Mutant NPM1-regulated IncRNA HOTAIRM1 promotes leukemia cell autophagy and proliferation by targeting EGR1 and ULK3

Yipei Jing†, Xueke Jiang†, Li Lei†, Meixi Peng†, Jun Ren†, Qiaoling Xiao†, Yao Tao†, Yonghong Tao†, Junpeng Huang†, Lu Wang†, Yuting Tang†, Zailin Yang‡, Zesong Yang§ and Ling Zhang∗

Abstract
Background: Acute myeloid leukemia (AML) with mutated nucleophosmin (NPM1), which displays a distinct long noncoding RNA (lncRNA) expression profile, has been defined as a unique subgroup in the new classification of myeloid neoplasms. However, the biological roles of key lncRNAs in the development of NPM1-mutated AML are currently unclear. Here, we aimed to investigate the functional and mechanistic roles of the lncRNA HOTAIRM1 in NPM1-mutated AML.

Methods: The expression of HOTAIRM1 was analyzed with a public database and further determined by qRT-PCR in NPM1-mutated AML samples and cell lines. The cause of upregulated HOTAIRM1 expression was investigated by luciferase reporter, chromatin immunoprecipitation and ubiquitination assays. The functional role of HOTAIRM1 in autophagy and proliferation was evaluated using western blot analysis, immunofluorescence staining, a Cell Counting Kit-8 (CCK-8) assay, a 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay, flow cytometric analyses and animal studies. The action mechanism of HOTAIRM1 was explored through RNA fluorescence in situ hybridization, RNA pulldown and RNA immunoprecipitation assays.

Results: HOTAIRM1 was highly expressed in NPM1-mutated AML. High HOTAIRM1 expression was induced in part by mutant NPM1 via KLF5-dependent transcriptional regulation. Importantly, HOTAIRM1 promoted autophagy and proliferation both in vitro and in vivo. Mechanistic investigations demonstrated that nuclear HOTAIRM1 promoted EGR1 degradation by serving as a scaffold to facilitate MDM2-EGR1 complex formation, while cytoplasmic HOTAIRM1 acted as a sponge for miR-152-3p to increase ULK3 expression.

Conclusions: Taken together, our findings identify two oncogenic regulatory axes in NPM1-mutated AML centered on HOTAIRM1: one involving EGR1 and MDM2 in the nucleus and the other involving the miR-152-3p/ULK3 axis in the cytoplasm. Our study indicates that HOTAIRM1 may be a promising therapeutic target for this distinct leukemia subtype.

Keywords: Acute myeloid leukemia, Nucleophosmin, Long noncoding RNA, HOTAIRM1, Autophagy, Proliferation, EGR1, ULK3

Background
Acute myeloid leukemia (AML) is a heterogeneous disease characterized by genetic abnormalities and epigenetic changes [1]. In the 2016 updated World Health Organization (WHO) classification of myeloid neoplasms...
neoplasms, AML with nucleophosmin (NPM1) mutations was defined as a distinct subtype [2]. NPM1 mutation results in cytoplasmic mislocalization of the mutant protein (NPM1c+), which is critical for its role in leukemogenesis [3]. To date, researchers have focused mainly on the effect of NPM1c+ on functional interactions with its nuclear protein interacting partners and have tried to target NPM1c+ by using CRM1 inhibitors or a combination of all-trans retinoic acid (ATRA) and arsenic trioxide (ATO) [4, 5]. Although most patients respond to the current therapy and achieve complete remission, relapse occurs frequently [6]. Hence, novel therapeutic targets should be investigated to improve the diagnosis and treatment of patients with NPM1-mutated AML.

Recently, with the development of high-throughput transcriptome analysis via next-generation sequencing, an increasing number of long noncoding RNAs (lncRNAs) have been discovered [7–9]. LncRNAs comprise a heterogeneous family of RNA molecules longer than 200 nucleotides with no or limited protein-coding potential [10, 11]. Originally considered mere transcriptional noise, lncRNAs are starting to be considered pivotal regulators in numerous biological processes, including cell proliferation, apoptosis, migration, autophagy, and other processes [12–14]. In parallel, lncRNAs are dynamically regulated by intracellular factors, such as transcription factors, as well as by extracellular factors [15]. Accumulating evidence indicates that abnormal expression of specific lncRNAs plays an important role in leukemogenesis [16], and a recent report provided evidence that NPM1-mutated AML displays a distinct lncRNA expression profile [17]. The lncRNA HOX antisense intergenic RNA myeloid 1 (HOTAIRM1), located on chromosome 7p15 between the human HOXA1 and HOXA2 genes, was originally considered a lncRNA associated with differentiation and expressed in myeloid lineage cells [18]. However, recently, HOTAIRM1 has been found to be expressed in multiple other cell types, such as neurons [19] and glioblastoma cells [20]. Dias Beya et al. [21] were the first to reveal in 2015 that HOTAIRM1 is upregulated in patients with NPM1-mutated AML compared to patients with NPM1 non-mutated patients and that expression of HOTAIRM1 is associated with overall survival. Therefore, a deeper exploration of the biological significance of HOTAIRM1 in leukemia is imperative.

In the present study, we first determined that HOTAIRM1 is highly expressed in NPM1-mutated AML. Moreover, HOTAIRM1 expression was at least partially upregulated by mutant NPM1 via Krüppel-like factor 5 (KLF5)-dependent transcriptional regulation. Importantly, HOTAIRM1 promoted leukemia cell autophagy and proliferation in vitro and in vivo. Furthermore, HOTAIRM1 exerted its oncogenic effects by acting as a scaffold to recruit MDM2 to EGR1 in the nucleus and serving as a miRNA sponge to regulate ULK3 in the cytoplasm. Collectively, our findings reveal the oncogenic role of HOTAIRM1 and provide novel insights for future treatment of this distinct leukemia subtype.

**Methods**

**Gene expression omnibus (GEO) and the cancer genome atlas (TCGA) analysis**

Gene profiling data of AML patients were downloaded from the NCBI Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/gds) under the accession number GSE15434 (n = 251) and The Cancer Genome Atlas (TCGA) (http://www.cancergenome.nih.gov) (n = 151). To filter out lncRNAs from gene expression matrix, we used R software (version 3.6.3) and R package biomaRt to annotate the obtained data. Differentially expressed lncRNAs were then identified using the limma package. The R packages ggplot2 and heatmap2 were used to generate lncRNA expression heatmaps and volcano plots, respectively. Moreover, the overall survival (OS) of AML and NPM1-mutated AML samples from the data of TCGA and BeatAML databases were analyzed according to Kaplan-Meier method.

**Clinical samples**

A total of peripheral blood and bone marrow samples of 34 AML patients, including 20 NPM1-unmutated AML and 14 NPM1-mutated AML cases, were obtained from the First Affiliated Hospital of Chongqing Medical University and the Third Affiliated Hospital of Chongqing Medical University. Details of the clinical characteristics of patients are provided in Additional file 1: Table S1. Approval was obtained from the ethics committee of Chongqing Medical University. This study was performed in compliance with the Declaration of Helsinki. Written informed consents were signed by all subjects for study purpose. The mononuclear cells were enriched by Ficoll lymphocyte separation solution (Hao Yang Biological Manufacture Co., Ltd., Tianjin, China). Briefly, the sample was diluted with an equal volume of PBS and mixed well. The blood suspensions were added into the surface of Ficoll and then centrifuged to form discrete layer. The mononuclear layers were collected and washed twice with 1 × PBS. Finally, total RNA from the mononuclear cells was isolated for the analysis of HOTAIRM1 expression.

