal hematopoietic progenitors. In proliferation assays, MNC from healthy donors maintained robust survival across a broad concentration range of TNIK-IN-1. Conversely, MNC in AML and five distinct AML cell lines exhibited pronounced, dose-dependent sensitivity. These findings underscore the translational potential of TNIK-IN-1 inhibitors, offering a favorable therapeutic window for clinical application. Furthermore, as TNIK signaling is corrected with neuronal health[39], our work suggests that it may be of interest to carefully consider the benefits of using TNIK inhibitors to reduce neurotoxicity induced by chemotherapeutic agents in patients with AML.

In addition, key questions remain unresolved. While mechanistic and therapeutic insights are restricted to AML, the efficacy of TNIK-IN-1 inhibition in other hematologic malignancies, such as myelodysplastic syndromes (MDS) and chronic myelogenous leukemia (CML), requires evaluation.

Furthermore, preclinical validation of TNIK-IN-1 is required, including pharmacokinetic profiling, safety evaluation, and efficacy assessments. It would be of considerable interest to explore how exactly NRBP2 regulate TNIK to suppress the proliferation of leukemia. Critically, longitudinal tracking of KLF4/NRBP2 expression during AML therapy may elucidate resistance mechanisms and yield predictive biomarkers.

In conclusion, our study demonstrates that the KLF4/MLL3 axis transcriptionally activates NRBP2 to suppress leukemia cell proliferation by promoting apoptosis. Furthermore, the small-molecule inhibitor TNIK-IN-1 inhibits AML cell proliferation by suppressing TNIK expression downstream of the KLF4/MLL3/NRBP2 axis. These findings elucidate a novel regulatory pathway for KLF4 in AML, identifying new therapeutic targets and a promising small-molecule drug for AML treatment.

Declarations

Data availability

The RNA-seq, ATAC-seq and CUT&Tag raw data supporting this study are available in the Gene Expression Omnibus (GEO) database at www.ncbi.nlm.nih.gov/geo, with the accession numbers GSE259333, GSE260724, GSE306746 and GSE306858.

Author infromation

Investigation, M.Y., C.C., T.L., Y.W., Q.J., S.Y..; data analysis: M.Y., C.C., T.L., X.X. and S.Y.; statistics: M.Y., C.C., T.L. and S.Y.; resources: Y.W., G.S., J.Y., H.D,X.C,X.Z., B.Z., Y.L., F.D.; writing, M.Y., S.Y., H.C. and T.C.; funding acquisition: T.C., H.C., and S.Y..; supervision, H.C., T.C..

Competing interests

The authors declare no competing interests.

Acknowledgments and Funding

This research was supported by the National Key Research and Development Program of China (2024YFA1107503, 2022YFA1106100, 2021YFA1103000), the National Natural Science Foundation of China (82525002, 92468206, 82270120), the Haihe Laboratory of Cell Ecosystem Innovation Fund (22HHXBSS00016), the CAMS Initiative for Innovative Medicine (2025-I2M-KJ-023, 2023-I2M-2-007,

2021-12M-1-040), and the CAMS Fundamental Research Funds for Central Research Institutes (3332021093).

