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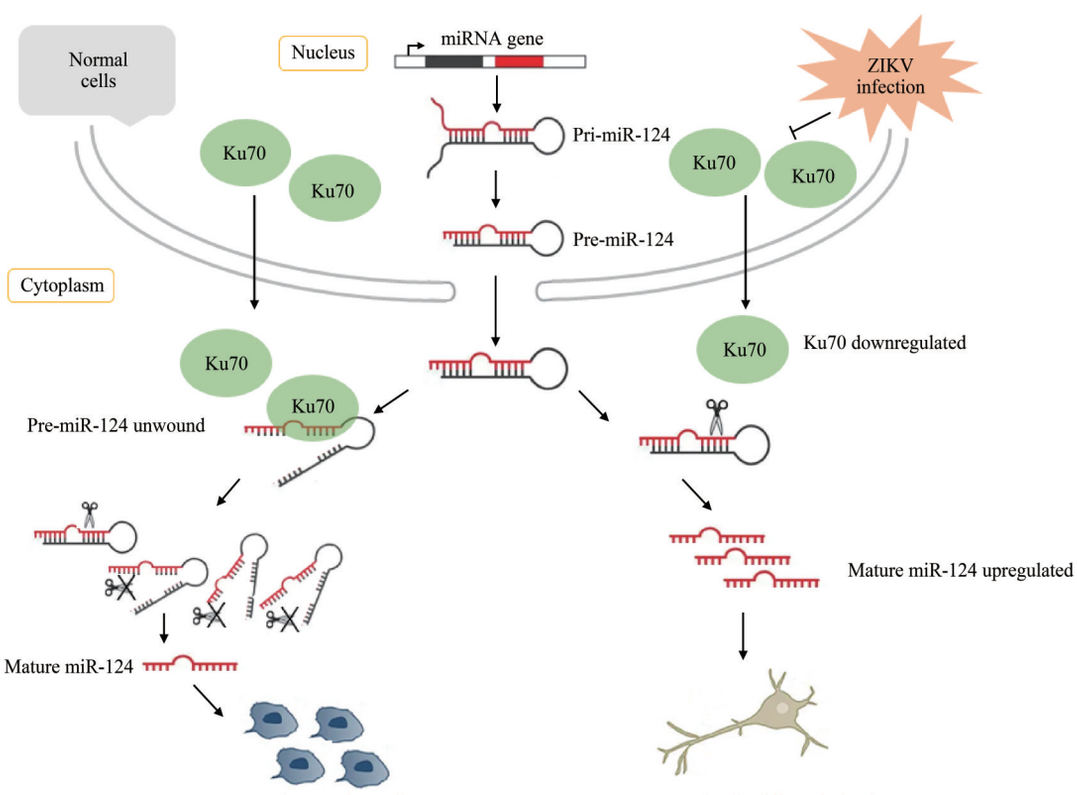
# Ku70 Functions as an RNA Helicase to Regulate miR−124 Maturation and Neuronal Cell Differentiation*

HUANG Ai-Xue[1)**], LI Rui-Ting[2)**], ZHAO Yue-Chao[1)**], LI Jie[1)], LI Hui[1)], DING Xue-Feng[1)], WANG Lin[1)], XIAO Can[1)], LIU Xue-Mei[1)***], QIN Cheng-Feng[2)***], SHAO Ning-Sheng[1)***]

([1)]*Beijing Institute of Basic Medical Sciences, Beijing* 100850, China;
[2)]*Beijing Institute of Microbiology and Epidemiology, Beijing* 100071, China)

## Graphical abstract

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** These authors contributed equally to this work.
*** Corresponding author.
LIU Xue-Mei. Tel: 86-10-66932313, E-mail: liuxuemei@bmi.ac.cn
QIN Cheng-Feng. Tel: 86-10-66948604, E-mail: qincf@bmi.ac.cn
SHAO Ning-Sheng. Tel: 86-10-66932313, E-mail: shaoningsheng@bmi.ac.cn
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**Abstract** **Objective** Human Ku70 protein mainly involves the non-homologous end joining (NHEJ) repair of double-stranded DNA breaks (DSB) through its DNA-binding properties, and it is recently reported having an RNA-binding ability. This paper is to explore whether Ku70 has RNA helicase activity and affects miRNA maturation. **Methods** RNAs bound to Ku protein were analyzed by RNA immunoprecipitation sequencing (RIP-seq) and bioinfomatic anaylsis. The expression relationship between Ku protein and miRNAs was verified by Western blot (WB) and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) assays. Binding ability of Ku protein to the RNAs was tested by biolayer interferometry (BLI) assay. RNA helicase activity of Ku protein was identified with EMSA assay. The effect of Ku70 regulated miR-124 on neuronal differentiation was performed by morphology analysis, WB and immunofluorescence assays with or without Zika virus (ZIKV) infection. **Results** We revealed that the Ku70 protein had RNA helicase activity and affected miRNA maturation. Deficiency of Ku70 led to the up-regulation of a large number of mature miRNAs, especially neuronal specific miRNAs like miR-124. The knockdown of Ku70 promoted neuronal differentiation in human neural progenitor cells (hNPCs) and SH-SY5Y cells by boosting miR-124 maturation. Importantly, ZIKV infection reduced the expression of Ku70 whereas increased expression of miR-124 in hNPCs, and led to morphologically neuronal differentiation. **Conclusion** Our study revealed a novel function of Ku70 as an RNA helicase and regulating miRNA maturation. The reduced expression of Ku70 with ZIKV infection increased the expression of miR-124 and led to the premature differentiation of embryonic neural progenitor cells, which might be one of the causes of microcephaly.

**Key words** Ku70, RNA helicase, miRNA maturation, miR-124, neuronal differentiation
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Human Ku protein is an evolutionarily conserved protein, which is a heterodimeric structure composed of two polypeptide chains through non-covalent bonds[1]. The two subunits of Ku protein are Ku70 (X-ray repair complementing defective repair in Chinese hamster cells 6, XRCC6) and Ku80 (X-ray repair complementing defective repair in Chinese hamster cells 5, XRCC5). Since Ku protein was discovered as a self-antigen in 1981[2-3], the functional investigation of Ku protein has consistently been focused on its DNA-binding properties and implications for non-homologous end joining (NHEJ) repair of double-stranded DNA breaks (DSB)[4].

However, the interactions of Ku protein with RNA have been rarely reported. Boulton *et al*.[5] were the first to report that Ku protein was able to bind to yeast telomerase RNA, who found that Ku protein was actually combined with a typical stem-loop double-stranded structure of telomerase RNA. Silvera used the yeast three-hybrid system to explore the interactions between the Ku protein and the internal ribosome entry sites (IRES) sequence. By analyzing the IRES sequence, they found it also had double-stranded features[6]. In 2020, Shadrina *et al*.[7] reported that Ku70 could bind to the stem-loop structure of the double-stranded RNA. Since the initial microRNA transcript (pri-miRNA), the precursor of miRNA (pre-miRNA), and the miRNA dimer shared a similar double-stranded structure[8], we wondered whether the pri-miRNA, the pre-miRNA, and the miRNA dimer will interact with the Ku protein and what will be the consequence of the interaction. Given that Ku protein can interact with Argonaute 2 (AGO2)[9], a key protein in the miRNA maturation, there is reason to speculate that Ku protein may be involved in the miRNA regulatory pathway.

miRNAs originate from the gene spacer of the genome, the intron or other unknown region, so their processing mechanisms vary, but they share strong commonality[10]. Several steps are involved in the classical miRNA maturation pathway. Firstly, the gene encoding miRNA in the nucleus is transcribed into a long pri-miRNA by RNA polymerase. Secondly, the pri-miRNA is cleaved into a 70−90 nucleotide length with a stem-loop structure called pre-miRNA through a complex of RNase III (Drosha enzyme) and its chaperone fraction DiGeorge syndrome critical region 8 (DGCR8). Pre-miRNAs are transported from the nucleus to the cytoplasm with the help of the Ran-GTP-dependent nucleoplasmic transporter Exportin-5. Then, the double-stranded structure of pre-miRNA is cleaved into 21−25 nucleotides by another RNase III (Dicer enzyme) with the 5' end phosphorylated and 2 nt protrusion in 3' end, like the incomplete paired

duplex siRNA[11]. The splicing process requires pre-miRNA to maintain a stable double-stranded structure and produces a double strand miRNA. Afterwards, the double-stranded RNA is unwound into mature single-stranded miRNA which binds to the RNA-induced silencing complex (RISC) to exert RNAi effect[8]. Mature miRNA represses translation or directly degrades mRNA by binding to the 3'-untranslated region (3'UTR) of target genes[12]. So far, the maturation mechanism of miRNA has been roughly elucidated, but some unknown mechanism regulates miRNA maturation and makes a difference to its downstream biological effects so that the functions of miRNA are not stable *in vivo*[13]. A better knowledge of the potential role of Ku protein in the regulation of miRNA maturation may inspire us with some new ideas.