**Cell culture**

Human myeloid leukemia cell lines OCI-AML2 and OCI-AML3 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, NI, Germany). The OCI-AML3 cells carry
NPM1 mutation type A (NPM1-mA). Stable NPM1-mA-GFP expressing OCI-AML2 cells (OCI-AML2 + NPM1-mA) were generated (Additional file 6: Figure S1a-c) using lentiviral transduction and selected with 5–10 μg/mL blasticidin and fluorescence-activated cell sorter using a FACSCalibur-Sort instrument (Becton Dickinson Biosciences, Copenhagen, Denmark) equipped with a 15-mW argon ion laser (488 nm) for excitation. Human myeloid leukemia cell lines KG-1a, NB4, THP-1, HL-60, U937 and human embryonic kidney cell line HEK293T were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). These myeloid leukemia cell lines were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific) and 1% penicillin/streptomycin solution (Beyotime, Shanghai, China). HEK293T cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin solution. All cell lines were incubated at 37°C in the presence of 5% CO2.

Quantitative real-time PCR (qRT-PCR)
Total RNA was isolated using the TRIzol reagent (Takara, Tokyo, Japan), and transcribed into cDNA using the Prime-Script™ RT Reagent Kit (Takara). The qRT-PCR analysis was performed on a CFX Connect™ real-time system (Bio-Rad, Hercules, CA, USA) with the SYBR Green reaction kit (KAPA Biosystems, MA, USA). Cycling conditions were 30s at 95°C for the initial denaturation, and amplification was performed with 39 cycles of 5s at 95°C, 30s at 59°C, 20s at 72°C, and finally 10min at 72°C for extension. The expression levels were analyzed using the 2-ΔΔCt method. The IncRNA and mRNA expression levels were normalized by GAPDH; the miRNA expression levels were normalized by U6. Details of the primer sequences used are shown in Additional file 2: Table S2. Nuclear export inhibitor KPT-330 (Selleck, Houston, TX, USA) was used to treat OCI-AML3 cells at the concentration of 2 μM for 10h. These treated cells were applied to detect HOTAIR1M1 expression by qRT-PCR.

Lentiviral vectors and cell infection
The target sequences of the lentivirus-based short hairpin RNA (shRNA) were as follows: shNPM1#1: 5′-GCC GACAAAAAGATTATCATCTTT-3′; shNPM1#2: 5′-AGC AAAGTTTCCAAGAAAA-3′; shHOTAIRM1#1: 5′-CTG GAGACTGTGTTACCTATAA-3′; shHOTAIRM1#2: 5′- AGCTGGGAGATTATCAACCAA-3′; shMDM2: 5′-TTGATATTTGGCATTGTGCTG-3′. These shRNA vectors and scramble lentiviral vectors were purchased from Gene Pharma (Shanghai, China). The leukemia cells were infected with lentivirus for 48h in the presence of 5μg/mL polybrene (Sigma-Aldrich), after which they were subjected to 2 μg/mL puromycin (Sigma-Aldrich) selection for 7 d. The puromycin-resistant cells were isolated and propagated for further analysis.

Cell transfection
Plasmids encoding Flag-NPM1-mA was kindly provided by Dr. C.J. Sherr (Genetics and Tumor Cell Biology, St, Jude Children's Research Hospital, Memphis, TN, USA). The pcDNA3.1 vector expressing HOTAIR1M1, EGR1, ULK3, and empty vector were designed and constructed by Genecreate (Wuhan, China). The HOTAIR1M1 sequences containing wild-type or mutant EGR1 binding sites were respectively cloned into the pcDNA3.1 vector to form the HOTAIR1M1-wild-type vector (HOTAIRM1-WT) and the HOTAIR1M1-mutated type vector (HOTAIRM1-Mut1 and HOTAIRM1-Mut2). These expression vectors were also purchased from Genecreate (Wuhan, China). The pCMV3-Flag-MDM2 plasmids were purchased from Sino Biological (Beijing, China). The short interfering RNA (siRNA) targeting EGR1, ULK3 and control siRNA were synthesized by Gene Pharma (Shanghai, China). The sequences of siRNA were as follows: siWWP1: 5′-GAGUUAGAUGCUU GAAG-3′; siEGR1: 5′-GCUUCAUGCUUCC UCU-3′; siULK3: 5′-CGAACUUUGUUCGGC-3′; siNC: 5′-UUCUUCGAAGCUACGU-3′. Additionally, miR-152-3p mimics (5′-UCAGUGCAUGACAGA ACUUGG-3′), mimics NC (5′- UUGUACUACAAAG GUACUG-3′), miR-152-3p inhibitor (5′- CCAAGUUUC GUCAUGCAUGA-3′), inhibitor NC (5′-CAGUAC UUUUGUGUAGAAA-3′) were synthesized by Gene Pharma. Cells (1 × 106 per well) were plated into a six-well plate and transfected with plasmids or siRNA using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol. The transfected cells were harvested (transfection efficiency >70%~80%) after 48h to 72h. These cells were then subjected to further functional assays.

Western blot analysis
The cultured cells were washed and lysed in cell lysis buffer (Beyotime) with protease inhibitor (Bimake, Houston, TX, USA). Then, total protein concentrations in different samples were measured by a BCA protein assay kit (Beyotime). Subsequently, equal amounts of protein were loaded on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After the proteins were separated, they were transferred onto polyvinylidene fluoride (PVDF) membranes and subsequently blocked in 5% non-fat milk (Boster Biological Technology, Wuhan, China). Thereafter, the PVDF membranes were immunoblotted with the indicated primary
antibodies overnight at 4°C. The following primary antibodies and respective dilutions were used in this study: anti-NPM1-mA (#PA1–46356, 1:1000; Thermo Fisher Scientific); anti-NPM1-wt (#32–5200, 1:1000; Thermo Fisher Scientific); anti-GAPDH (sc-47,724, 1:5000; Santa Cruz Biotechnology, Dallas, TX, USA); anti-β-tubulin (ab18207, 1:1000; Abcam, Cambridge, United Kingdom), anti-PCNA (ab29, 1:1000; Abcam), anti-KLF5 (ab137676, 1:1000; Abcam), anti-WWP1 (ab43791, 1:1000; Abcam), anti-Cyclin D1 (ab185241, 1:1000; Abcam), anti-Ago2 (ab186733, 1:1000; Abcam), anti-MDM2 (ab16895, 1:1000; Abcam); anti-LC3 (A5179, 1:1000), anti-p62 (A5180, 1:1000), anti-CDK4 (A5189, 1:1000), anti-Bax (A5131, 1:1000), anti-Bcl-2 (A5010, 1:1000) and anti-ULK3 (A5208, 1:1000) were purchased from Bimake; anti-EGR1 (#4154, 1:1000) and anti-ubiquitin (#3936, 1:1000) were purchased from Cell Signaling Technology (Danvers, MA, USA). Then the PVDF membranes were incubated with anti-rabbit secondary antibody (#SA00001–2, 1:4000; Proteintechn, Rosemont, IL, USA) or anti-mouse secondary antibody (#A9917, 1:4000; Milipore, Burlington, MA, USA) for 1h, and subsequently exposed to enhanced chemiluminescence substrate (Milipore). GAPDH was used as the internal control. Protein synthesis inhibitor CHX (Millipore) was incubated with cells for the indicated periods of time. Proteasome inhibitor MG132 (Selleck) was used to treat cells at the concentration of 5 μM for 8h. These treated cells were applied to detect EGR1 and KLF5 expression by western blotting.