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Figures

Fig. 1 KLF4 interacts with MLL3 complex in AML cells

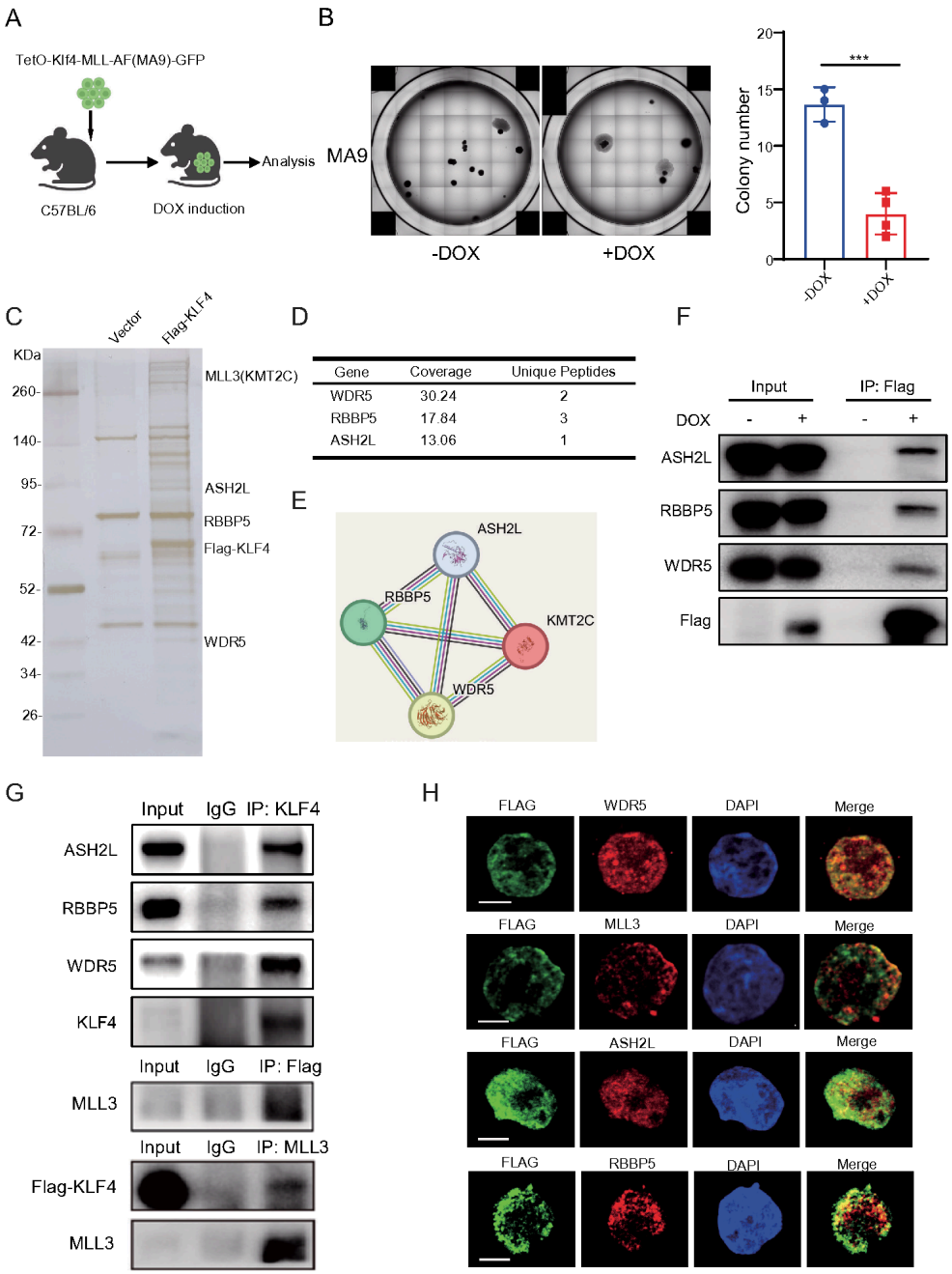


Figure 1

KLF4 interacts with MLL3 complex in AML cells.

(A) The schematic of the experimental procedure. Briefly, MA9-KLF4 cells were injected into recipient mice. 1 mg/ml of doxycycline was added to drinking water for 6 days to induce KLF4 expression. BM MA9 cells were collected for analyzed. (B) Colony assays of MA9 cells with KLF4 overexpression

induced with 2.5ug/ml doxycycline (DOX). n=3-4. The colony numbers from the left panel were analyzed. Data are represented as mean ± SD. ***$p < 0.001$, unpaired two-tailed Student's t test. (C) Analysis of FLAG-KLF4-associated proteins. Differential protein bands of the immunoprecipitated from THP-1 cell extracts were retrieved from silver-stained SDS-PAGE and analyzed by mass spectrometry. The MLL3, ASH2L, RBBP5, WDR5 and FLAG-KLF4 are shown. (D) Prediction of interacting protein complexes among KMT2C(MLL3), ASH2L, RBBP5 and WDR5 (https://cn.string-db.org/) (upper panel). (E) The parameters of the representatively WDR5, ASH2L and RBBP5 are shown by mass spectrometry. (F) Immunoprecipitation (IP) followed by immunoblotting (IB) with cellular extracts from THP-1 cells expressing FLAG-KLF4 with or without DOX. (G) Co-IP analysis of the interaction between KLF4 and MLL3, ASH2L, RBBP5, and WDR5 with cellular extracts from THP-1 cells. (H) Representative images of immunofluorescence labeling for FLAG, MLL3, ASH2L, RBBP5 and WDR5 in THP-1 cells, scale bars, 5μm.