In this paper, we found for the first time that Ku70 protein had RNA helicase activity and affected miRNA maturation, particularly the neuronal specific miRNA, such as miR-124. The knockdown of Ku70 both in SH-SY5Y cells and human neural progenitor cells (hNPCs) promoted neuronal differentiation by boosting miR-124 maturation. We also found that Zika virus (ZIKV) infected hNPCs had decreased expressions of Ku70 but increased expressions of miR-124, which resulted in morphologically neuronal differentiation. Our results suggested that Ku70 might be involved in microRNA maturation regulation and neuronal cell differentiation, which was possibly a novel function of Ku70.

## 1 Materials and methods

### 1.1 Cell lines, cell culture and cell transfection

Human cells NCI-H1299 and NCI-H460 were provided by Procell Life Science & Technology Co., LTD. (China) and cultured in RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS, Hyclone, USA). Human cells SH-SY5Y were purchased from the Cell Resource Center of Peking Union Medical College (China) and cultured in RPMI-1640 medium (Gibco, USA) containing 15% FBS (Hyclone, USA). Neural Progenitor Cell Origin ATCC-BXS0117 Normal (ATCC#ACS-5003) was obtained from Dr. QIN Cheng-Feng's Lab and cultured in NeuroCult™-XF Basal medium (StemCell, USA) containing 20 μg/L rhEGF (recombinant human epidermal growth factor, StemCell, USA), 10 μg/L rh-bFGF (recombinant human fibroblast growth factor-basic, StemCell, USA), 0.2% Heparin (StemCell, USA). All medium contained 100 unit/ml penicillin and 100 mg/L streptomycin. The cells were maintained in cell culture flasks (Thermo Fisher Scientific, USA) in a humidified chamber at 37℃ with 5% $CO_2$. Cells at 70% confluency were transfected with vectors or RNAs. All transfections were performed using Lipofectamine 2000 (Thermo Fisher Scientific, USA) according to the manufacturer's protocol.

### 1.2 Animals

Pregnant BALB/c mice at gestation day 16 were obtained from Vital River, China. Mouse experiments were conducted and approved by the Institutional Animal Care and Use Committee at Beijing Institute of Basic Medical Sciences. The accreditation number of this study was IACUC-DWZX-2021-641.

### 1.3 Nucleotides and proteins

Recombinant human Ku70 protein and recombinant human Ku80 protein were purchased from OriGene Technologies (USA). Plasmids pCMV3-C-His-NCV, pCMV3-Ku70-His and pCMV3-Ku80-His were purchased from Sino Biological (China). Ku70 siRNA, Ku80 siRNA, miR-124 mimics and scrambled RNA as negative control (NC) were synthesized by RiboBio (China). Single strand RNAs, such as pre-miR-124, miR-124 and ssRNA-17 with biotin conjugated, were synthesized by Biosyntech (China). Primers were synthesized by Sangon (China). All chemically synthesized oligonucleotides (Table 1, 2) were purified electrophoretically.

### 1.4 RNA immunoprecipitation

H1299 cells ($1\times10^7$) were cross-linked with UV and lysed for 15 min on ice by radioimmunoprecipitation assay (RIPA) buffer consisting of 100 mmol/L KCl, 5 mmol/L $MgCl_2$, 10 mmol/L HEPES (pH 7.0), and 0.5% NP-40 detergent freshly supplemented with 1 mmol/L dithiothreitol (DTT), 100 U/ml RNase-Out (Invitrogen, USA), and a protease inhibitor cocktail (Sigma, USA). The cell lysate was diluted in 1∶10 with NT2 Buffer consisting of 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L $MgCl_2$, and 0.05% NP-40 freshly supplemented with 200 U/ml RNase-Out, 1 mmol/L DTT, 20 mmol/L EDTA, and a protease inhibitor cocktail. The insoluble particles in the lysate were precipitated by centrifugation at

**Table 1　The primers used in this article**

| Name | Forward | Reverse |
|---|---|---|
| mRNA RT primer | 5'-TTTTTTTTTTTTTTTTT-3' | |
| β-actin | 5'-GGCATCGTGATGGACTCCG-3' | 5'-GCTGGAAGGTGGACAGCGA-3' |
| Ku70 | 5'-GTTGATGCCTCCAAGGCTATG-3' | 5'-CCCCTTAAACTGGTCAAGCTCT-3' |
| Ku80 | 5'-GTGCGGTCGGGGAATAAGG-3' | 5'-GGGGATTCTATACCAGGAATGGA-3' |
| miRNA RT primer | 5'-GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTTTTTTVN-3' | |
| hsa-miR-124 | 5'-TAAGGCACGCGGTGAATGCCAA-3' | 5'-GTGCAGGGTCCGAGGT-3' |
| hsa-pre-miR-124-1 | 5'-CCGTGTTCACAGCGGACC-3' | 5'-GCCCCATTCTTGGCATTCA-3' |
| hsa-pri-miR-124-1 | 5'-CTCACCTCAGCCTCCCAAAG-3' | 5'-GAAGACAAACCACCAGGAAACA-3' |
| hsa-miR-138 | 5'-AGCTGGTGTTGTGAATCAGGCCG-3' | 5'-GTGCAGGGTCCGAGGT-3' |
| hsa-pre-miR-138-1 | 5'-AATCAGGCCGTTGCCAATCA-3' | 5'-GCCCTGGTGTTGTGAAGTAGCC-3' |
| hsa-pri-miR-138-1 | 5'-CTCACCTCAGCCTCCCAAAG-3' | 5'-GAAGACAAACCACCAGGAAACA-3' |
| hsa-let-7a | 5'-TGAGGTAGTAGGTTGTATAGTT-3' | 5'-GTGCAGGGTCCGAGGT-3' |
| hsa-miR-105 | 5'-TCAAATGCTCAGACTCCTGTGGT-3' | 5'-GTGCAGGGTCCGAGGT-3' |
| hsa-miR-448 | 5'-TTGCATATGTAGGATGTCCCAT-3' | 5'-GTGCAGGGTCCGAGGT-3' |
| hsa-miR-515 | 5'-TTCTCCAAAAGAAAGCACTTTCTG-3' | 5'-GTGCAGGGTCCGAGGT-3' |
| hsa-miR-544a | 5'-ATTCTGCATTTTTAGCAAGTTC-3' | 5'-GTGCAGGGTCCGAGGT-3' |
| hsa-miR-544b | 5'-ACCTGAGGTTGTGCATTTCTAA -3' | 5'-GTGCAGGGTCCGAGGT-3' |
| hsa-miR-548a | 5'-CAAAACTGGCAATTACTTTTGC-3' | 5'-GTGCAGGGTCCGAGGT-3' |
| hsa-miR-548b | 5'-AAAAGTAATTGTGGTTTTGGCC-3' | 5'-GTGCAGGGTCCGAGGT-3' |
| hsa-miR-562 | 5'-AAAGTAGCTGTACCATTTGC-3' | 5'-GTGCAGGGTCCGAGGT-3' |
| hsa-miR-569 | 5'-AGTTAATGAATCCTGGAAAGT-3' | 5'-GTGCAGGGTCCGAGGT-3' |
| hsa-miR-584 | 5'-TTATGGTTTGCCTGGGACTGAG-3' | 5'-GTGCAGGGTCCGAGGT-3' |
| hsa-miR-649 | 5'-GTGTTGTTCAAGAGTCAAAA-3' | 5'-GTGCAGGGTCCGAGGT-3' |
| hsa-miR-767 | 5'-TGCACCATGGTTGTCTGAGCATG-3' | 5'-GTGCAGGGTCCGAGGT-3' |
| hsa-miR-941 | 5'-CACCCGGCTGTGTGCACATGTGC-3' | 5'-GTGCAGGGTCCGAGGT-3' |
| hsa-miR-1225 | 5'-GTGGGTACGGCCCAGTGGGGGG-3' | 5'-GTGCAGGGTCCGAGGT-3' |
| hsa-miR-767 | 5'-TGCACCATGGTTGTCTGAGCATG-3' | 5'-GTGCAGGGTCCGAGGT-3' |
| U6 snRNA | 5'-CGCTTCGGCAGCACATATACTA-3' | 5'-CGCTTCACGAATTTGCGTGTCA-3' |