Luciferase reporter assay
For the HOTAIRM1 promoter luciferase reporter assay, the predicted binding sites of HOTAIRM1 and KLF5 were obtained from JASPAR (http://jaspar.genereg.net/). The different KLF5-binding fragment sequences or the corresponding promoter constructs in the promoter region of HOTAIRM1 were synthesized and then inserted into the pGL3-basic firefly luciferase reporter vector (E1751, Promega), named HOTAIRM1-pGL3-F, HOTAIRM1-pGL3-S1, HOTAIRM1-pGL3-S2. These vectors were co-transfected into AML cells with KLF5 construct or empty vector using transfection reagent vectors (E1751, Promega), named HOTAIRM1-pGL3-WT (HOTAIRM1-WT) and the HOTAIRM1-mutated type vector (HOTAIRM1-Mut). Similarly, the vectors of ULK3-WT and ULK3-Mut were constructed according to the above method. Next, these vectors were co-transfected into HEK293T cells with miR-152-3p mimics or mimics NC using Lipofectamine 2000 (Invitrogen) following the manufacturer’s procedures. Measurement of luciferase activity was conducted with a Dual-Luciferase Report Assay System (Promega, Madison, WI, USA) at 48h post-transfection. The relative luciferase activity was calculated by dividing firefly luciferase activity by renilla luciferase activity. All the expression vectors mentioned above were purchased from Genecreate (Wuhan, China).

Chromatin immunoprecipitation (ChIP) assays
ChIP assays were performed to verify the interaction between KLF5 and the promoter region of HOTAIRM1. EZ-Magna ChIP kit (Millipore) was utilized to carry out this assay. In brief, cells were treated with 1% formaldehyde at 37°C. After cells were cross-linked for 10min, 125nM glycine was added for the termination of crosslinking for 5min. Thereafter, cells were harvested and sonicated to generate DNA fragments in length of 200 to 1000bp. Subsequently, the cell lysis was immunoprecipitated overnight at 4°C with KLF5 antibody (ab137676; Abcam) or the negative control IgG antibody (#12–371; Millipore), respectively. The ChIP primer sequences are listed in Additional file 3: Table S3. These primers were then used for qRT-PCR according to the manufacturer’s instructions. Using the formula $2^{\Delta \text{InputCt} - \Delta \text{TargetCt}} \times 0.1 \times 100$, the ChIP data were calculated as percentages with respect to the input DNA. The RCR products were loaded into electrophoresis on a 2% agarose gel and observed using ultraviolet light.

Immunofluorescence staining
Leukemia cells were washed with PBS and cytospun onto coverslips, then fixed with 4% paraformaldehyde for 20min under room temperature, permeabilized with 0.3% Triton X-100 for 15 min and then blocked for 30min with 5% of goat serum. The cells were rinsed with PBS and incubated overnight at 4°C in dilution buffer containing primary antibody rabbit monoclonal antibody LC3 (NB100-2220, 1:200; Novus, Littleton, CO, USA). The cells were washed 3 times with PBS. Then, the cells were incubated with Alexa Fluor fragment of goat anti-rabbit IgG (Beyotime) for 1h, and 4′,6-diamino-2-phenylindole (DAPI, Beyotime) was used for the nucleus counterstaining. Finally, the cells were visualized using a fluorescence microscope (Nikon, Tokyo, Japan) at 400× magnification.

Cell proliferation assays
Cell viability was measured with a Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto,
Cells were seeded into a 96-well plate (Corning, NY, USA) at a density of $3 \times 10^3$ cells per well with RPMI-1640 containing 10% FBS. Subsequently, 10 μl CCK8 reagent was added into each well and incubated with cells for 2h. Finally, the absorbance was measured at 450 nm using the microplate reader (BioTeck, CA, USA). When performing a 5-ethyl-2'-deoxyuridine (EdU) incorporation assay (Ribobio, Guangzhou, China), we seeded cells into a 24-well plate at a density of $2 \times 10^4$ cells per well and each well was added with 50 μM EdU at 37°C for 4h, followed by fixation in 4% formaldehyde at room temperature for 15min. Next, the cells were treated with 0.2% Triton X-100 at room temperature for 5min and 100μL Apollo® mixture (RiboBio) for 30min, and then DAPI was added for 30min in order to label the cell nuclei. The ratio of EdU-stained cells (with green fluorescence) to DAPI-stained cells (with blue fluorescence) was used to evaluate cell proliferation. Images were taken and analyzed with a fluorescence microscope at 100× magnification.

Flow cytometric analyses

Cell cycle and apoptosis were analyzed by flow cytometry. Propidium (PI) staining was adopted to assess the cell cycle of leukemia cells. Briefly, cells were washed 3 times with PBS, centrifuged, and the supernatant was discarded. The cell concentration was adjusted to approximately $1 \times 10^6$ cells/mL after cells were resuspended in PBS, and added with 1 mL of precooled 75% ethanol (−20°C) to fix cells at 4°C for 2h, followed by centrifugation. The ice ethanol and the supernatant were discarded, and added with 100 μL of RNase A in the dark, water bathed for at 37°C for 30min, and then added with 400 μL of PI (Millipore). The cells were then incubated in dark conditions at 4°C for 30min, and the cell cycle was determined using flow cytometry at 488nm. The Annexin V FITC-PI staining assay was used to detect apoptosis. Cytarabine (Ara-C, Sigma) was used to treat OCI-AML3 cells at the concentration of 10 μM for 48h, OCI-AML2 + NPM1-mA cells at the concentration of 200 μM for 48h [22]. These cells were harvested and washed with PBS. Apoptosis staining was performed using an Annexin V FITC-PI apoptosis detection kit (BD Biosciences, Piscataway, NJ, USA) according to the manufacturer’s instructions. Stained cells were analyzed by FACSCalibur™ Flow cytometry (BD Biosciences) with CellQuest software.

Subcellular fractionation and fluorescence in situ hybridization (FISH)

To observe the sublocation of HOTAIRM1 in leukemia cells, nuclear and cytoplasmic RNA substrate were separated using PARIS Kit (Ambion, Austin, TX). HOTAIRM1 expression was determined by qRT-PCR with U6 as the nuclear control and GAPDH as the cytoplasmic control. For FISH assay, cells were first grown in 24-well plates for 24h and then fixed with 4% paraformaldehyde for 20 min at room temperature. After permeabilization with paraformaldehyde, cells were hybridized with 2 μM Cy3-labeled HOTAIRM1 FISH probe mix (Gene Pharma), and DAPI was used for nuclear counterstaining. Images were taken and analyzed with a fluorescence microscope at 400× magnification.