Fig. 2 TRD and ZnF domain of KLF4 mediates association of KLF4 and MLL3 complex

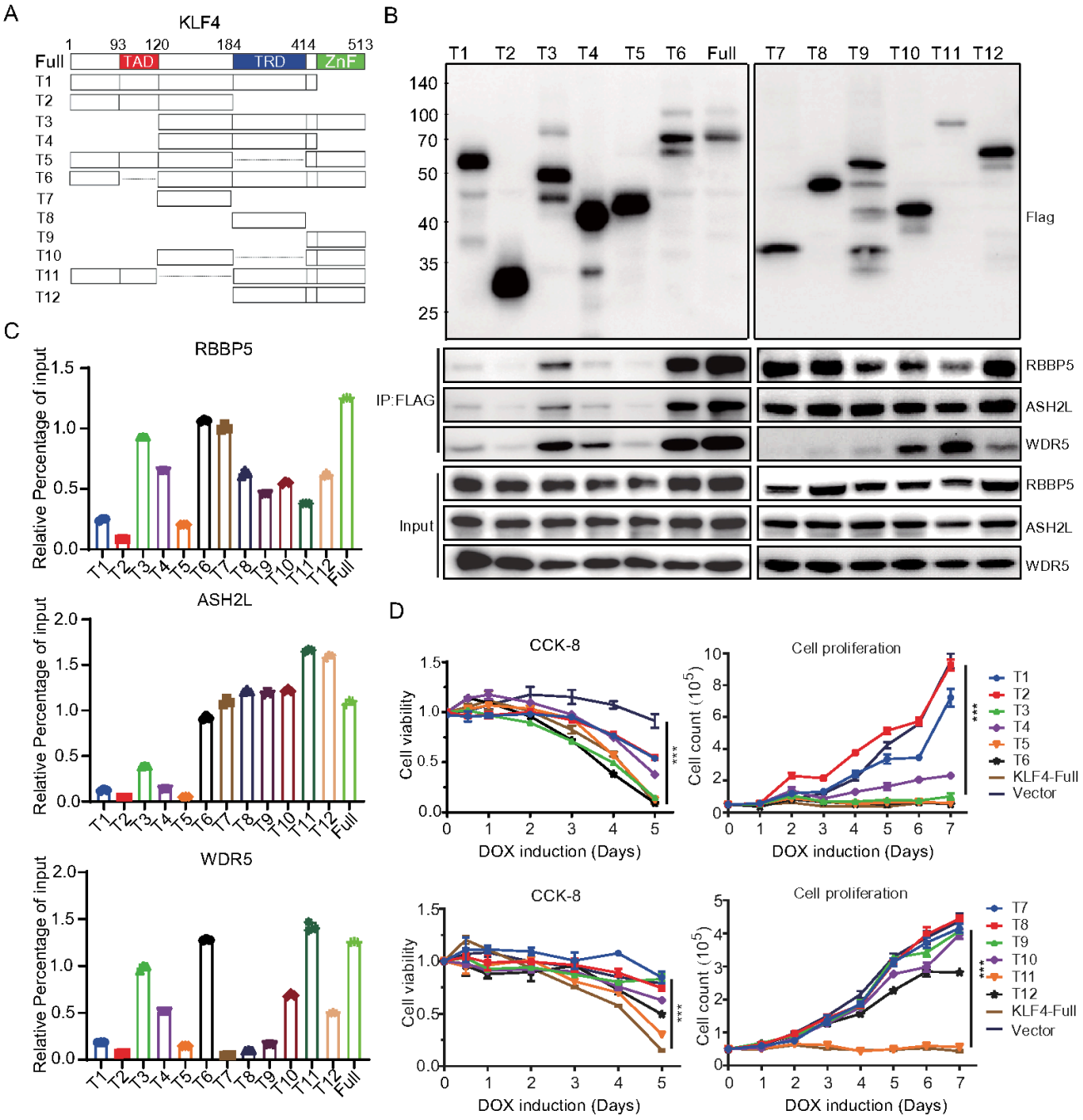


Figure 2

TRD and ZnF domain of KLF4 mediates association of KLF4 and MLL3 complex

(A) Schematic of 12 (T1-T12) the KLF4 truncation mutants. TAD, transactivation domain; TRD, transrepression domain; ZnF: Zinc finger domain. (B) Immunoprecipitation (IP) followed by immunoblotting (IB) anti-FLAG with cellular extracts from THP-1 cells expressing FLAG-KLF4 to identify