**Table 2　The RNA sequences used in this article**

| Name | | Sequence |
|---|---|---|
| Ku70 siRNA | Sense | 5'-CCAACAGGCUGCAUGAAAU-3' |
| | Antisense | 5'-GGUUGUCCGACGUACUUUA-3' |
| Ku80 siRNA | Sense | 5'-GAAGAGGCAUAUUGAAAUA-3' |
| | Antisense | 5'-UAUUUCAAUAUGCCUCUU-3' |
| MiR-124 mimics | Sense | 5'-UAAGGCACGCGGUGAAUGCCAA-3' |
| | Antisense | 5'-UUGGCAUUCACCGCGUGCCUUA-3' |
| Negative Control | Sense | 5'-UUCUCCGAACGUGUCACGU-3' |
| | Antisense | 5'-ACGUGACACGUUCGGAGAA-3' |
| pre-miR-124 | | Biotinynated 5'-AGGCCUCUCUCUCCGUGUUCACAGCGGACCUUGAUUUAAA UGUCCAUACAAUUAAGGCACGCGGUGAAUGCCAAGAAUGGGGCUG-3' |
| miR-124 | | Biotinynated 5'-UAAGGCACGCGGUGAAUGCCAA-3' |
| ssRNA-17 | | Biotinynated 5'-GUGUAGUAAUCGUCCAU-3' |
| ssRNA-39 | | 5'-AGUUUAAAACGCACGAGACACAAUGGACGAUUACUACAC-3' |

15 000 *g* for 15 min at 4℃. The Ku70 antibody (1∶100), Ku80 antibody (1∶100) or control IgG was added to protein-A Sepharose beads (Sigma, USA) pre-incubated in 5% bovine serum albumin-NT2 Buffer for 1 h at 4℃. After gentle rotation for 4 h at 4℃, the beads were washed four times in cold NT2 buffer and added to the cell lysates (10 μl beads/ml lysate). Immunoprecipitation was performed by gentle rotation overnight at 4℃. The immunoprecipitated complexes were washed four times in NT2 Buffer and resuspended in 100 μl NT2 buffer containing 30 μg proteinase K (Sigma, USA) to release the RNP complex. Total RNA was extracted using TRIzol reagent (Invitrogen, USA) and performed next steps or stored in −80℃.

### 1.5 RNA sequencing and data analysis

Sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (#E7530L, NEB, USA) following the manufacturer's recommendations and index codes were added to attribute sequences to each sample. The libraries were sequenced on Illumina Novaseq6000 platform and 150 bp paired-end reads were generated. After sequencing, contaminated reads for adapters and low-quality reads were removed. Clean reads were aligned to the human genome (GRC38.94. chr) using Hisat2 v2.1.0. The number of reads mapped to each gene in each sample was counted by HTSeq v0.6.0. TPM (transcripts per kilobase of exon model per million mapped reads) was then calculated to estimate the expression level of genes and transcripts in each sample.

### 1.6 Quantitative reverse transcriptase−polymerase chain reaction

RNA was isolated using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions, and 1 μg total RNA was subjected to reverse transcription using the Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Promega, USA). For quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis, the Mx3000p Real-Time PCR system was used (Stratagene, USA) with SYBR Green Realtime PCR Master Mix (Toyobo, Japan). All measurements were performed in triplicate and the relative mRNA levels were analyzed using Mx3000p software (version 4.1). After mRNA was normalized with β-actin mRNA and miRNA normalized with U6 snRNA, Ku70 mRNA, Ku80 mRNA, miRNA levels in each group were expressed as fold change relative to the expression levels in cells.

### 1.7 Western blot

Rabbit polyclonal anti-Ku70 antibody was purchased from Abcam, USA. Rabbit anti-Ku80 antibody and rabbit anti-NeuN antibody were purchased from ProteinTech, China. GAPDH and β-actin were used as an internal control, mouse anti-GAPDH and mouse anti-β-actin antibody was purchased from Abclonal Technology, China. $10^7$ cells were lysed in 100 μl RIPA buffer (Applygen, China) with protease inhibitor cocktail (Thermo Fisher Scientifc, USA). Protein concentration was determined using Pierce BCA Protein Assay Kit (CoWin Biotech, China), and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred *via* 100 voltages onto 0.45 μm polyvinylidene difluoride (PVDF) membranes (Millipore, USA). After being blocked with 5% non-fat milk in 0.1% PBST, the membranes were incubated with primary antibodies overnight at 4℃, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h. Immunoreactivity was detected *via* electrochemiluminescence (ECL) by using the SuperSignal® West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, USA). Each experiment was conducted in three biological replicates.

### 1.8 RNA helicase assay

The RNA helicase assay measures the displacement of a labelled oligonucleotide fragment from a partial duplex molecule according to reported research[21]. Thereaction mixture (10 μl) contained 20 mmol/L Tris-HCl (pH 8.0), 1 mmol/L $MgCl_2$, 4 mmol/L ATP, 100 mmol/L KCl, 4 mmol/L DTT and the helicase fraction. Incubations were performed at 37℃ for 30 min. The reaction was terminated by the addition of 1.5 μl of 75 mmol/L EDTA, 2.25% SDS, 37.5% glycerol and 0.3% bromophenol blue; products were separated by 6% non-denaturing PAGE. The gels were transferred *via* 100 voltages to Hybond-N® Nylon Membrane (GE Healthcare, USA) and detected *via* ECL by using the LightShift® Chemiluminescent EMSA Kit (Thermo Fisher Scientific, USA).

### 1.9 Biolayer interferometry assay

Biolayer interferometry (BLI) assays were performed on a GatorPrime (GatorBio, China) instrument at 37℃ with shaking at 1 000 r/min. The biotinylated RNA was immobilized to streptavidin probes (GatorBio, China) at 100 nmol/L in Q-buffer (0.2% BSA in 0.1% TBST) and then were exposed to Ku protein for 120 s of association and 180 s of dissociation with Q-buffer. The data were baseline subtracted prior to fitting performed using a 1∶1 binding model. Mean $K_d$ values were determined with a global fit applied to all data.

### 1.10 Virus infection

ZIKV used in this study is GZ01 strain (GenBank accession no: KU820898), which was originally isolated from a Chinese patient returned from Venezuela in 2016. All viral stocks were prepared in C6/36 cells and titrated by standard plaque forming assay. hNPC line were infected with either GZ01 at multiplicity of infection (*MOI*)=1 or treated with medium.

### 1.11 Immunofluorescence microscopy

One thousand cells were planted on glass cover slips, fixed with methanol, washed with PBS, and blocked with 2% normal rabbit serum. The coverslips were then incubated with a mouse anti-ZIKV Envelope antibody (BioFront Technologies, USA) overnight and the corresponding FITC-conjugated secondary antibodies at room temperature for 1 h. After antibody incubation, cells were washed twice with PBS and stained with 4, 6-diamidino-2-phenylindole (DAPI) for 10 min. The cells were then viewed under an LSM 510 META confocal microscope (Zeiss, Germany).

### 1.12 Statistical analysis

At least three independent experiments were performed for each assay. Data are shown as means± SD. GraphPad prism 8.0 (GraphPad Software, USA) was used for statistical analysis. Statistical differences were analyzed by unpaired Student's *t* test, and *P*-values less than 0.05 were considered statistically significant.

