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CircKEAP1 serves as a ceRNA to suppress lung adenocarcinoma progression by activating KEAP1 signal pathway

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Abstract

Background

Circular RNAs (circRNAs) are widely expressed noncoding RNAs, and plays a key role in the biological function of competitive endogenous RNA (ceRNA) network in various human diseases, especially in cancer. However, the regulatory roles of circRNAs in lung adenocarcinoma (LUAD) remains largely unknown.

Methods

The expression profiles of circRNAs in LUAD tissues and adjacent non-tumor tissues were analyzed by Agilent Arraystar Human CircRNA microarray. The level and prognostic values of circKEAP1 in tissues and cancer cell lines were determined by quantitative real-time PCR. Then, the effects of circKEAP1 on tumor growth were investigated by functional experiments *in vitro* and *in vivo*. Mechanistically, dual luciferase reporter assay, RNA pull-down and RNA immunoprecipitation experiments were performed to confirm the interaction between circKEAP1 and miR-141-3p in LUAD.

Results

We found circKEAP1 was significantly downregulated in LUAD tissues, and repressed tumor growth both *in vitro* and *in vivo*. Mechanistically, circKEAP1 competitively binds to miR-141-3p and relive miR-141-3p repression for its target gene KEAP1, which activated the KEAP1/NRF2 signal pathway, and finally suppress the cell proliferation.

Conclusions

Our findings suggest that circKEAP1 inhibits LUAD progression through circKEAP1/miR-141-3p/KEAP1 axis and it may serve as a new target for treatment of LUAD patients.

Introduction

LUAD is the most common histopathological subtype of lung cancer, which is the most commonly diagnosed cancer and the leading cause of cancer death worldwide [1]. The morbidity of LUAD is continuously raised during the past years, especially in women, never-smokers and young adults [1]. The overall 5-year survival of LUAD is less than 20% due to the lack of effective early diagnosis and treatment options [2]. Therefore, a more comprehensive understanding of the molecular mechanisms of LUAD is important to develop reliable early biomarkers and novel therapies for LUAD.

miRNA was a novel kind of about 22nt non-coding RNAs which could repress gene expression by binding with their target mRNAs [3]. It is well known that the downregulation of some tumor-suppressive genes in cancer may cause by the increase of miRNAs (named as oncomiR), whereas the decrease of tumor-suppressive miRNAs (named as tumor suppressor miRNAs) lead to the upregulation of oncogenes [4].

CircRNAs is a novel class of endogenous RNAs, which have been discovered ubiquitously expressed in mammalian cells and played an important role in the regulation of gene expression [5]. CircRNAs are characterized by the covalently closed loop structure, which result to resistant to the degradation of exonuclease RNase R [5], and used to be just considered as a by-product of a splicing error for a long time. However, numerous of reports recently revealed circRNAs could act as miRNA sponges, protein sponges, transporters or scaffolds, which were in turn involved in the initiation and progress of a variety of diseases, especially cancers [5, 6]. Consequently, circRNAs that function as sponge for miRNA are likely to be involved in cancer [7]. In LUAD, several circRNAs have been revealed to be significantly dysregulated [8, 9, 10, 11, 12, 13, 14] and could act as ceRNA to sponge oncomiR or tumor suppressor miRNAs [8, 9, 13, 15, 16, 17]. For instance, circHIPK3 was found to modulate autophagy by sponging miR-124-3p [15]. Circ100146 could directly bind to miR-361-3p and miR-615-5p to promote the progress of non-small cell lung cancer [13]. CircPTK2 functions as a sponge of miR-429/miR-200b-3p to inhibit TGF-β-induced EMT and LUAD cell invasion by relieving the repression of miR-429/miR-200b-3p for TIF1γ [9]. CircPRKCI was confirmed to act as a sponge for both miR-545 and miR-589 and abrogated their suppression of the protumorigenic transcription factor E2F7 to promote tumor growth in LUAD [17].