truncation was built successfully. CO-IP by KLF4 truncation mutant baits were analyzed by Western blotting with antibodies RBBP5, ASH2L and WDR5. (C) Comparison of grayscale values between IP protein (RBBP5, ASH2L and WDR5) and input. (D) CCK-8 assays of DOX-induced KLF4 expression in different truncations (left panel). Cell proliferation assays of DOX-induced KLF4 expression in different truncations (right panel). Data are represented as mean ± SD. ***$p < 0.001$, two-way ANOVA.

Fig. 3 KLF4/MLL3 axis promotes NRBP2 transcription in AML cells

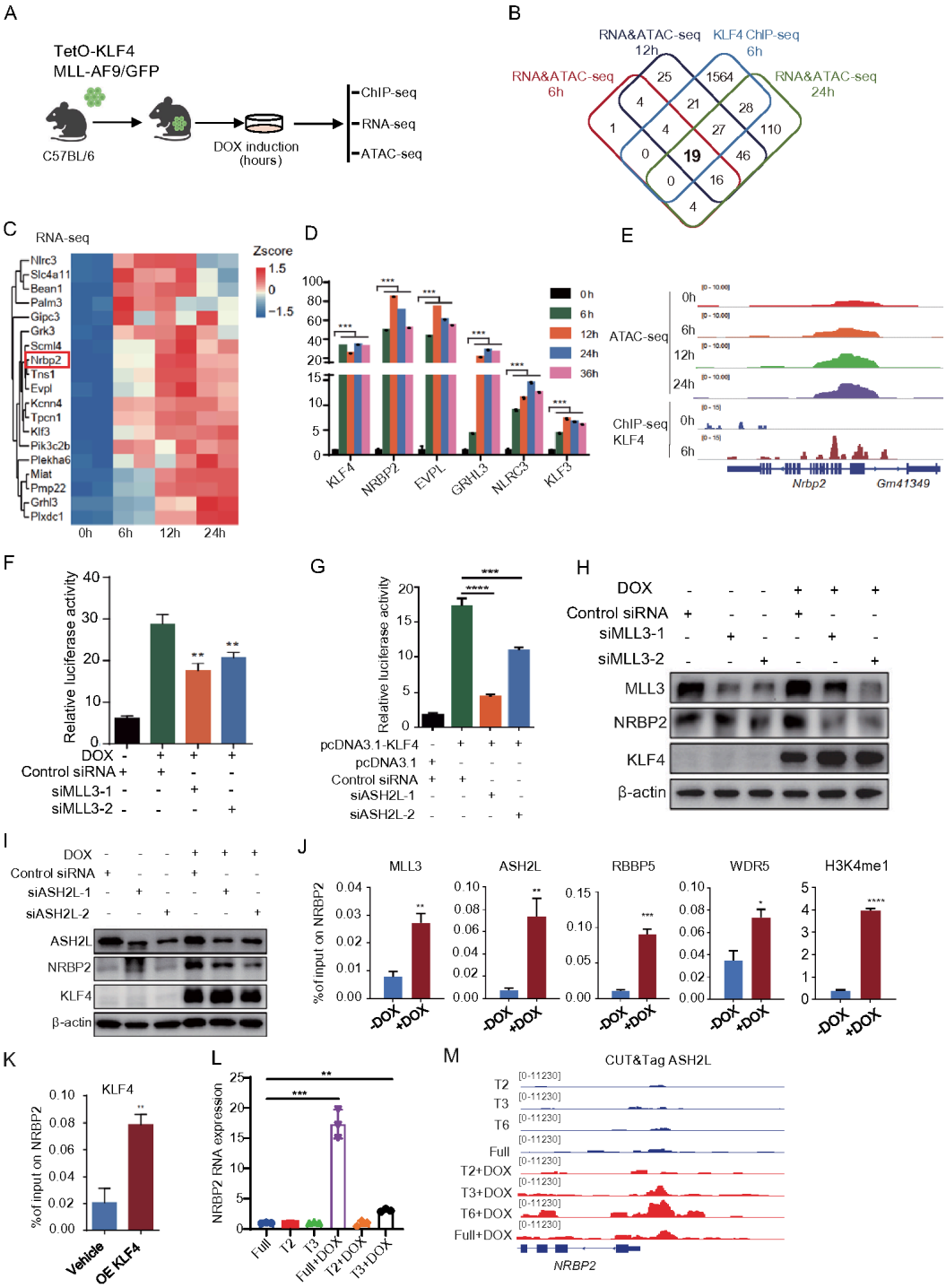


Figure 3

KLF4/MLL3 axis promotes NRBP2 transcription in AML cells

(A) Schematic diagram illustrating the workflow for RNA-seq, ATAC-seq and ChIP-seq. First, TetO-KLF4 MA9 cells were isolated from mouse bone marrow. Second, DOX were added to TetO-KLF4 MA9 cells for 0, 6,12,24 hours. (B) Number of overlapping upregulated differentially expressed genes (DEGs) and peak genes in overexpression KLF4 MA9 cells according to RNA-seq (6h,12h,24h), ATAC-seq (6h,12h,24h), and ChIP-seq(6h). (C) Heatmap of DEGs in 19 overlapping gene from Fig. 3B. (D) qRT-PCR analysis of KLF4, NRBP2, EVPL, GRHL3, NLRC3 