## 2 Results

### 2.1 Identified RNAs binding to Ku protein through RNA−IP sequencing

To find out whether Ku protein could bind to some RNAs, RNA immunoprecipitation (RNA-IP) experiments were performed in H1299 cells using the Ku70 and Ku80 antibodies respectively. RNAs specifically binding to Ku protein obtained through sequencing were listed in Table 3. The results

**Table 3 Identified RNAs binding to Ku protein through RNA−IP sequencing in H1299 cells**

| Ku70 RNA-IP outcome | | Ku80 RNA-IP outcome | |
|---|---|---|---|
| Class | Number | Class | Number |
| Telomerase-vert | 58 | 5S_rRNA | 34 |
| 5S_rRNA | 58 | IRES_Hsp70 | 33 |
| IRES_Hsp70 | 40 | Telomerase-vert | 30 |
| pre-miR-562 | 12 | pre-miR-548 | 9 |
| pre-miR-492 | 11 | pre-miR-492 | 9 |
| pre-miR-649 | 6 | pre-miR-544 | 6 |
| pre-miR-548 | 4 | pre-miR-562 | 6 |
| pre-miR-692 | 3 | pre-miR-649 | 5 |
| pre-miR-544b | 3 | pre-miR-692 | 3 |
| pre-let-7 | 2 | pre-miR-684 | 2 |
| pre-miR-584 | 1 | pre-miR-1255 | 1 |
| pre-miR-941 | 1 | pre-miR-569 | 1 |
| pre-miR-515 | 1 | pre-miR-448 | 1 |
| pre-miR-138 | 1 | pre-miR-105 | 1 |
| pre-miR-124 | 1 | pre-miR-767 | 1 |
| | | pre-miR-584 | 1 |
| | | pre-miR-138 | 1 |
| | | pre-miR-124 | 1 |

indicated that not only telomerase RNA and IRES were bound to Ku protein, as was reported[5-7], but many pre-miRNAs such as pre-miR-562, pre-miR-548, pre-miR-492, pre-miR-138 and pre-miR-124 were found to have high scores. Thus, we confirmed that Ku protein have pre-miRNA binding ability.

### 2.2　Ku protein−bound RNA has a typical double−stranded structure

After the Ku protein-bound RNAs were analyzed with an online database (https://miRbase. org), it was found that the secondary structures of those RNAs tended to be typical double-stranded (Figure 1a). Sequence analysis of these miRNA precursors with WebLogo3.3 (https://weblogo. berkeley. edu/logo. cgi) showed that there was no obvious identity among those sequences (Figure 1b). These results suggested that Ku70 might bind to the double-stranded structure.

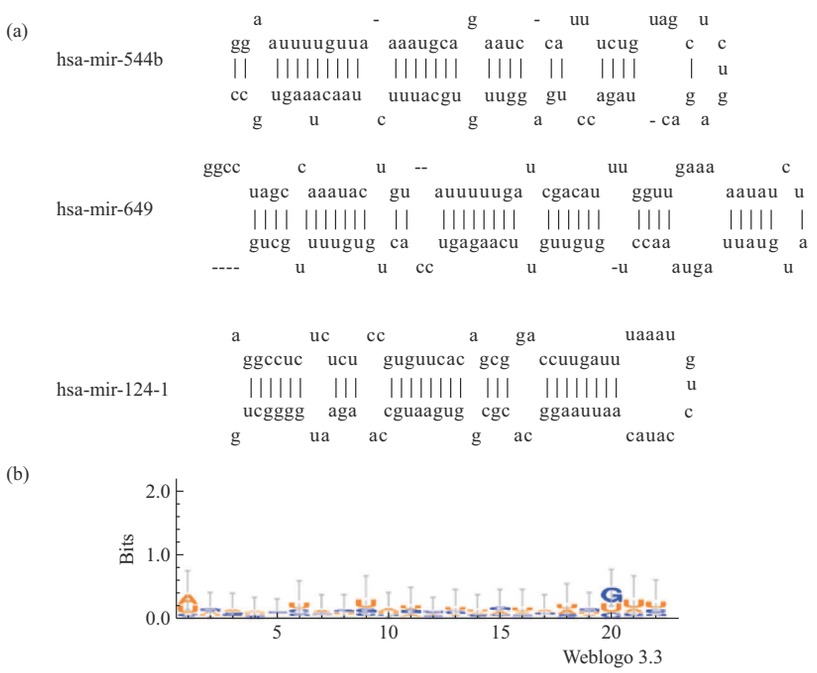


**Fig. 1　Ku protein−bound RNA has a typical double−stranded structure**

(a) pre-miRNAs specifically bound to Ku protein tended to be complementary pairing on their secondary structure. (b) Ku protein-bound RNA did not have obvious sequence specificity by WebLogo 3.3 (https://weblogo.berkeley.edu/logo.cgi).

### 2.3　Ku protein affects the expressions of related miRNAs

To investigate the correlation between Ku protein and miRNAs, two cell lines were selected as research models. One was radioresistant H1299 cells, and the other was radiosensitive H460 cells[14-15]. The expressions of Ku protein and miRNAs were detectedwith Western blot (WB) and qRT-PCR assays. Results showed that the higher the expressions of Ku70 or Ku80 protein (Figure 2a−c) in the cells, the lower the expressions of related miRNAs, or *vice versa* (Figure 2d). Here, we illustrated that the different basal expression of Ku protein correlated with the expression level of miRNA maturation, suggesting that altered Ku protein expressions might affect the miRNA processing.

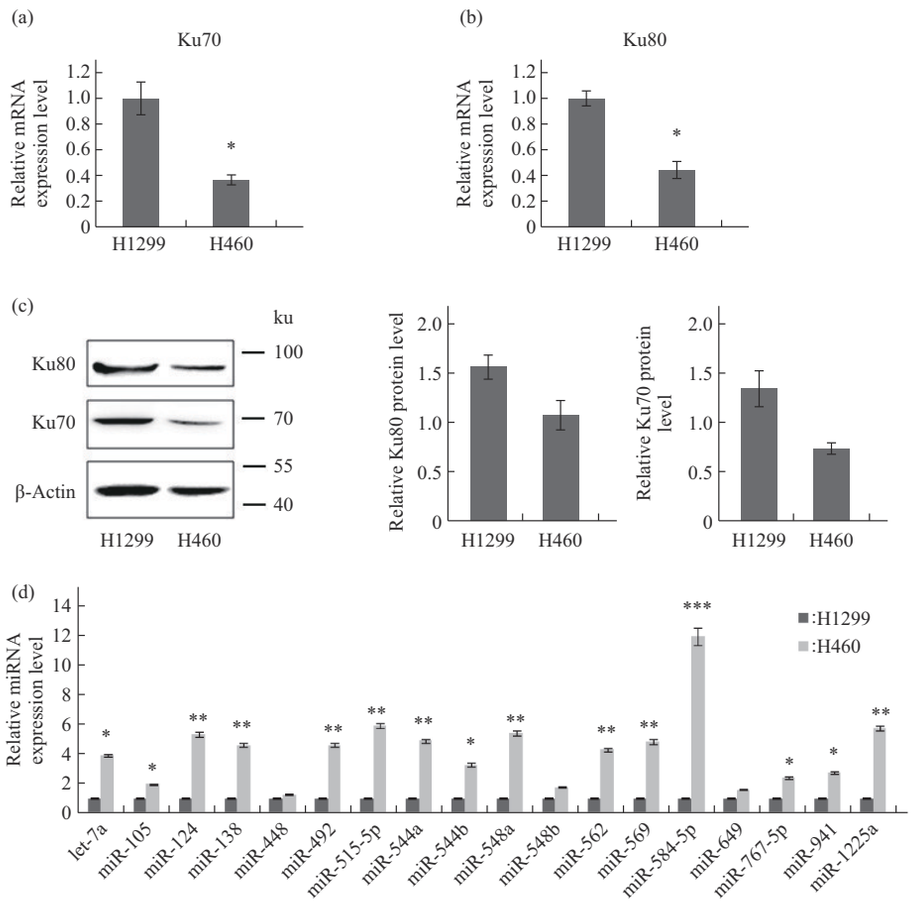


**Fig. 2　Ku protein affects expressions of related miRNAs**

(a,b) qRT-PCR analysis of relative expressions of Ku70 mRNA (a), Ku80 mRNA (b) in H1299 and H460 cells. β-actin was used as a normalized control. (c) Ku70/Ku80 protein expression in H1299 and H460 cells was detected by Western blot. β-Actin was used as a loading control. (d) Ku protein-related miRNA experession in H1299 and H460 cells was measured by qRT-PCR. U6 snRNA was used as a normalized control. Values represented means of three independent experiments. The qPCR data are shown as the mean±SD (Student's *t* test, **P*<0.05, ***P*<0.01, ****P*<0.001).