RNA pulldown assays

RNA pulldown assays were performed using the RNA-Protein Pull-Down Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. The cells were crosslinked in 1% formaldehyde for 10min, equilibrated in glycine buffer for 5min, washed with cold PBS 3times, scraped with 1 mL of lysis buffer and incubated for 10min. The cell samples were sonicated and then centrifuged, after which the supernatant was transferred to a 2-mL tube, and 50 μL cell lysis was saved for input analysis. The lystate supernatant was incubated with HOTAIRM1 probes or a negative probe for 3h at room temperature with rotation. Then, 100 μL of Streptavidin Magnetic Beads was added, and the mixture was incubated for 1h with stirring. The bead/sample mixture was washed twice, after which 10% of the mixture was subjected to RNA purification, while the remaining 90% was subjected to protein purification. After washing for 3–4times, HOTAIRM1-associated proteins, which were retrieved from beads, were subjected to SDS-PAGE and silver staining. Differential protein bands were excised and identified by mass spectrometry and western blotting.

RNA immunoprecipitation (RIP) assays

RIP assays were performed using an EZ-Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer’s instructions. The cells were rinsed with cold PBS and fixed in 1% formaldehyde for 10min. After centrifugation (1500×g for 15min at 4°C), cell pellets were collected and re-suspended in RIP lysis buffer. Then 100 μL of the lystate was incubated with RIP buffer containing magnetic beads, which were conjugated with human anti-EGR1 (Cell Signaling Technology), anti-Ago2 (Abcam) and normal rabbit IgG (Millipore). Among the antibodies, IgG was considered a negative control (NC). Protease K buffer was then added to the samples. Finally, the target RNA was extracted and purified for further study by qRT-PCR.
Immunoprecipitation (IP) assay
The cells were harvested and washed twice with ice-cold PBS buffer. Cells were then sonicated in IP buffer [20 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na3VO4 and protease inhibitor cocktail (Bimake), pH 7.5] at 4 °C for 3 times per 5 s by Bio-ruptor UCD-200 (Diagenode, Belgium), followed by centrifuged at 13,300 g at 4 °C for 10 min to remove the supernatants were each incubated with specific antibodies to normalize total amounts of the inputs. The cell debris. Expressing of the indicated proteins in the lysates was checked by western blotting using relevant antibodies to normalize total amounts of the inputs. The supernatants were each incubated with specific antibodies or normal IgG (as control), and equal amounts of protein A/G beads (Bimake) overnight at 4 °C. The protein A/G beads with the bound antibodies and interacting proteins were pelleted and washed 3 times with IP buffer before boiled in 2× SDS-PAGE sample. The boiled samples were then resolved in SDS-PAGE and subject to western blot analysis.

Animal experiments
Five-week-old female NOD/SCID mice were purchased from The Animal Resources Centre (Canning Vale WA, Australia). NOD/SCID mice were randomly split in 3 groups (n = 5/group). Total of 2 × 107 engineered OCI-AML3 cells stably transfected with shNC, shHOTAIRM1 and HOTAIRM1-silenced OCI-AML3 cells followed by HOTAIRM1 plasmid introduction (shHOTAIRM1 + HOTAIRM1) were injected into mice via the tail vein. Mice were monitored weekly for signs of weight loss or lethargy. Peripheral blood was obtained from mice and white blood cell counts (WBC) were analyzed. Mice were killed after 8 weeks, and bone marrow cells were harvested from femurs for Wright’s staining and western blotting. Mouse livers and spleens were excised and serially sectioned into 4 μm sections, and then the infiltration of liver and spleen was analyzed by hematoxylin and eosin staining (HE) and Immunohistochemistry (IHC) analysis of Ki-67. The Kaplan-Meier method was used to analyze the survival curves of mice in each group. Immunoprecipitation (IP) assay

Volcano plots showing the expression profiles of lncRNAs (top, samples compared to NPM1-wt AML samples (left, GSE15434; right, TCGA). c HOTAIRM1 expression levels in patients with NPM1-wt AML (n = 113) and patients with NPM1-mutated AML (n = 138) were obtained from the GSE15434 dataset. d HOTAIRM1 expression levels in AML patients without (n = 118) and with NPM1 mutation (n = 33) were obtained from the TCGA database. e The expression levels of HOTAIRM1 in primary AML blasts without (n = 20) and with NPM1 mutation (n = 14). f qRT-PCR was conducted to determine the level of HOTAIRM1 in AML cell lines. GAPDH was used as the endogenous control. g-h Survival analysis based on HOTAIRM1 expression in AML (g) and NPM1-mutated AML samples (h) using the data from TCGA and BeatAML. The data are presented as the mean ± SD of three independent experiments. ***P < 0.001

Results
HOTAIRM1 is highly expressed in NPM1-mutated AML cells
To identify the lncRNAs that potentially participate in the progression of NPM1-mutated AML, we analyzed gene expression profiles using public databases (GSE15434 and TCGA datasets). As expected, many lncRNAs were identified to be differentially expressed in NPM1-mutated AML, among which HOTAIRM1 was one of the most substantially changed (Fig. 1a-b). Notably, HOTAIRM1 was significantly upregulated in AML with NPM1 mutation compared to AML without NPM1 mutation (Fig. 1c-d). Consistent with these findings, the expression level of HOTAIRM1 was measured by qRT-PCR in 34 primary AML blasts, and HOTAIRM1 was found to be highly expressed in NPM1-mutated AML cases (n = 14) compared with NPM1-unmutated AML cases (n = 20) (Fig. 1e). Moreover, the HOTAIRM1 expression level was measured in a panel of AML cell lines, and relatively high expression of HOTAIRM1 was observed in OCI-AML3 cells, which naturally carry NPM1 mutation type A (NPM1-mA) (Fig. 1f). Subsequently, the prognostic value of HOTAIRM1 was assessed using data from TCGA and BeatAML. Kaplan-Meier survival analysis of AML patients revealed a significantly shorter overall survival time in the cohort with high HOTAIRM1 expression (Fig. 1g). Moreover, high HOTAIRM1 expression was evidently associated with poorer prognosis in...
Fig. 1 (See legend on previous page.)
NPM1-mutated AML (Fig. 1h). These findings demonstrated the high expression of HOTAIRM1 in NPM1-mutated AML.