and KLF3 expression in THP-1 cells (n = 3 per group). (E) IGV software visualization of ATAC-seq and ChIP-seq results for KLF4 in relation to NRBP2. (F and G) For reporter assays, THP-1 cells were co-transfected with KLF4 and siMLL3 or KLF4 and siASH2L together with Renilla and NRBP2 cis-regulatory region(CRRs) luciferase. The relative luciferase activity was determined by sequential normalization to Renilla and pGL3-vector activity (n = 3). (H) Western blots of the expression of MLL3, NRBP2 and KLF4 are shown in overexpression KLF4 and siMLL3 THP-1 cells. (I) Western blots of the expression of ASH2L, NRBP2 and KLF4 are shown in overexpression KLF4 and siASH2L THP1 cells. (J, K) ChIP-qPCR analysis showing MLL3, ASH2L, RBBP5, WDR5, H3K4me1 and KLF4 binding to NRBP2 promoter regions upon KLF4 expression (n = 3). (L) qRT-PCR of NRBP2 in full length, T2 and T3 truncation cells with or without DOX treatment. (M) IGV software visualization of CUT&Tag of ASH2L results in relation to NRBP2 form indicate cells. Data are represented as mean ± SD. *p< 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA for D, F-G and L; unpaired two-tailed Student’s t test for J-K.