## 2.4　Ku protein inhibits miRNA maturation

To verify our hypothesis, Ku70 or Ku80 was knocked down with Ku protein specific siRNAs in H1299 cells (Figure 3a, b). The results showed dramatic increases in the relative expressions of some tested miRNAs, among which miR-124 and miR-138 changed the most significantly (Figure 3c). Then, miR-124 and miR-138 were chosen to analyze the effect of Ku protein on the process of miRNA maturation. After knockdown of Ku70 or Ku80, the mature miRNA (miR-124) was increased (Figure 3d), while the expression of precursor miR-124 (pre-miR-124) was decreased (Figure 3e). There was no significant change in the primary transcript of miR-124 (pri-miR-124) expression (Figure 3f). Over-expression of Ku70 or Ku80 in H1299 cells yielded contrary results (Figure 3g−l). Similar changes of expressions of pri-miR-138, pre-miR-138 and mature miR-138 were observed in knockdown or over-expressed Ku70 or Ku80 in H1299 cells (data not shown). These results indicated that both Ku70 and Ku80 protein might cause the accumulation of pre-miRNAs and affect the splicing of pre-miRNAs into mature miRNAs.

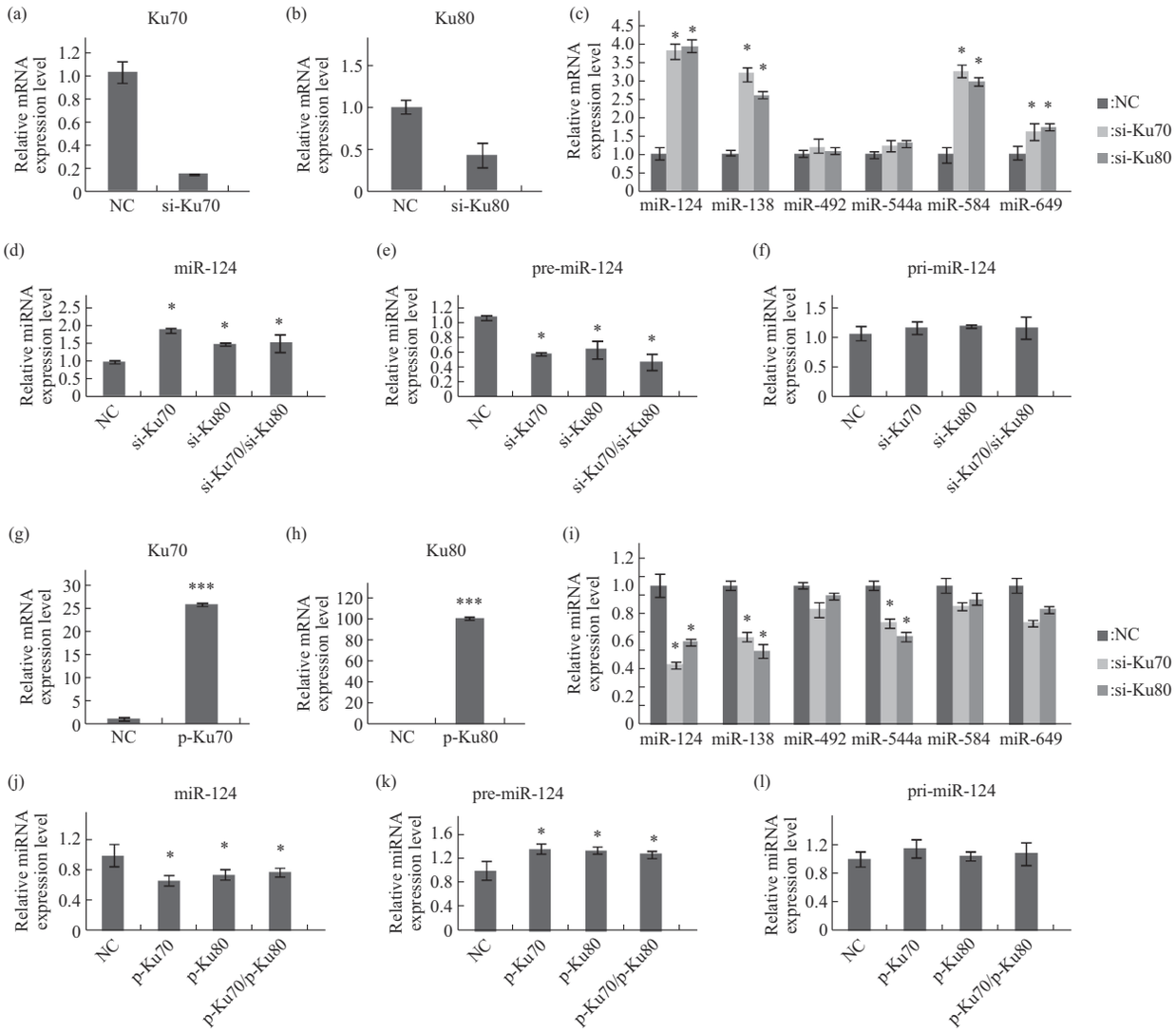


**Fig. 3 Ku protein inhibits miRNA maturation**

qRT-PCR analysis of Ku70 and Ku80 mRNA expression in H1299 cells (a,b). Ku70 (a) and Ku80 (b) mRNA expression levels were decreased after transfection with Ku70 or Ku80 siRNAs respectively. (c) Expressions of Ku protein-related miRNAs after knockdown of Ku protein. (d−f) Different expressions of mature miR-124 (d), precursor pre-miR-124 (e), and primary transcript pri-miR-124 (f) after knockdown of Ku protein. (g,h) Ku70 (g) and Ku80 (h) mRNA expression levels were upregulated after transfection with plasmids of Ku70 or Ku80 respectively. (i) Expressions of Ku protein-related miRNAs after over-expression of Ku protein. (j−l) Different expressions of mature miR-124 (j), precursor pre-miR-124 (k), and primary transcript pri-miR-124 (l) after over-expression of Ku protein. β-Actin and U6 snRNA were used as an internal control. Values represented means of three independent experiments. The qRT-PCR data are shown as the mean±SD (Student's *t* test, **P*<0.05, ****P*<0.001).

## 2.5 Ku protein can bind to pre−miR−124 directly

To test whether Ku protein could interfere in pre-miRNA splicing into mature miRNA, the direct interactions between Ku protein and pre-miR-124 or miR-124 were detected with BLI assays as previously reported[16-17]. Results showed that both Ku70 and Ku80 could bind to pre-miR-124 directly (Figure 4a, b), except the single strand of mature miR-124. Binding kinetics of Ku70 or Ku80 with pre-miR-124 was also analyzed. The $K_d$ value of Ku70 or Ku80 was 2.92 μmol/L and 86 nmol/L respectively (Figure 4c, d).