**High expression of HOTAIRM1 is maintained by mutant NPM1 via KLF5-dependent transcriptional regulation**

Next, we explored the reason for the high HOTAIRM1 expression in NPM1-mutated AML cells. The NPM1 mutation is an AML driver lesion [23], and mutant NPM1 aberrantly mislocalized in the cytoplasm may induce mislocalization of its interacting partners, such as proteins and RNA molecules [22]. We first observed the subcellular localization of HOTAIRM1 in OCI-AML2 cells, OCI-AML3 cells and engineered OCI-AML2 + NPM1-ma cells. Cell fractionation experiments showed that the cellular localization of HOTAIRM1 remained the same regardless of the NPM1 mutational status (Additional file 7: Figure S2). Subsequently, we investigated the impact of this mutation on the expression of HOTAIRM1. Knockdown of NPM1-ma (Additional file 8: Figure S3a-b) significantly decreased the HOTAIRM1 level (Fig. 2a), whereas overexpression of NPM1-ma (Additional file 8: Figure S3c) had the opposite effect (Fig. 2b). Based on the effects of NPM1 mutation on the aberrant cytoplasmic localization of the NPM1 protein [24], KPT-330, a selective CRM-1/exportin-1 (XPO-1) inhibitor, was used to relocate cytoplasmic NPM1-ma to the nucleus (Additional file 8: Figure S3d), resulting in a decrease in the level of HOTAIRM1 (Fig. 2c). Furthermore, we sought to determine the molecular mechanism by which NPM1-ma upregulates HOTAIRM1 expression. We first found that either NPM1-ma knockdown or KPT-330 treatment decreased (Fig. 2d, f) but NPM1-ma overexpression increased (Fig. 2e) the luciferase activity in cells transfected with the HOTAIRM1 promoter. NPM1 may bind directly to the promoters of genes (i.e., c-MYC) to regulate their expression [25]. However, in our study, neither NPM1-wt nor NPM1-ma was able to bind the promoter of the HOTAIRM1 gene, as determined by ChIP (Additional file 9: Figure S4). We then sought to determine whether the regulation of HOTAIRM1 by NPM1-ma requires a cooperative event involving in this process. KLF5 was predicted to bind to the HOTAIRM1 promoter region with a high score (Additional file 4: Table S4), and the potential KLF5 binding sites in the HOTAIRM1 promoter sequence are shown in Fig. 2g. The ChIP assays showed that the E2 fragment of HOTAIRM1 (from −541 to −550bp) was responsible for the affinity of KLF5 for the HOTAIRM1 promoter (Fig. 2h, Additional file 10: Figure S5). Then, luciferase reporter assays were performed by cotransfecting three different luciferase reporter plasmids (Fig. 2i) with the KLF5 construct. Only the HOTAIRM1-pGL3-S2 group exhibited high HOTAIRM1 promoter activity (Fig. 2j). Notably, knockdown of NPM1-ma led to significant inhibition of the binding between KLF5 and the HOTAIRM1 promoter (Fig. 2k) and thus reduced HOTAIRM1 promoter activity (Fig. 2l), indicating that HOTAIRM1 expression is at least partially upregulated by mutant NPM1 via KLF5-dependent transcriptional regulation.

Finally, we sought to determine the mechanism by which NPM1-ma regulates KLF5 transcriptional activity. The IP-western blotting results showed a lack of binding between NPM1 and KLF5 (Additional file 11: Figure S6a-b). Notably, NPM1-ma knockdown caused a decrease in the KLF5 protein level but not the KLF5 mRNA level (Fig. 2m). In addition, we evaluated KLF5 expression in OCI-AML3 cells incubated with the proteasome inhibitor MG132 (Fig. 2n) and the protein synthesis inhibitor cycloheximide (CHX) (Fig. 2o) after knockdown of NPM1-ma. The results indicated that NPM1-ma regulates KLF5 protein stability via the proteasome pathway. WWP1 is a well-known E3 ubiquitin ligase that targets the KLF5 protein for ubiquitin-mediated degradation [26], and we investigated whether NPM1-ma regulates KLF5 via WWP1. IP experiments showed that NPM1-ma (but not NPM1-wt, Additional file 11: Figure S6c) bound to WWP1 (Fig. 2p) and that WWP1 bound to KLF5 (Fig. 2q). Importantly, NPM1-ma deficiency promoted the protein interaction between WWP1 and KLF5 (Fig. 2r) and increased WWP1-induced KLF5 ubiquitination (Fig. 2s). Collectively, these data showed that high expression of HOTAIRM1 was maintained by mutant NPM1 via KLF5-dependent transcriptional regulation and that WWP1 may be involved in this process.

**HOTAIRM1 promotes leukemia cell autophagy and proliferation**

Then, we investigated the biological functions of HOTAIRM1 in NPM1-mutated AML cells. Two individual shRNAs targeting HOTAIRM1 suppressed HOTAIRM1 expression in OCI-AML3 cells (Additional file 12: Figure S7a) and OCI-AML2 + NPM1-ma cells (Additional file 12: Figure S7c). Next, western blot analysis showed that HOTAIRM1 knockdown significantly decreased the microtubule-associated protein 1 light chain 3 (LC3-II) level and increased the p62 level (Fig. 3a, Additional file 13: Figure S8a). Additionally, immunofluorescence staining indicated that depletion of HOTAIRM1 reduced the accumulation of LC3 puncta (Fig. 3b, Additional file 13: Figure S8b). Then, CCK-8 assays showed that suppression of HOTAIRM1 expression decreased cell viability (Fig. 3c, Additional file 13: Figure S8c). Correspondingly, the number of EdU-positive cells was decreased in the HOTAIRM1-silenced group (Fig. 3d, Additional file 13: Figure S8d).
Furthermore, we examined whether HOTAIRM1 affects cell proliferation by altering cell cycle progression and apoptosis. Flow cytometric analyses showed that loss of HOTAIRM1 significantly increased the percentage of cells in G0/G1 phase and reduced that of cells in S phase (Fig. 3e, Additional file 13: Figure S8e). In addition, both the basal (Fig. 3f, Additional file 13: Figure S8f) and Ara-C-induced (Fig. 3g, Additional file 13: Figure S8g) apoptosis rates were markedly increased in HOTAIRM1-silenced cells. Moreover, HOTAIRM1 knockdown significantly decreased the levels of Cyclin D1, CDK4 and Bcl-2 but increased the level of Bax (Fig. 3h, Additional file 13: Figure S8h).

In complementary gain-of-function studies, the HOTAIRM1 expression vector was transfected into OCI-AML3 cells (Additional file 12: Figure S7b) and OCI-AML2 + NPM1-mA cells (Additional file 12: Figure S7d), and these cells were used for further analysis. As anticipated, forced expression of HOTAIRM1 increased the LC3-II level, decreased the p62 level (Fig. 3i, Additional file 12: Figure S7c).
Additional file 13: Figure S8i) and induced the accumulation of LC3 puncta (Fig. 3j). Moreover, overexpression of HOTAIRM1 enhanced cell viability and increased the number of EdU-positive cells (Fig. 3k-l). In addition, gain of HOTAIRM1 promoted cell cycle progression (Fig. 3m) and inhibited both basal (Fig. 3n) and Ara-C-induced apoptosis (Fig. 3o). Furthermore, HOTAIRM1 overexpression significantly increased the levels of Cyclin D1, CDK4, Bax and Bcl-2 but decreased the level of Bax (Fig. 3p). Additionally, overexpression of HOTAIRM1 in OCI-AML2 (Additional file 13: Figure S8) promoted cell autophagy and proliferation (Additional file 14: Figure S9). Collectively, these results indicated an important role of HOTAIRM1 in promoting autophagy and proliferation in NPM1-mutated AML cells.

**Nuclear HOTAIRM1 promotes EGR1 degradation through MDM2-mediated ubiquitination**

We further explored the potential mechanisms by which HOTAIRM1 contributes to the malignant phenotypes of NPM1-mutated AML cells. First, cellular fractionation and FISH assays were conducted to observe the subcellular localization of HOTAIRM1 in OCI-AML3 cells, and the data showed that HOTAIRM1 was expressed in both the nucleus and cytoplasm (Fig. 4a). Since nuclear
IncRNAs can perform their functions by binding to proteins [27, 28]. We conducted RNA pulldown assays followed by mass spectrometry in OCI-AML3 cells using an in vitro biotinylated HOTAIRM1 transcript (Fig. 4b). Mass spectrometry analysis (Additional file 5: Table S5) revealed that early growth response 1 (EGR1) bound to HOTAIRM1, and this binding was further confirmed by RNA pulldown followed by western blot analysis (Fig. 4c). EGR1, a zinc finger transcription factor, plays an important role in controlling inflammation, growth, differentiation, apoptosis and tumor progression [29]. Our RIP assays demonstrated that HOTAIRM1 RNA was precipitated with the anti-EGR1 antibody (Fig. 4d). To further identify the EGR1-interacting region in HOTAIRM1, we first generated HOTAIRM1-WT, HOTAIRM1-Mut1 and HOTAIRM1-Mut2 constructs (Additional file 15: Figure S10) based on the JASPAR database (Fig. 4e). Subsequent RIP assays showed that overexpression of HOTAIRM1-Mut1 reversed the inhibition of binding between HOTAIRM1 and EGR1 induced by HOTAIRM1 knockdown (Fig. 4f). Similarly, RNA pulldown assays revealed that EGR1 bound to HOTAIRM1-Mut1 (Fig. 4g). These data demonstrated that HOTAIRM1 directly binds to the EGR1 protein.