In this study, the expression profile of circRNAs in LUAD was investigated by Human CircRNA microarray V1 and a novel LUAD-related circRNA circKEAP1 was identified to be significantly downregulated in LUAD tissues. Subsequently, the function and molecular mechanism of circKEAP1 in LUAD development and progression was explored. As the results in the Human CircRNA microarray, the data showed circKEAP1 was remarkably downregulated in LUAD tissues, compared to the adjacent non-cancerous tissues. Additionally, circKEAP1 could inhibit cell proliferation by acting as a sponge for miR-141-3p to relieve miR-141-3p repression for its target gene KEAP1, which in turn downregulate the expression of NRF2 and repress the NRF2 signal pathway. In summary, our data show that circKEAP1 might act as a tumor suppressor via miR-141-3p-KEAP1-NRF2 signaling in LUAD.

Method And Materials

Patients' characteristics

CircRNA and miRNA expression profiles for seven paired LUAD cancer tissues and adjacent normal tissues were generated using the Agilent-069978 Arraystar Human CircRNA microarray and NanoString nCounter Human miRNA Expression Assay. The clinicopathological features of these seven patients are described in Table s1. We collected 105 cancer tissues and paired distal normal tissues from patients who were firstly diagnosed with LUAD without any treatment at the Harbin Medical University Cancer Hospital (Harbin, China). The clinicopathological features are described in Table s2. The Ethics Committee of the Harbin Medical University Cancer Hospital authorized the study, and we conducted it in conformity to the Declaration of Helsinki.

Cell culture

We purchased A549 (human LUAD cell line) and BEAS-2B (normal human bronchial epithelial cell line) from American Type Culture Collection (ATCC) (Manassas, VA, USA). A549 was cultured in DMEM Medium (Gibco, Carlsbad, CA, USA) with 10% FBS, 10,000 units/ml penicillin, and 10,000 μg/ml streptomycin. BEAS-2B was cultured in RPMI 1640 Medium (Gibco, Carlsbad, CA, USA) with 10% FBS, 10,000 units/ml penicillin, and 10,000 μg/ml streptomycin. All the cells were maintained in a 5% CO_2 humidified atmosphere at 37 °C.

RNA/ gDNA extraction and RT-PCR/ qRT-PCR assay

Total RNAs were isolated by TRIzol reagent (Takara, Dalian, China) according to the manufacturer's instruction. gDNA was isolated by Genomic DNA Isolation Kit (Sangon Biotech, Shanghai, China). The quality and quantity of RNA and DNA were detected by Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The nuclear and cytoplasmic fractions were purified by PARIS Kit (Ambion, Life Technologies). RNA was reverse transcribed by HiScript II Q RT SuperMixfor qPCR (+gDNA wiper) (Vazyme, Nanjing, China). The AmpliTaq DNA Polymerase (Life Technologies) was used for PCR. The 2% agarose gel electrophoresis was performed to observe the cDNA and gDNA PCR products. AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China) was used for qRT-PCR, and GAPDH was used to normalize the level of circRNA and mRNA. Hydrolysis probe-based RT-qPCR assay of miRNA was performed according to the manufacturer's instructions (Applied Biosystems). The miRNA level was normalized by small nuclear U6. Primers are listed in Table s3.

RNase R treatment

Total RNA (about 2mg) was incubated with 5 U/μg RNase R (Epicentre Technologies) for 30 min at 37 °C. Then, the RNA was purified by RNeasy MinElute Cleaning Kit (Qiagen) according to the the manufacturer's protocols.

Actinomycin D assay

BEAS-2B cells were exposed to 2 μg/ml actinomycin D (Sigma) at indicated time point. Subsequently, the cells were harvested to extract the total RNA, and the stability of circKEAP1 and KEAP1 mRNA was analyzed using qRT-PCR.