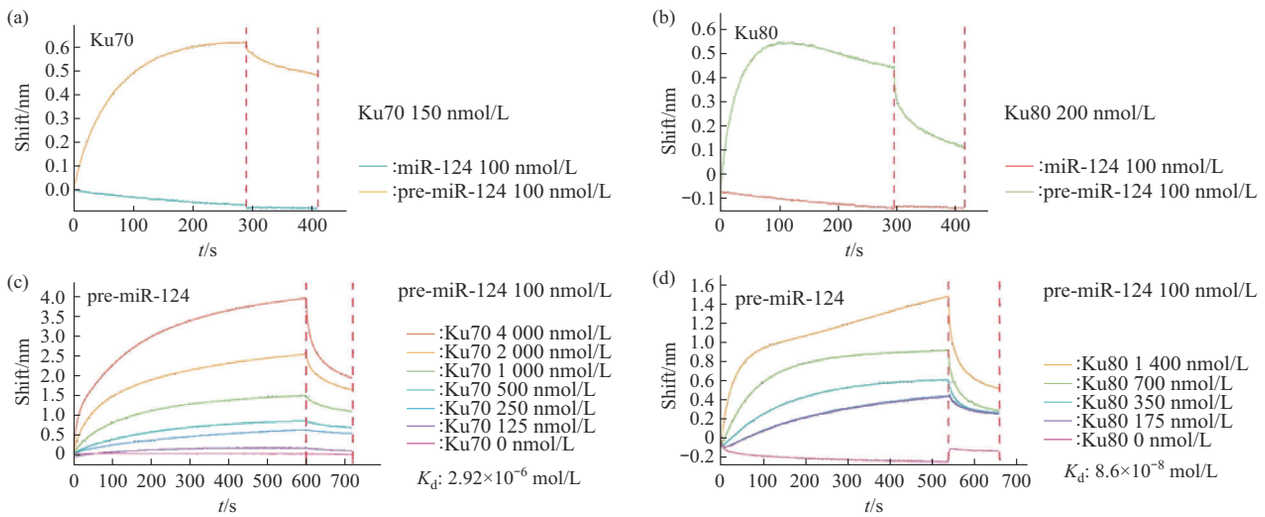


**Fig. 4　Ku protein can directly bind to pre−miR−124**

(a,b) The binding of Ku70 (a) or Ku80 (b) with pre-miR-124 or mature miR-124 respectively detected by BLI technology. (c,d) Binding kinetics of Ku70 (c) or Ku80 (d) with pre-miR-124. The $K_d$ value of Ku70 was 2.92 μmol/L and that of Ku80 was 86 nmol/L. The light-wave shifts were recorded, curves were fitted and $K_d$ values were calculated by GatorPrime (GatorBio).

### 2.6　Ku70 functions as an RNA helicase

It is reported that for Dicer dicing pre-miRNA to form mature miRNA, pre-miRNA has to maintain its double-stranded structure. Once the double-strand stability is affected, the dicing of Dicer enzyme will be affected inevitably[18]. Considering the DNA helicase function of Ku protein[19], it was assumed that Ku protein might function as an RNA helicase to break the double-stranded structure of pre-miRNA, resulting in Dicer cleavage inhibition. The RNA helicase activity of Ku protein was also investigated by using a dsRNA model as the helicase substrate[20]. The model was composed of two pieces of partially complementary nucleotide chains, which were 17 nt and 39 nt in size respectively (Figure 5a). The dsRNA substrate with 5' free ends could be obtained *via* annealing and proved to bind to both Ku70 and Ku80 proteins by EMSA (Figure 5b, c). The unwinding reaction system that contained ATP reacted at 37°C for 20 min. Surprisingly, the results showed that only Ku70 protein was able to unwind the dsRNA (Figure 5d) in a dose-dependent manner (Figure 5e). Subsequent studies demonstrated that Ku70 could also function as a helicase capable of unwinding the advanced structure of pre-miR124 (Figure 5f).

### 2.7　Ku70 regulates miR−124 maturation and neuronal cell differentiation

MiR-124 is a well-known neuron-specific miRNA and a regulator of RE1 silencing transcriptional factor (REST) based transcriptional silencing complex that inhibits the expressions of neuron-specific genes in non-neuronal cells, thereby forming auto-regulatory loops during neuronal differentiation[21-23]. In our study, it was found that the expression of Ku70 decreased gradually (Figure 6a) along with the differentiation of hNPCs into neurons, while the level of miR-124 increased (Figure 6b). To explore the effects of Ku70 on miR-124 maturation and neuronal differentiation, miR124 mimics or Ku70 siRNA was transfected into hNPCs and SH-SY5Y. Results showed that both hNPCs and SH-SY5Y had morphologically turned into differentiated neurons

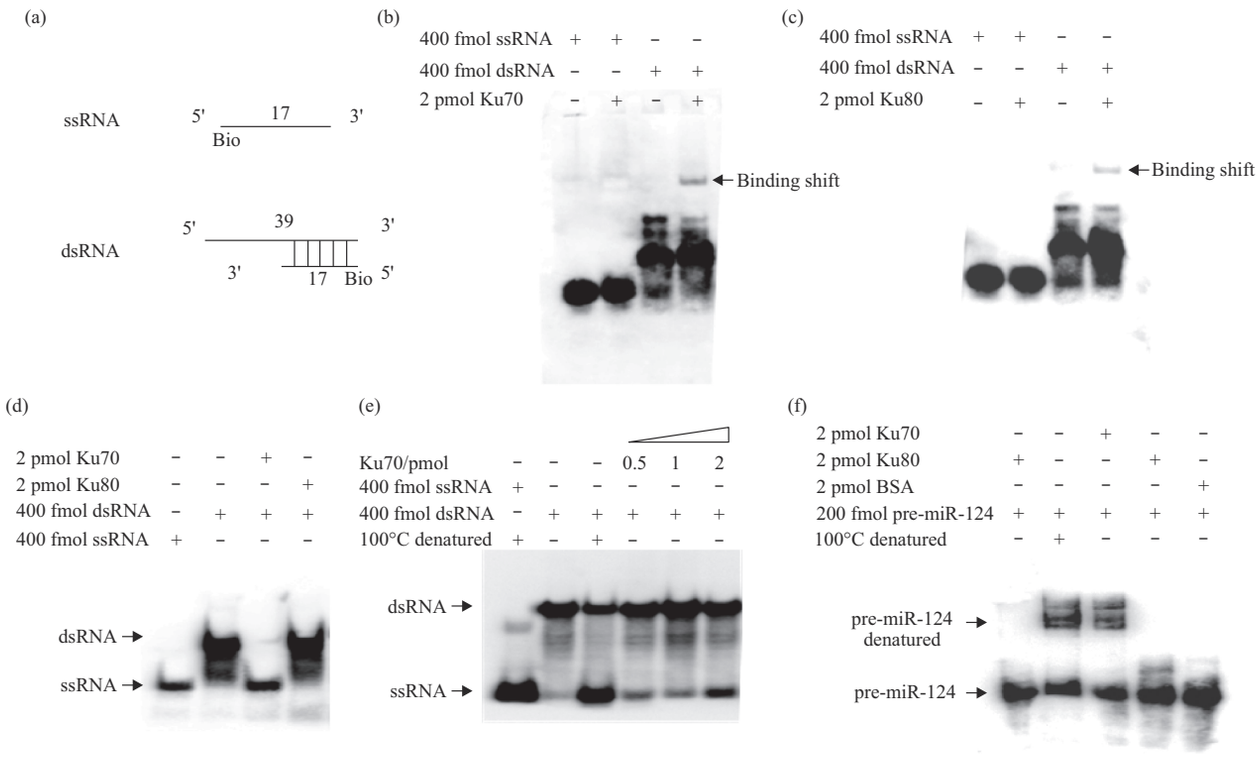


**Fig. 5　Ku70 functions as an RNA helicase**

(a) Scheme of dsRNA substrate. (b,c) EMSA demonstrated that Ku70 (b) and Ku80 (c) interacted with dsRNA, but not with ssRNA. (d) Validation of the helicase activity of Ku70 and Ku80. (e) The RNA helicase activity of Ku70 was concentration dependent. (f) Validation of the helicase activity of Ku70 for pre-miR-124.

when miR-124 was over-expressed or Ku70 was knocked down (Figure 6c, d). Ku70 and miR-124 expression was validated by qRT-PCR after transfection in hNPCs (Figure 6e) and SH-SY5Y (Figure 6f). The expression of NeuN, a neonatal neuron marker, was also up-regulated under the same conditions (Figure 6g, h). Knockdown of Ku70 or over-expression of miR-124 showed consistent effect on neuronal progenitor cells differentiation, which further illustrated that the alteration in Ku70 is functionally led to miR-124 expression change.

### 2.8　ZIKV infection up−regulates the expression of miR−124 and results in differentiation of hNPCs

ZIKV is a typical mosquito-borne flavivirus with neural tropism, and vertical transmission of ZIKV lead to severe microcephaly by targeting NPCs[24-25]. To clarify the potential interaction between ZIKV infection and Ku70, we infected hNPCs with ZIKV and determined the effect *via* immunofluorescence assay before and after 24 h ZIKV infection (Figure 7a). 6 d after infection, the morphological changes of the cells were observed. As shown in Figure 7b, hNPCs tended to be morphologically neuronally differentiated. We wondered whether miR-124 and Ku70 expression changed after ZIKV infection, so that we performed qRT-PCR experiments and results showed that miR-124 was found to be significantly up-regulated (Figure 7c) and Ku70 was significantly down-regulated (Figure 7d) after ZIKV infection. Consequences to our previous data, we proved that ZIKV infection affects the expressions of miR-124 and Ku70 and results in differentiation of hNPCs. We demonstrated for the first time that Ku70 was down-regulated in neuronal progenitor cells while ZIKV infection. And changes in Ku70 could cause an increase in miR-124, leading to a prematurely differentiation in neuronal cells, which provides a new explanation for the disease mechanism of microcephaly.