Next, we focused on the biochemical consequences of the interaction between HOTAIRM1 and EGR1. Overexpression of EGR1 (Additional file 16: Figure S11a) had no effect on the HOTAIRM1 level (Additional file 16: Figure S11b). Conversely, HOTAIRM1 expression negatively regulated the EGR1 protein level (Fig. 5a) without altering the EGR1 mRNA level (Fig. 5b). On the basis of these data, we further investigated whether HOTAIRM1 is involved in the regulation of EGR1 protein stability. As shown in Fig. 5c, the proteasome inhibitor MG132 antagonized the decrease in EGR1 level caused by HOTAIRM1. In addition, treatment with the protein synthesis inhibitor CHX demonstrated that HOTAIRM1 decreased the half-life of the EGR1 protein (Fig. 5d). Subsequent ubiquitination assays revealed that HOTAIRM1 knockdown decreased the level of ubiquitinated EGR1 protein, whereas HOTAIRM1 overexpression increased EGR1 ubiquitination (Fig. 5e). These findings suggested that HOTAIRM1 reduces the stability of EGR1 by promoting its ubiquitination.

Later, we identified which ubiquitin ligase might target EGR1 for degradation in leukemia cells. Murine double minute 2 (MDM2), a well-known E3 ubiquitin-protein ligase, was predicted to interact with EGR1 through bioinformatics analysis (UbiBrowser) (Additional file 17: Figure S12a-b). Indeed, we detected the interaction of endogenous MDM2 and EGR1 in OCI-AML3 cells by IP (Fig. 5f). Importantly, knockdown of MDM2 increased but overexpression of MDM2 decreased the EGR1 protein levels (Fig. 5g). We further confirmed that MDM2 increased EGR1 ubiquitination (Fig. 5h). Finally, we examined the role of HOTAIRM1 in MDM2-mediated EGR1 degradation. The association of HOTAIRM1 with both EGR1 and MDM2 was verified by RNA pulldown assays (Fig. 5i). Correspondingly, IP assays showed that downregulation of HOTAIRM1 inhibited the interaction between EGR1 and MDM2, whereas HOTAIRM1 overexpression had the opposite effect (Fig. 5j). Considering these results collectively, we concluded that HOTAIRM1 promotes EGR1 degradation through MDM2-mediated ubiquitination.

Nuclear HOTAIRM1 promotes autophagy and proliferation by downregulating EGR1 expression

EGR1 has been linked to tumor suppression during cancer progression [30]. However, the role of EGR1 in leukemia cells is still unclear. We found that forced expression of EGR1 significantly inhibited autophagy (Fig. 6a), proliferation (Fig. 6b) and cell cycle progression (Fig. 6c) but resulted in a significant increase in apoptosis (Fig. 6d). Furthermore, to determine whether HOTAIRM1 regulates leukemia cell autophagy and proliferation via EGR1, we performed rescue experiments. Silencing EGR1 partially reversed the HOTAIRM1 knockdown-induced reductions in autophagy (Fig. 6e) and proliferation (Fig. 6f, Additional file 18: Figure 13a-b). Conversely, EGR1 overexpression partially reversed the promotive effects of HOTAIRM1 on autophagy (Fig. 6g) and proliferation (Fig. 6h, Additional file 18: Figure 13c-d). Taken together, these data revealed that nuclear HOTAIRM1 promotes autophagy and proliferation by downregulating EGR1.

(See figure on next page.)

**Fig. 4** HOTAIRM1 interacts with EGR1 in NPM1-mutated AML cells. a The subcellular localization of HOTAIRM1 in OCI-AML3 cells was detected by FISH. Scale bar: 25 μm. b Schematic diagram of the identification of HOTAIRM1 binding proteins by RNA pulldown coupled with mass spectrometry. c The interactions between EGR1 or Ago2 and HOTAIRM1 in OCI-AML3 cells were validated by RNA pulldown and subsequent western blotting. d HOTAIRM1 RNA was measured by qRT-PCR in IgG control, EGR1, and Ago2 RIP samples. e Schematic diagram of the putative EGR1-binding sites in HOTAIRM1. f The effect of overexpression of HOTAIRM1-WT, HOTAIRM1-Mut1 and HOTAIRM1-Mut2 in HOTAIRM1-silenced OCI-AML3 cells was assessed by RIP assays. g RNA pulldown and western blotting were used to detect EGR1 expression in HOTAIRM1-silenced OCI-AML3 cells transfected with different vectors. The data are presented as the mean±SD of three independent experiments. *P<0.05, **P<0.01, ***P<0.001. n.s. indicates no significant difference.
Fig. 4 (See legend on previous page.)
Cytoplasmic HOTAIRM1 promotes autophagy and proliferation by sponging miR-152-3p to upregulate ULK3 expression

Given that HOTAIRM1 was also expressed in the cytosome, we then explored the role of cytoplasmic HOTAIRM1 in NPM1-mutated AML cells. HOTAIRM1 has already been identified as a microRNA sponge, including in AML [31, 32]. In this study, we observed that Ago2, the core component of the RNA-induced silencing complex, bound to HOTAIRM1 (Fig. 4c-d). A total of 12 miRNAs binding to HOTAIRM1 were identified by intersecting the predictions of lncRNA target prediction bioinformatics software tools (DIANA and starBase) (Fig. 7a), and miR-152-3p was found to exhibit the greatest change in expression upon HOTAIRM1 modulation (Fig. 7b-c). Next, the negative correlation between HOTAIRM1 and miR-152-3p expression in NPM1-mutated cell lines (Fig. 7d) and patients with NPM1-mutated AML (Fig. 7e) was verified by qRT-PCR. In addition, we analyzed the association between miR-152-3p expression and overall survival in patients with NPM1-mutated AML through the TCGA database and found that low expression of miR-152-3p was associated with poor overall survival in patients (Fig. 7f).

To further confirm the role of HOTAIRM1 involves in modulating miR-152-3p expression, we transfected cells with the miR-152-3p mimics. As expected, HOTAIRM1 knockdown significantly reduced the luciferase activity in the presence of miR-152-3p, whereas HOTAIRM1-Mut-transfected cells did not show a significant response to miR-152-3p (Fig. 7g). These findings clarified that HOTAIRM1 directly targets miR-152-3p.