Vector construction and cell transfection

To overexpress circKEAP1, the full-length cDNA of circR-KEAP1 was synthesized and then cloned into pLCDH-ciR plasmid (Geneseed, Guangzhou, China). For luciferase reporter vector, the sequence of circKEAP1, mutated circKEAP1 (the seed sequence mutated from UGGUGUU to ACCACAA for binding site 1, and CAGCGUUG to GUCGCAAC for binding site 2), KEAP1 3'UTR and mutated KEAP1 3'UTR (the seed sequence mutated from CAGUGUU to GUCACAA) was synthesized and then cloned into the pGL3-promoter vector (Geneseed, Guangzhou, China). The Dual Luciferase Assay Kit (Promega, Madison, WI, USA) was used to examine the luciferase activity accordance to the manufacturer's protocols. SiRNAs of

circKEAP1, miRNA mimics, miRNA inhibitors and corresponding negative control (NC) were synthesized by GenePharma (Shanghai, China). Cells were transfected using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instruction.

Cell proliferation assays

The proliferation activity of A549 cells was tested by both Cell-Light™ EdU DNA Cell Proliferation Kit (Ribobio, Guangzhou, China) and Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) following the manufacturer's protocols.

RNA immunoprecipitation (RIP) and Biotin-coupled miRNA capture

The Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA) was used for RIP experiments according to the manufacturer's instructions. AGO2 antibody used for RIP was purchased from Cell Signaling Technology. The 3' end biotinylated miR-141-3p mimics or control RNA (Ribio, Guangzhou, China) were transfected into BEAS-2B cells for 48 h before harvest. Then, we added cell lysis buffer (5 mM MgCl2, 100 mM KCl, 20 mM Tris (pH 7.5), 0.3% NP-40, 50U of RNase OUT (Invitrogen, USA)) and complete protease inhibitor cocktail (Roche Applied Science, IN) into the cell pellets, and incubated them on ice for 30 min. The biotin-coupled RNA complex was pulled down by incubating the cell lysates with streptavidin-coated magnetic beads (Life Technologies) by centrifugation at 10,000×g for 20 min.

Western blot analysis

The protein extraction reagent (Thermo Scientific) with a cocktail of proteinase inhibitors (Roche Applied Science, Switzerland) was used to isolate the total protein from cells or tissue samples. Equal amount of total protein was separated by 10% SDS–PAGE and transferred onto a PVDF membrane. Then, the membranes were blocked with 5% skimmed milk powder and incubated the membranes with primary antibodies at 4 °C overnight and then incubated with secondary antibodies at room temperature for 2 h. The bands were examined by Immobilob™ Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA). The primary antibody and secondary antibodies were purchased from Cell Signaling Technology, and the detailed information list below: KEAP1(KEAP1 (D6B12) Rabbit mAb #8047, Cell Signaling Technology, Beverly, MA, USA), GAPDH (GAPDH (D16H11) XP® Rabbit mAb #5174, Cell Signaling Technology, Beverly, MA, USA), NRF2 (NRF2 (D1Z9C) XP® Rabbit mAb #12721, Cell Signaling Technology, Beverly, MA, USA), HDAC4 (HDAC4 (D8T3Q) Rabbit mAb #15164, Cell Signaling Technology, Beverly, MA, USA) and the secondary antibodies (Anti-rabbit IgG, HRP-linked Antibody #7074, Cell Signaling Technology, Beverly, MA, USA).

Animal experiments

Stably over-expressed cell lines were established by transfecting A549 cells with *circKEAP1* overexpressed plasmid or control plasmid and selected with puromycin. Then, we suspended 10^6 A549 cells in 100 μl PBS and injected into the right flank of 6-week-old male BALB/c nude mice. We measured

the volume of tumors every five days. After 20 days later, the mice were sacrificed and the tumors were collected for further analysis.

Statistical analyses

Adequate sample size was determined according to the previous studies that performed analogous experiments. A two-sided test was applied. The raw data applying *t-test* was normally distributed. Data are expressed throughout the manuscript as mean±SD. The SPSS 18.0 software was performed to the statistical analyses, and the GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA) was used to generate the graphs. A P-value <.0.05 was regarded as statistically significant.