Fig. 4 KLF4 suppresses AML cell proliferation through NRBP2

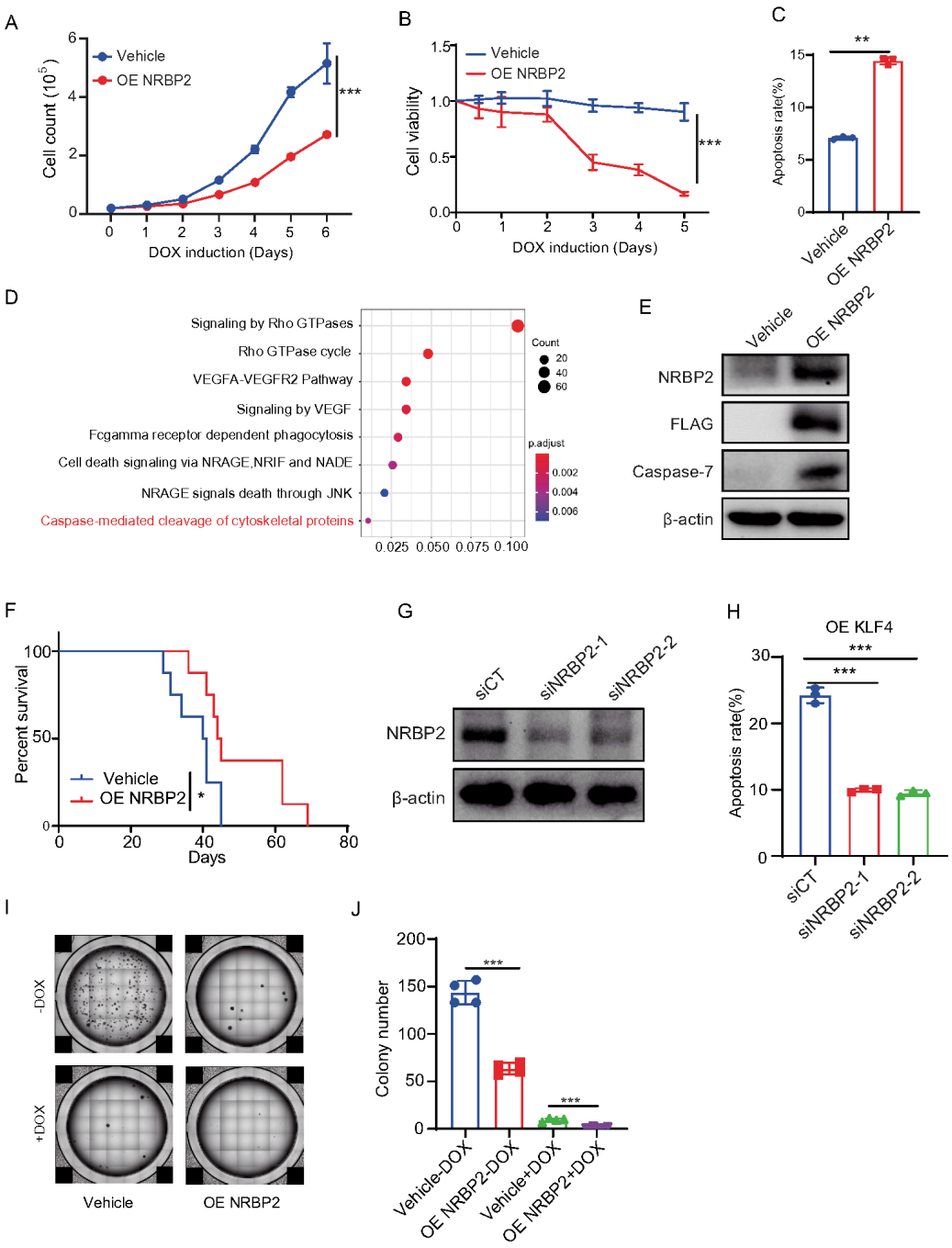


Figure 4

KLF4 suppresses AML cell proliferation through NRBP2

(A) Cell proliferation assays of NRBP2 overexpression in THP-1 cells from D0-D6. (B) CCK-8 assays of NRBP2 overexpression in THP-1 cells from D0-D6. (C) Apoptosis assays of NRBP2 overexpression in THP-1 cells at 48h. (D) GO analysis was enriched in Caspase-mediated cleavage of cytoskeletal proteins

in MA9 cells. (E) Western blots of the expression of NRBP2, Flag, BAX, Caspase-7 and BCL-2. (F) The survival curves of recipient mice. The red trace is the survival curves of overexpression NRBP2 and the blue curves is vehicle, respectively *p< 0.05, log - rank test. (G) THP-1 cells were transduced with control or NRBP2 siRNAs. The cellular extracts were collected for western blots analysis. (H) Apoptosis assays of overexpression KLF4 and siNRBP2 in THP-1 cells at 24h. (I, J) It showed colony assays in THP-1 cells overexpressed KLF4 and NRBP2. Data are represented as mean ± SD. ***p < 0.001, two-way ANOVA for A-B, unpaired two-tailed Student's t test for C, one-way ANOVA for H and J.

Fig. 5 KLF4/NRBP2 axis downregulates TNIK and TNIK inhibitor suppresses AML progression