Later, we investigated the putative target genes of miR-152-3p and found a trend toward a negative correlation between miR-152-3p and unc-51-like kinase 3 (ULK3) expression in the TCGA AML dataset (Fig. 7h). ULK3 is a kinase that induces autophagy [33–35] and was found to be highly expressed in NPM1-mutant AML (Fig. 7i). Importantly, miR-152-3p mimics significantly attenuated the luciferase activity of ULK3-L3-WT (Fig. 7j). Moreover, miR-152-3p upregulation led to decreased ULK3 mRNA and protein levels (Fig. 7k), and the opposite results were found for miR-152-3p downregulation (Fig. 7l). Furthermore, ULK3 expression was downregulated by HOTAIRM1 knockdown, while simultaneous miR-152-3p knockdown reversed the inhibition of ULK3 induced by HOTAIRM1 knockdown in OCI-AML3 cells (Fig. 7m). In contrast, the promotion of ULK3 expression by HOTAIRM1 upregulation was alleviated after miR-152-3p overexpression (Fig. 7n). Finally, forced expression of ULK3 reversed the inhibition of autophagy and proliferation (Additional file 19: Figure S14a-b) and the increase in apoptosis (Additional file 20: Figure S15a-b) induced by HOTAIRM1 knockdown. On the other hand, ULK3 silencing reversed the autophagy-enhancing, cell proliferation-promoting (Additional file 19: Figure S14c-d) and apoptosis-inhibiting (Additional file 20: Figure S15c-d) effects of HOTAIRM1 upregulation. Taken together, these results implied that cytoplasmic HOTAIRM1 promotes autophagy and proliferation by sponging miR-152-3p to upregulate ULK3 expression.

HOTAIRM1 knockdown impairs leukemogenesis in vivo

Based on our in vitro findings regarding HOTAIRM1, we speculated that HOTAIRM1 might play an important role in leukemogenesis in vivo. A xenograft model was established in NOD/SCID mice by injection of engineered OCI-AML3 cells (shNC, shHOTAIRM1 and shHOTAIRM1 + HOTAIRM1). As shown in Fig. 8a-c, HOTAIRM1 knockdown led to lower white blood cell (WBC) counts and liver and spleen weights. Wright’s staining of bone marrow smears showed that HOTAIRM1 knockdown significantly reduced the number of leukemia cells (Fig. 8d). In addition, HE staining revealed that fewer leukemia cells had infiltrated the liver and spleen in the shHOTAIRM1 group (Fig. 8e). IHC staining of tissues infiltrated by leukemia cells with anti-Ki-67 antibodies further implied that knockdown of HOTAIRM1 can inhibit leukemia cell proliferation (Fig. 8f). Western blot analysis showed that HOTAIRM1 knockdown decreased the LC3-II level and increased the p62 level (Fig. 8g). Moreover, the survival of mice implanted with shHOTAIRM1 cells was significantly prolonged compared with...
Fig. 5  (See legend on previous page.)
that of mice implanted with shNC cells (Fig. 8h). Notably, forced expression of HOTAIRM1 reversed the inhibitory effects of HOTAIRM1 knockdown on leukemogenesis (Fig. 8a-h). In conclusion, these data suggested that HOTAIRM1 knockdown impairs the progression of NPM1-mutated AML.

Discussion

Accumulating evidence implies that aberrant expression of IncRNAs plays a critical role in leukemia initiation and prognostic outcomes [36–38]. However, only a very small number of IncRNAs have been experimentally validated and functionally annotated in NPM1-mutated AML [22, 39, 40]. Our data herein demonstrated the high level of IncRNA HOTAIRM1 in NPM1-mutated leukemia cells and indicated that HOTAIRM1 was upregulated at least partially by mutant NPM1 via KLF5-dependent transcriptional regulation. Furthermore, nuclear HOTAIRM1 promoted EGR1 ubiquitination by enhancing the MDM2-EGR1 interaction, while cytoplasmic HOTAIRM1 increased ULK3 expression by competitively sponging miR-152-3p, therefore contributing to leukemia cell autophagy and proliferation (Fig. 9).

In the current study, we analyzed gene expression profiles using public databases and found that HOTAIRM1, an IncRNA transcribed from the antisense strand of the HOXA gene [20], was one of the most significantly upregulated IncRNAs. High expression of HOTAIRM1 was subsequently confirmed by qRT-PCR in both NPM1-mA-positive OCI-AML3 cells and primary AML blasts. In addition, our findings were supported by those of a previous study indicating that HOTAIRM1 expression was elevated in myeloid leukemia cells [18, 21]. Additionally, other HOX cluster IncRNAs, such as HOXA10-AS and HOXB-AS3, have been reported to be significantly overexpressed in NPM1-mutated leukemia cells [39, 41]. Deregulated IncRNAs have prognostic applications in AML [42]. Our results derived from public databases showed that a high level of HOTAIRM1 indicated a poorer prognosis in NPM1-mutated AML, consistent with a previous study that established that patients with NPM1-mutated AML with high HOTAIRM1 expression have worse prognoses [21]. Indeed, the clinical prognostic role of HOTAIRM1 in patients with NPM1-mutated AML awaits further study.

Next, we focused on the molecular mechanism underlying the high expression of HOTAIRM1 in NPM1-mutated AML. Recently, Gourvest M et al. revealed that the localization of the IncRNA LONA is dependent on the NPM1 mutational status [22]. Here, the cellular localization of HOTAIRM1 in leukemia cell lines remained the same regardless of the mutational status of NPM1. Notably, NPM1-mA significantly increased the HOTAIRM1 level and the luciferase activity of the HOTAIRM1 promoter. We next sought to identify the specific transcription factors involved in this process. Bioinformatics analysis and mechanistic experiments suggested that KLF5 directly activated the transcription of HOTAIRM1 by functioning as a transcriptional activator. More importantly, NPM1-mA knockdown inhibited KLF5-mediated transcriptional activation of HOTAIRM1. In addition, we investigated the mechanism by which NPM1 regulates KLF5 transcriptional activity and found that NPM1-mA weakened the KLF5-WWP1 interaction, thereby diminishing WWP1-mediated ubiquitination and degradation of KLF5. Collectively, our data revealed that the mutant form of NPM1 is responsible for the upregulation of HOTAIRM1 in AML cells via stabilization of the KLF5 transcription factor. Interestingly, IncRNAs have also been reported to regulate NPM1c+−mediated transcription. Zhu et al. [40] reported that the IncRNA HOXBLINC loss attenuates NPM1c+−driven leukemogenesis by rectifying the signature of NPM1c−leukemic transcription programs. In addition, IncRNA expression is susceptible to diverse factors at different levels. For example, epidermal growth factor rapidly attenuates the expression of the IncRNA LIMT by enhancing histone deacetylation in its promoter region [43]. N6-methyladenosine (m6A) methylation mediated by METTL3 and METTL14 contributes to the increased stability of LNCAROD [44]. A recent study described that the DNA methyltransferase DNMT3b can reduce HOTAIRM1 expression in AML [45]. Thus, other possible regulatory mechanisms contributing to HOTAIRM1 expression in NPM1-mutated AML merit further exploration.
Fig. 6 (See legend on previous page.)
Next, we explored the biological effects of high HOTAIRM1 expression in NPM1-mutated leukemia. Our previous work showed high autophagic activity in NPM1-mutated AML cells [46]. Therefore, we first investigated the role of HOTAIRM1 in regulating autophagy and found that HOTAIRM1 elevated autophagic activity. Accumulating evidence suggests that the functional relationship among autophagy, proliferation, and apoptosis is complex [47]. Here, we observed that HOTAIRM1 promoted cell proliferation and inhibited apoptosis. In vivo experiments further suggested that HOTAIRM1 promoted the malignant phenotype of NPM1-mutated AML. Collectively, these findings implied that HOTAIRM1 might play an oncogenic role in NPM1-mutated AML. Our idea was supported by a research showing that knockdown of HOTAIRM1 facilitated Ara-C-induced apoptosis in the AML cell lines HL-60 and THP-1 [48]. Notably, Chen et al. [31] reported that HOTAIRM1 plays a role as a tumor suppressor gene in acute promyelocytic leukemia (APL). In that study, HOTAIRM1 enhanced the autophagy pathway, which promoted ATRA-induced degradation of the PML-RARA oncoprotein and resulted in cell differentiation. These findings and our data help to identify HOTAIRM1 as a general regulator of autophagy and survival processes in leukemia cells.