Results

CircKEAP1 is downregulated in LUAD

The expression profile of circRNAs in seven paired samples of cancer tissues and adjacent normal tissues from LUAD patients was analyzed by Human CircRNA microarray. There were 32 dysregulated circRNAs with p-value < 0.05, of which 15 circRNAs were downregulated and 17 circRNAs were upregulated (Fig. 1A and B and Table s4). Since the downregulated circRNAs in cancer tissues might play tumor suppressive roles and could serve a novel method to treat cancer, the 15 downregulated circRNAs were chosen for furth study. Firstly, we analyzed these downregulated circRNAs in another two circRNA expression profile (GSE112214 and GSE101586) of LUAD in GEO database. The results showed hsa_circRNA_104126 and hsa_circRNA_102442 were significantly downregulated in all the three circRNA expression profile (Fig. 1C). Then, the two circRNAs were determined in the seven paired samples of cancer tissues and adjacent normal tissues from LUAD patients by qRT-PCR. Consistent with the results of circRNA expression profile, hsa_circRNA_104126 and hsa_circRNA_102442 were downregulated in the cancer tissues (Fig. 1D). Compared to the hsa_circRNA_104126, the expression level of hsa_circRNA_102442 in the normal tissues was much higher (Fig. 1D).

Hsa_circRNA_102442 (termed circKEAP1) is located at chr19: 10610070-10610756, and derived from exon2 of KEAP1 gene with a length of 686nt by alternative splicing (Fig. 2A). We amplified the back-spliced junction of circKEAP1by divergent primers and confirmed by Sanger sequencing (Fig. 2A). Then, we performed Northern blot analysis by a probe targeted the back-spliced junction, and the result showed that circKEAP1 could be observed at 686nt (Fig. 2B). Additionally, PCR analysis for reverse-transcribed RNA (cDNA) and genomic DNA (gDNA) showed that divergent primers could amplify products from cDNA of adjacent normal tissues but not from cDNA of cancer tissues and gDNA (Fig. 2C). Next, the expression level of circKEAP1 in another 105 paired LUAD samples was analyzed by quantitative reverse transcription PCR (qRT-PCR). As showed in Fig.2D, the expression of circKEAP1 was obviously decreased in cancer tissues (Fig. 2D).

The same results were revealed in cell experiments. CircKEAP1 was amplified from cDNA of the human normal lung epithelial cell line BEAS-2B, but not from cDNA of LUAD cancer cell line A549 and gDNA (Fig.

2E). Subsequently, we treated the BEAS-2B cells with Actinomycin D (an inhibitor of transcription) to explore the stability of circKEAP1 and KEAP1. As showed in Figure 2F, the half-life of circKEAP1 transcript exceeded 24 h and was much longer than KEAP1. Finally, qRT-PCR analysis was conducted for nuclear and cytoplasmic circKEAP1 to observe cellular localization of circKEAP1. As showed in Fig.1H, circKEAP1 transcript was preferentially located in the cytoplasm (Fig. 2G).

Taken together, circKEAP1 mainly existed in the cytoplasm, and significantly decreased in LUAD cancer tissues.

circKEAP1 inhibits tumor growth *in vivo* and *in vitro*

To study the function of circKEAP1 in LUAD progression, *we* synthesized the full-length cDNA of circKEAP1 and cloned into the pLCDH-ciR plasmid, which contained a front and back circular frame. *We found that* circKEAP1 plasmid *could successfully increase* circKEAP1 *expression in A549 cells (Fig. 3A).* A nude mice xenograft model by implanting A549 cells transfected with control plasmid or circKEAP1 plasmid was established to identify the effect of circKEAP1 on tumor growth *in vivo*. The A549 cells were subcutaneously injected into the right flank of 6-week-old male BALB/c nude mice. The tumor volumes were monitored from the 5 days after A549 cell injection, and the mice were sacrificed after 20 days. We found overexpression of circKEAP1 drastically suppress tumor growth of A549 cells (Fig. 3B). The tumor weights were remarkably decreased by circKEAP1 (Fig. 3C and D). As showed in figure 3E, both H&E staining and Ki-67 staining of these tumors showed decreased cell mitosis and lower percentage of proliferative cells in the circKEAP1 overexpression group. As the previous results in the cells *(Fig. 3A)*, the expression level of circKEAP1 was much higher in the circKEAP1 overexpression group, compared to the control group (Fig. 3F). These data indicated that circKEAP1 repressed tumor growth *in vivo*. To identify the effect of circKEAP1 on cancer cell proliferation *in vitro*, the EDU assay and CCK-8 assay were performed to the A549 cells transfected with control plasmid or circKEAP1 plasmid. As the results showed in Fig. 3G and H, both the EDU assay and CCK-8 assay revealed circKEAP1 significatly suppressed the cancer cell growth *in vivo*.