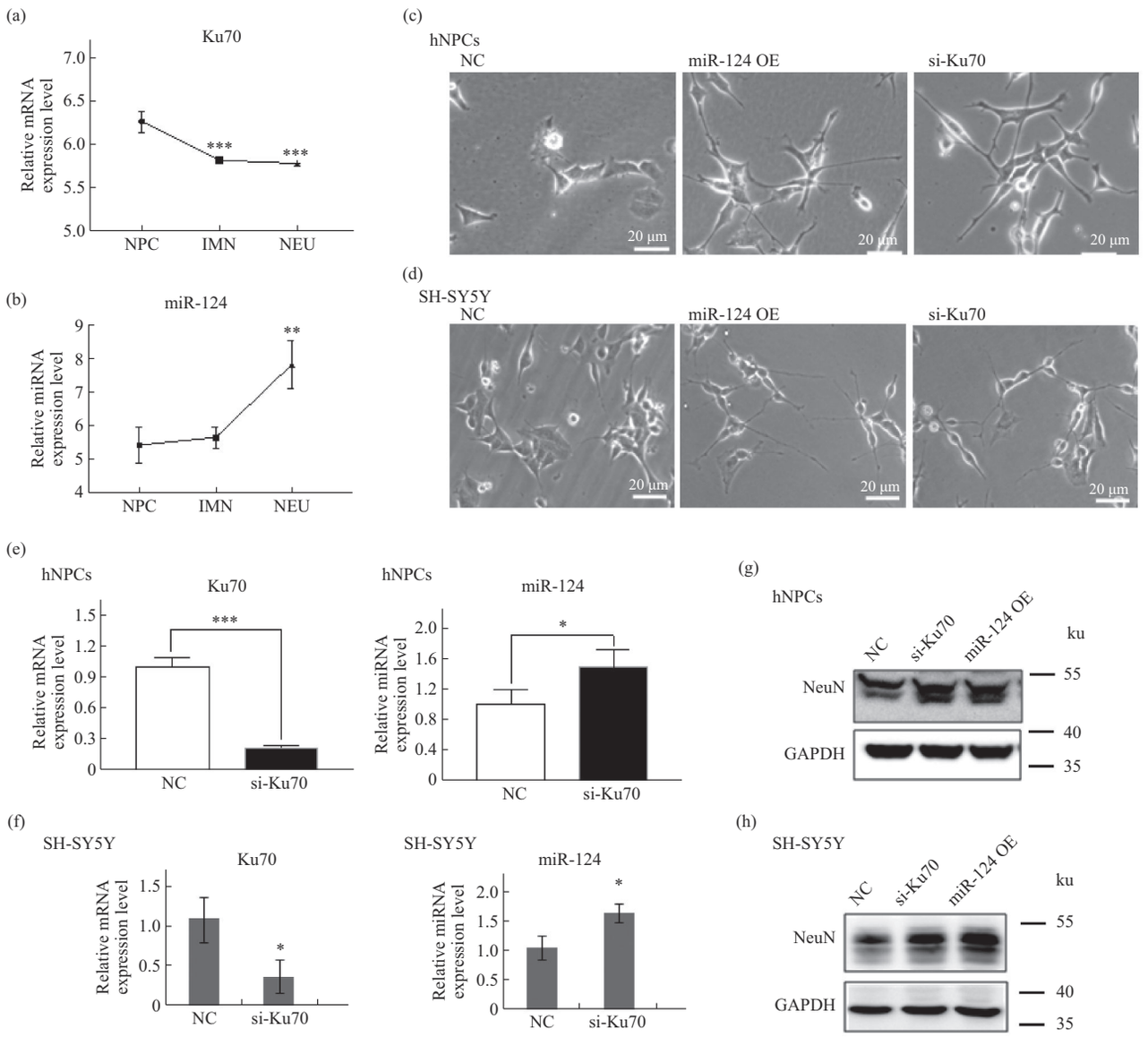


**Fig. 6　Ku70 affects miR−124 maturation and neuronal cell differentiation**

(a,b) miR-124 (a) and Ku70 (b) expression increased along with hNPCs differentiation were analyzed by qRT-PCR. IMN, immature neurons; NEU, mature neurons. (c, d) Morphological analysis showed over-expression (OE) of miR-124 or knockdown of Ku70 (si-Ku70) promotes the differentiation of hNPCs (c) and SH-SY5Y cells (d) to neurons. (e) Ku70 and miR-124 expression were validated after transfection with Ku70 siRNA in hNPCs. β-Actin mRNA and U6 snRNA were used as internal control. (f) Ku70 and miR-124 expression were validated after transfection wtih Ku70 siRNA in SH-SY5Y. β-Actin mRNA and U6 snRNA were used as internal control. (g, h) The expression of NeuN was detected by Western blot in hNPCs (g) and SH-SY5Y cells (h). GAPDH was used as an internal control. Values represented means of three independent experiments. Data are shown as the mean±SD (Student's *t* test, **P*<0.05, ***P*<0.01, ****P*<0.001).