To investigate the potential mechanisms by which HOTAIRM1 contributes to the malignant phenotype of NPM1-mutated leukemia cells, we examined its subcellular localization. HOTAIRM1 was localized in both the nucleus and cytoplasm. We first tested the role of nuclear HOTAIRM1 in leukemia cells. EGR1, a Cys2-His2-type zinc finger transcription factor, was identified as a specific interacting partner of HOTAIRM1. Further research showed that the proteasome pathway contributed to HOTAIRM1-induced EGR1 protein degradation. Then, MDM2 was confirmed to target EGR1 for ubiquitin-mediated degradation. More notably, nuclear HOTAIRM1 promoted EGR1 degradation by enhancing the MDM2-EGR1 interaction. Previous studies have suggested that lncRNAs can function as scaffolds to influence protein expression. For example, Lnczc3h7a acts as a scaffold to facilitate the interaction between TRIM25 and activated RIG-I, consequently increasing TRIM25-mediated K63-linked ubiquitination of RIG-I [49]. Additionally, the lncRNA CRNDE acts as a scaffold for DMBT1 and C-IAP1 complexes to activate the PI3K-AKT pathway [50]. Moreover, lncRNAs play roles as signals, decoys, and guides for regulatory proteins. LncRNA-Gas5 binds to the DNA-binding domain of the glucocorticoid receptor by acting as a decoy [51]. The lncRNA PACER dissociates the repressive NF-kB subunit p50 from the COX-2 promoter to activate COX-2 gene expression [52]. Recently, a study reported that HOTAIRM1 binds to ILF3 and blocks the effects of ILF3 on stabilizing pre-miR-144 [53]. Thus, whether other HOTAIRM1-associated proteins are involved in NPM1-mutated AML requires further investigation. After investigating the role of nuclear HOTAIRM1, we explored the function of cytoplasmic HOTAIRM1 in leukemia cells. The miR-152-3p level was significantly changed after modulation of HOTAIRM1, and miR-152-3p was found to bind directly to HOTAIRM1. More importantly, miR-152-3p also bound to ULK3 and regulated its expression. In addition, our data identified cytoplasmic HOTAIRM1 as a competing endogenous RNA (ceRNA) for miR-152-3p and antagonized its repression of ULK3 protein translation. These results are similar to the observation recently reported by Lin et al. [54], which indicated that HOTAIRM1 increases ZEB2 expression by sponging miR-873-5p. A recent study described that the lncRNA LBCS interacts with heterogeneous nuclear ribonucleoprotein K (hnRNPK) to suppress androgen receptor (AR) translation efficiency by forming a complex with hnRNPK and AR mRNA [55]. Thus, the other potential mechanisms underlying the oncogenic role of cytoplasmic HOTAIRM1 need to be determined. Most importantly, we are the first to reveal that HOTAIRM1 plays different regulatory roles in the nucleus and cytoplasm.
Fig. 7 (See legend on previous page.)
Fig. 8 HOTAIRM1 knockdown impairs leukemogenesis in vivo. a Blood was collected from all animals via the tail vein 4 weeks after cell transplantation, and the total WBC count was determined. b–c The mean liver (b) and spleen (c) weights of mice transplanted with shNC, shHOTAIRM1 and shHOTAIRM1 + HOTAIRM1 cells were quantified. d Immature cells from bone marrow were evaluated by Wright's staining. e Representative images of histologic sections of livers and spleens from mice transplanted with cells treated as indicated. Scale bar: 50 μm. f IHC staining of Ki-67 in liver and spleen tissue from mice transplanted with cells. Scale bar: 50 μm. g The protein levels of LC3 and p62 in each group were determined by western blotting. h Kaplan-Meier survival curves of mice in each group.
in NPM1-mutated AML. Prompted by these findings, we further investigated the role of the downstream HOTAIRM1 target genes EGR1 and ULK3 in leukemia cells. These results revealed that EGR1 and ULK3 participated in the modulation of autophagy and proliferation induced by HOTAIRM1. Our findings were consistent with the implication of EGR1 in the regulation of cell proliferation [56], migration [57] and apoptosis [58]. In addition, ULK3, an ATG1-related isoform [59], is involved in many biological processes, including autophagy [33], cytokinesis [34] and Hedgehog signaling [35]. Therefore, these findings demonstrate that HOTAIRM1 promotes leukemia cell autophagy and proliferation by regulating EGR1 and ULK3 expression.

In summary, the results of this study indicate that HOTAIRM1 functions as an oncogene in NPM1-mutated AML. Upregulation of HOTAIRM1 is induced by mutant NPM1 via KLF5-dependent transcriptional regulation. Importantly, high expression of HOTAIRM1 promotes autophagy and proliferation via the functions of HOTAIRM1 as a scaffold to recruit MDM2 to EGR1 in the nucleus and as a sponge for miR-152-3p to upregulate ULK3 in the cytoplasm. These findings shed new light on the lncRNAs involved in the pathogenesis of NPM1-mutated AML and indicate that HOTAIRM1 may be an important target for future treatment of this distinct leukemia subtype.

**Abbreviations**

AML: Acute myeloid leukemia; WHO: World Health Organization; NPM1: Nucleophosmin; NPM1c+: Cytoplasmic mutant NPM1; CRM1: Chromosome maintenance region 1; ATRA: All-trans retinoic acid; ATO: Arsenic trioxide; LncRNA: Long noncoding RNA; ceRNA: Competing endogenous RNA; HOTAIRM1: HOX antisense intergenic RNA myeloid 1; Ara-C: Cytarabine; KLF5: Kruppel like factor 5; WWP1: WW Domain-Containing Protein 1; MDM2: Murine double minute 2; EGR1: Early growth response protein 1; ULK3: Unc-51 like kinase 3; GEO: Gene Expression Omnibus; TCGA: The Cancer Genome Atlas; OS: Overall survival; DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; NPM1-mA: NPM1 mutation type A; ATCC: American Type Culture Collection; FBS: Fetal bovine serum; qRT-PCR: Quantitative real-time PCR; shRNA: Lentivirus-based short hairpin RNA; siRNA: Short interfering RNA; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF: Polyvinylidene fluoride; Ag2o: Argonaute 2; CDK4: Cyclin-dependent kinase 4; LC3: Microtubule-associated protein 1 light chain 3; CHX: Cycloheximide; ChIP: Chromatin immunoprecipitation; CCK-8: Cell Counting Kit-8; EdU: 5-ethynyl-2′-deoxyuridine; FISH: Subcellular fractionation and fluorescence in situ hybridization; RIP: RNA immunoprecipitation;
Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13046-021-02122-2.
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