In summary, these findings suggest that circKEAP1 could repress the tumor growth *in vivo* and *in vivo*.

circKEAP1 act as a sponge for miR-141-3p

Recently, circRNA has been increasingly found to be involved in cancer by releasing downstream molecules by competing with miRNA [5, 6]. As showed in Fig.2G, *circKEAP1* was mainly located in the cytoplasm *(Fig. 2G)*. C*ircKEAP1 was* speculated to be *severed as a miRNA sponge. In order to verify the hypothesis, we firstly analyzed the miRNA expression profiles of the seven paired* LUAD cancer tissues and non-tumor tissues by Human miRNA Expression Assay. The volcano plot showed significantly different profiles of miRNAs between the seven paired samples of LUAD and adjacent normal tissues (Fig. 4A and Table s6). Then, we predicted the potential binding sites of miRNAs in the *circKEAP1by Targetscan[18], and found* a total of 135 miRNAs were identified as potential targets of *circKEAP1 (Table s7)*. As the *circKEAP1 has been found to be downregulated in LUAD cancer tissues, compared to* adjacent

normal tissues (Fig. 2D), the miRNA which was upregulated in the LUAD tissues and had the binding site with *circKEAP1* was choose for further study. The resulted showed 13 miRNAs, including miR-141-3p, miR-106b-3p, miR-93-3p, miR-21-3p, miR-19b-3p, miR-29b-5p, miR-3445-5p, miR-375 and miR-200c-3p, contained at least one binding site with *circKEAP1 and significantly upreg*ulated in LUAD cancer tissues (Fig. 4B). A dual-luciferase reporter assay was performed to further confirm the interaction between *circKEAP1* and its predicted 13 miRNAs in A549 cells. Among the 13 predicted miRNAs, all the miRNAs significantly attenuated the luciferase activity of A549 cells (Fig. 4C), and the miR-141-3p was more obvious than other miRNAs and selected for deeper investigation. It has been proved that circRNAs bind with miRNAs through AGO2 (Argonaute 2). Therefore, we conducted the anti-AGO2 RNA immunoprecipitation (RIP) assay in BEAS-2B cells to pull down the RNA transcripts which bind to AGO2 with anti-AGO2 antibody. Interestingly, both *circKEAP1* and miR-141-3p were efficiently pulled down by anti-AGO2 antibodies (Fig. 4D). Thus, it's supposed that *circKEAP1* might act as a sponge for miR-141-3p. Subsequently, we examined the expression levels of miR-141-3p in the 105 pairs of LUAD cancer tissues and adjacent non-cancerous tissues, and found miR-141-3p was markedly upregulated in LUAD cancer tissues compared with adjacent non-tumor tissues (Fig.4E). As showed in Fig.4F, there are two binding sites between miR-141-3p and *circKEAP1* (Fig. 4F). We performed the dual-luciferase reporter assay to confirm the bioinformatics prediction analysis in A549 cells. The full-length of *circKEAP1* and muted *circKEAP1* with muted miR-141-3p binding sites were subcloned into luciferase reporter pGL3 plasmid. We found miR-141-3p mimic could effectively increase the expression level of miR-141-3p in A549 cells (Fig S1), and significantly decreased the luciferase activity of wildtype group, however it had no effect on the mutant group (Fig. 4G). These results suggested there might be a direct interaction between *circKEAP1* and miR-141-3p. Then, the miRNA pull-down assay with specific biotin-labeled miR-141-3p was performed to further verify the binding of *circKEAP1* and miR-141-3p. As expected, *circKEAP1*was significantly enriched in the biotin-labeled miR-141-3p group compared with control (Fig. 4H). Additionally, we also found the expression level of miR-141-3p in both A549 cells (Fig. 4I) and tumors (Fig. S2A) was markedly decreased when we overexpressed the *circKEAP1 by circKEAP1 plasmid*. The decrease could be attenuated by miR-141-3p mimic in A549 cells (Fig. 4J).