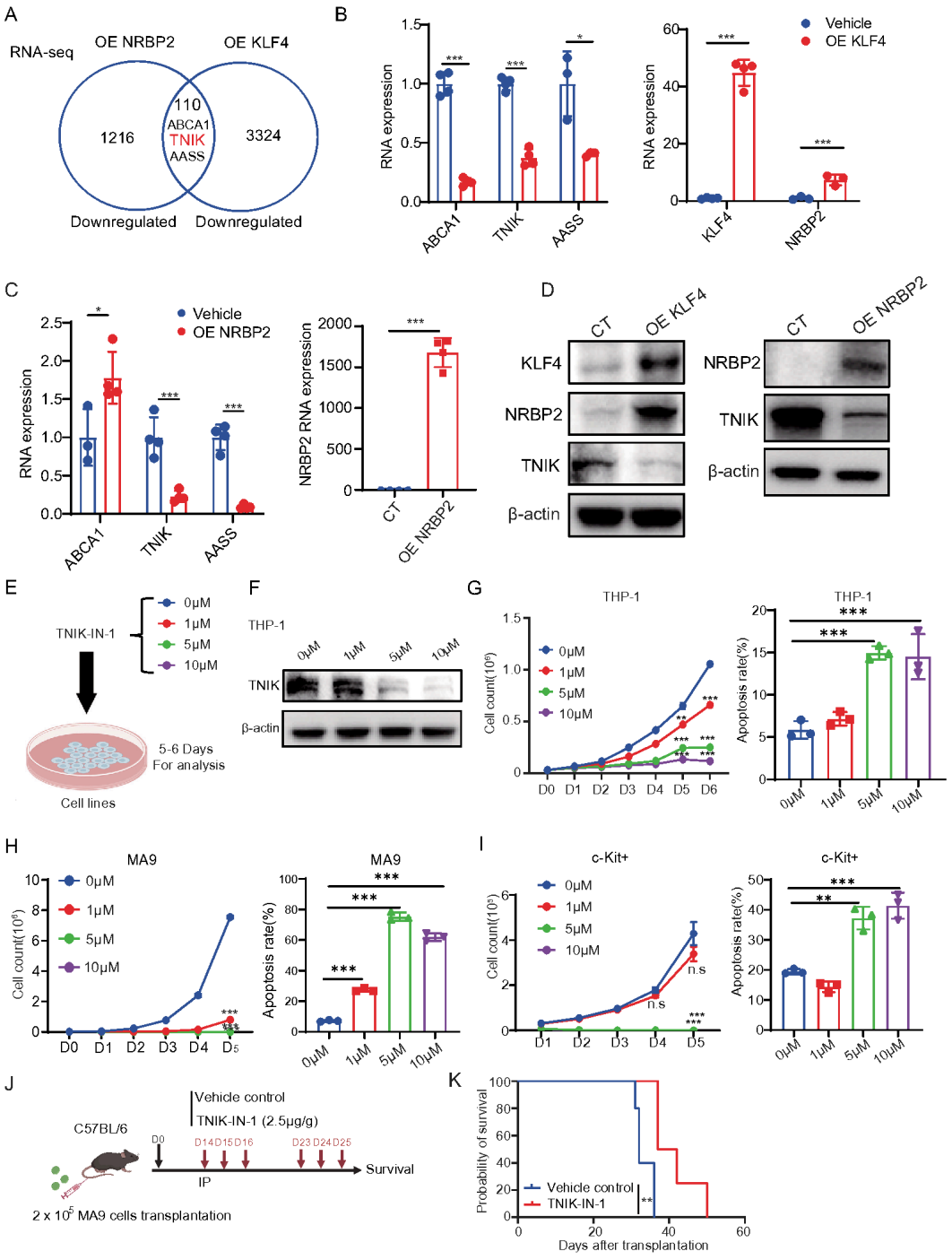


Figure 5

KLF4/NRBP2 axis downregulates TNIK and TNIK inhibitor suppresses AML progression

(A) Number of overlapping downregulated differentially expressed genes DEGs in overexpression KLF4 and NRBP2 THP-1 cells according to RNA-seq. (B) qRT-PCR analysis of KLF4, NRBP2, ABCA1, TNIK and AASS expression in control and OE KLF4 THP-1 cells. (C) qRT-PCR analysis of NRBP2, ABCA1, TNIK and AASS expression in control and OE NRBP2 THP-1 cells. (D) Western blots of the expression of NRBP2, KLF4 and TNIK in overexpressed KLF4 and NRBP2 cells, respectively. (E) Schematic diagram of TNIK-IN-1 treatment of cell lines. (F) Western blots of the expression of TNIK, BCL-2 and BAX in THP-1 cells added with TNIK-IN-1. (G-I) Proliferation and apoptosis assays in THP-1, MA9 and c-kit cells with TNIK-IN-1 at 0, 1, 5, 10μM. (J) Schematic diagram of TNIK-IN-1 treatment of MA9 mouse model. (K) The survival curves of recipient mice. The red trace is the survival curves of TNIK-IN-1 treatment and the blue curves is vehicle control, respectively. ***p* < 0.01, log-rank test. Data are represented as mean ± SD. **p* < 0.05, ***p*< 0.01, ****p* < 0.001, one-way ANOVA for B-C and G-I.