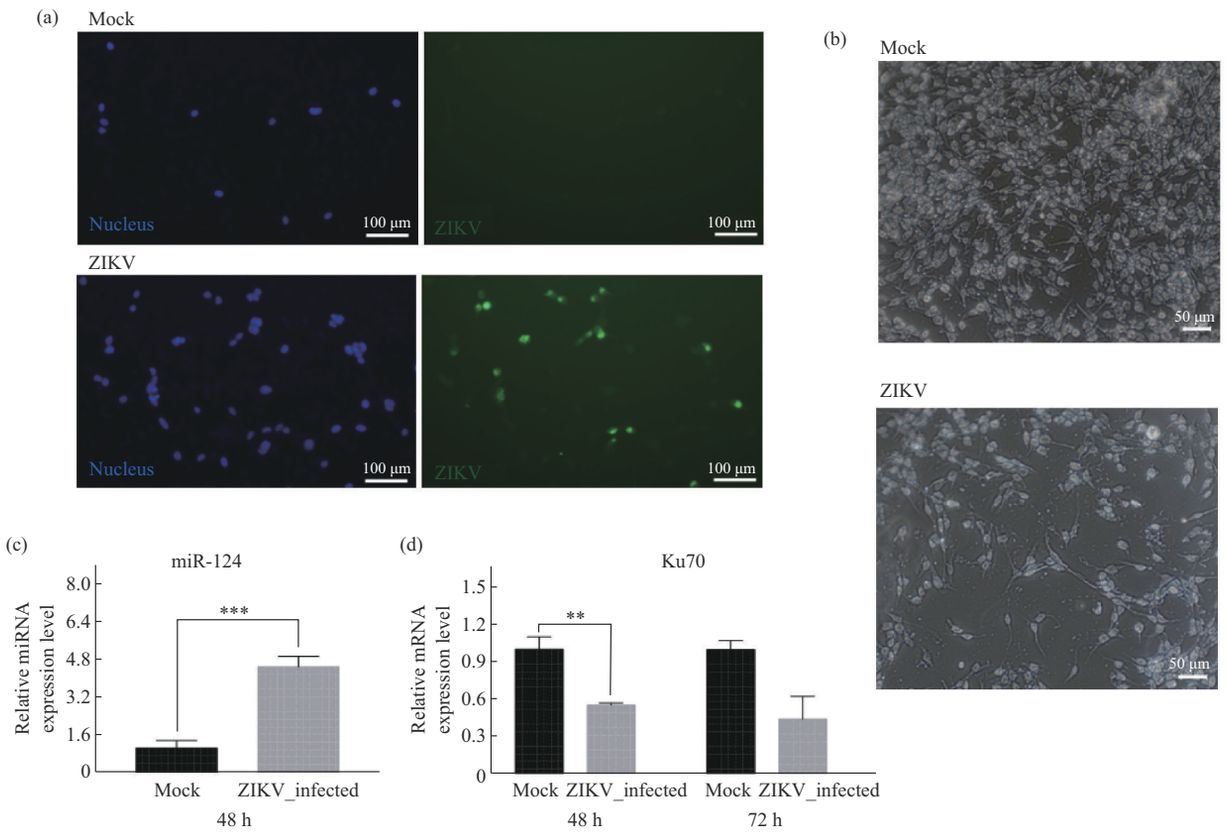


**Fig. 7　ZIKV infection up−regulates the expression of miR−124 resulting in differentiation of hNPCs**

(a) Immunofluorescence assay of ZIKV in hNPCs shows before and after 24 h ZIKV infection. (b) Morphological changes of hNPCs infected with or without ZIKV for 6 d. (c) qRT-PCR analysis of miR-124 expression. U6 snRNA was used as an internal control. (d) qRT-PCR analysis of Ku70 mRNA after ZIKV infection. β-Actin mRNA was used as an internal control. Values represented means of three independent experiments. The qRT-PCR data are shown as the mean±SD (Student's *t* test, ***P*<0.01, ****P*<0.001).

## 3　Disscussion

Ku70 is considered to be a multifunctional protein, for it is not only a crucial DNA binding protein in NHEJ repair of DSBs, but also plays a key role in apoptosis, virus defense, senescence, and tumorigenesis[26]. Our research revealed for the first time that Ku70 protein had RNA helicase activity and was involved in the process of miRNA maturation.

Ku70 could unwind the double-stranded chains of pre-miRNA, thus may cause pre-miRNA accumulation and disturbing the dicing of pre-miRNA by Dicer enzyme, which needs to be investigated in the future study.

Deficiency of Ku protein leads to the up-regulation of mature miRNA, down-regulation of pre-miRNA, and unchanged level of pri-miRNA.

Two subunits of Ku protein, Ku70 and Ku80, are heterodimers that can recognize and bind to the break ends of DNA chain respectively. Meanwhile, it unchains the two broken ends through the DNA helicase activity of Ku protein in an ATP-dependent manner. Then, the Ku-DNA complex recruits DNA-protein kinases C（DNA-PKc) to the broken site to repair the damaged DNA[27]. While both Ku70 and Ku80 can bind double-stranded RNAs or pre-miRNA with hairpin structure *in vitro*, it was found that only Ku70 was confirmed as possessing RNA helicase activity. The exact mechanism by which Ku80 affects the expression of miRNAs is far from clear, and Ku80 may be involved in the expression of Ku70, because Ku70 and Ku80 normally exist as heterodimers in cells. All these need to be investigated in the future.

Ku70 protein mainly occurs in the nucleus during DNA repair[28]. It is reported that Ku70 could also located both in the nucleus and cytoplasm of several types of cultured human and monkey cells[29]. Ku70 could translocate from the nucleus to the

cytoplasm in case of stress stimuli such as infection or exogenous DNA[30-31]. This can serve as evidence for an unidentified function of Ku70 protein. Our research revealed that Ku70 protein was involved in pre-miRNA cleavage, which happened to occur in the cytoplasm. The effect of miRNA maturation was negatively regulated by Ku70 protein, suggesting that the abnormal expression of housekeeping gene Ku70 might lead to miRNA processing disorders. Our findings here pointed to a new function of Ku70 protein accompanied by its subcellular translocation.

miR-124 is a well-known neuron-specific microRNA and a regulator of the REST-based transcriptional silencing complex. Over-expression of miR-124 can drive the differentiation of neural progenitor cells into neurons and C2C12 cells into neuron-like cells[32-33]. Inhibition of miR-124 expression by the RNA-binding protein polypyrimidine-tract-binding (PTB) can reverse differentiation[23]. The finding that Ku70 protein could act on miR-124 maturation and alter neuronal differentiation indicated that Ku70 played important roles in neuronal development. Bakalkin *et al*. [34] reported that Ku protein was gradually reduced during brain development and maintained stability of neuronal differentiation. Our data also suggested that the expression of Ku70 protein diminished along with the embryonic development of mice (Figure S1). All this led to the assumption that Ku70 could regulate the neuronal development of the brain by negatively regulating miR-124 maturation.

The essential roles of Ku protein in viral infection and neurodevelopment remind us of its association with neurotropic viruses, such as ZIKV. Recent reports reveal that ZIKV-induced microcephaly is due to down-regulation of neurogenesis and up-regulation of apoptosis in hNPCs, which leads to neurological impairment and neuronal differentiation disorders[35], but the precise mechanism remains unclear. Our research confirmed that ZIKV infected hNPCs can decrease Ku70 expressions and increase miR-124 levels, which results in differentiation of neural progenitor cells. Our findings suggested that the absence of RNA helicase Ku70 could possibly lead to abnormal processing of miRNA, which causes developmental abnormalities of neurons in case of ZIKV infection. The premature differentiation of embryonic neural progenitor cells might be one of the causes of microcephaly. Our results might provide new insights into the mechanism of microcephaly.

## 4 Conclusion

Here, we revealed that the Ku70 protein had RNA helicase activity and affected miRNA maturation. Deficiency of Ku70 led to the up-regulation of a large number of mature miRNAs, especially neuronal specific miRNAs like miR-124. Importantly, ZIKV infection reduced the expression of Ku70 whereas increased expression of miR-124 and led to the premature differentiation of embryonic neural progenitor cells, which might be one of the causes of microcephaly.

**Supplementary** Available online (http://www.pibb.ac.cn or http://www.cnki.net): PIBB_20230456_Figure_S1.pdf

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# Ku70作为RNA解旋酶调节miR−124的加工成熟及神经细胞的分化*

黄皑雪[1)**]　李睿婷[2)**]　赵越超[1)**]　李　洁[1)]　李　慧[1)]　丁学锋[1)]　王　琳[1)]
肖　参[1)]　刘雪梅[1)***]　秦成峰[2)***]　邵宁生[1)***]
（[1)] 北京基础医学研究所，北京 100850；[2)] 北京微生物流行病研究所，北京 100071）

**摘要　目的**　Ku70蛋白主要通过其DNA结合特性参与双链DNA断裂（DSB）的非同源端连接（NHEJ）修复，有报道称其具有RNA结合功能，本文探索Ku70是否具有RNA解旋酶活性并影响miRNA加工成熟。**方法**　利用RNA免疫共沉淀（RIP）测序结合生物信息学分析Ku蛋白结合的RNA；蛋白质印迹法（Western blot，WB）结合定量反转录PCR（qRT-PCR）检测Ku蛋白与miRNAs的表达关系；生物膜干涉技术（BLI）实验分析Ku蛋白与RNA的结合能力；电泳迁移率变动分析（EMSA）实验确定Ku70及Ku80的RNA解旋酶活性；形态学检测结合WB分析Ku70调节miR-124引起的神经细胞功能变化；免疫荧光结合形态学分析寨卡病毒（ZIKV）感染后Ku70及miR-124的变化与神经元分化关联。**结果**　研究发现，Ku70蛋白具有RNA解旋酶活性，并通过其RNA解旋酶活性影响miRNA加工成熟。Ku70缺失引起许多miRNAs上调，其中包括神经细胞特异的miR-124。在人神经前体细胞（hNPCs）和人神经母细胞瘤细胞（SH-SY5Y）中敲低Ku70可促进miR-124的成熟，从而导致上述细胞向神经元分化。本文进一步发现，ZIKV感染影响了Ku70及miR-124的表达，导致细胞形态的分化。**结论**　本研究揭示了Ku70的一种新功能，即Ku70有可能参与miRNA的成熟调控和神经细胞的分化，并且可能是ZIKV病毒致小头症的原因之一。

**关键词**　Ku70，RNA解旋酶，miRNA加工成熟，miR-124，神经元分化
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** 并列第一作者。
*** 通讯联系人。
刘雪梅　Tel：010-66932313，E-mail：liuxuemei@bmi.ac.cn
秦成峰　Tel：010-66948604，E-mail：qincf@bmi.ac.cn
邵宁生　Tel：010-66932311，E-mail：shaoningsheng@bmi.ac.cn
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