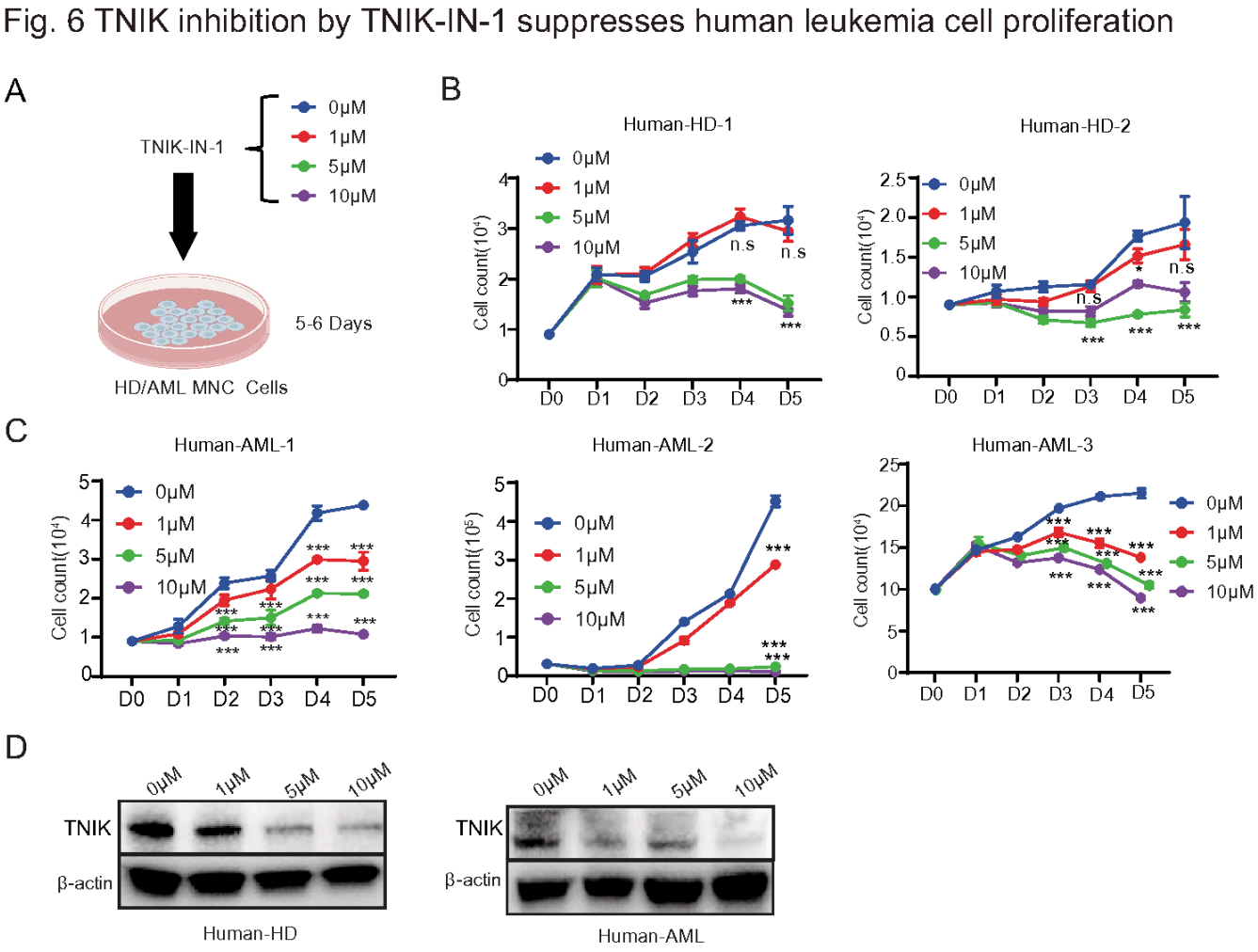


Figure 6

TNIK inhibition by TNIK-IN-1 suppresses human leukemia cell proliferation.

(A) Schematic diagram of TNIK-IN-1 treatment of HD (healthy donor)/AML MNC cellsfrom BM samples. (B-C) Proliferation assays of HD or AML MNC cells with TNIK-IN-1 treatment at 0, 1, 5, 10μM. (D) Western blots of the expression of TNIK in HD or AML MNC cellstreated with TNIK-IN-1 at 0, 1, 5, 10μM. Data are represented as mean ± SD. *$p < 0.05$, ***$p < 0.001$, one-way ANOVA for B-C.

Supplementary Files

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