These results reveal that *circKEAP1 could* server as a sponge for miR-141-3p in LUAD cancer cells.

miR-141-3p repress the KEAP1 expression in tumors

It's well known that circRNAs can act as miRNAs sponge to regulate downstream targets [5, 6], by sharing the same miRNAs with mRNA [7, 19]. Three bioinformatics software TargetScan [18], miRDB [20] and miRanda [21] were used to predict the target genes of miR-141-3p. Bioinformatics analysis showed that the 3'-UTRs of ZEB1, ZEB2, and KEAP1 contained miR-141-3p complementary sequences by all the three algorithm (Fig. 5A). In order to confirm the repression of miR-141-3p, we firstly performed dual luciferase reporter assay in A549 cells. We found miR-141-3p remarkably suppressed the luciferase activity of luciferase reporter vector containing the *KEAP1* 3'UTR sequence, while it has no effect on ZEB1 and ZEB2 in A549 cells (Fig. 5B). As predicted, miR-141-3p have one binding sites in the 3'-UTR of KEAP1 (Fig.5C). In order to further confirm the repression of miR-141-3p on KEAP1, we firstly performed dual luciferase reporter

assay in A549 cells. We found miR-141-3p remarkably suppressed the luciferase activity of luciferase reporter vector containing the *KEAP1* 3'UTR wild type sequence (Fig. 5D), while had no influence on the muted vector containing the muted *KEAP1* 3'UTR sequence (Fig. 5D). Then, we performed the miRNA pull-down assay with specific biotin-labeled miR-141-3p, and the result showed *KEAP1* mRNA was also specific enrichment in the biotin-labeled miR-141-3p group, like circKEAP1 (Fig. 5E). The IHC staining for KEAP1 protein in 105 paired LUAD cancer tissues and adjacent normal tissues and western blotting for KEAP1 protein in 12 paired LUAD cancer tissues and adjacent normal tissues was performed to analysis the protein level of KEAP1. The results showed the protein level of KEAP1 was remarkably downregulated in LUAD cancer tissues, compared to the adjacent non-cancerous tissues (Fig. 5F and G). Pearson correlation analysis revealed a significant negative correlation between KEAP1 protein level and miR-141-3p. These results suggested miR-141-3p might regulat the protein level of KEAP1. To determine whether miR-141-3p could inhibit KEAP1 expression, BEAS-2B cells were transfected with mimics of miR-141-3p to increase the level of miR-141-3p, while A549 cells were transfected with inhibitors of miR-141-3p to downregulate the cellular levels of miR-141-3p. As anticipated, mimics of miR-141-3p could inhibit KEAP1 expression, while inhibitors increased KEAP1 levels (Fig. 5H). KEAP1 is confirmed to bind to NRF2 (nuclear factor erythroid 2-related factor 2) and promote its degradation by the ubiquitin proteasome pathway, which could in turn repress the HDAC4 (histone deacetylase 4) expression in LUAD [22].We found *miR-141-3p* mimics significantly increased the NRF2 and HDAC4 expression by repressing the KEAP1 expression, and *miR-141-3p inhibitors remarkably suppressed the* NRF2 and HDAC4 expression by reliving the suppression of miR-141-3p for KEAP1.

Actually, KEAP1 has been proved to be a target gene of miR-141-3p in LUAD by many previous studies [23, 24, 25, 26, 27, 28]. Our results confirmed again that miR-141-3p can inhibit KEAP1 by binding to its 3'-UTR.

circKEAP1 relieved repression of miR-141-3p for KEAP1 expression

To confirm *circKEAP1 could regulate the repression of miR-141-3p for* KEAP1, the luciferase reporters containing *KEAP1* 3'UTR wild type sequence or muted sequence was co-transfected with circKEAP1 plasmid or miR-141-3p mimic in A549 cells. The results showed circKEAP1 significantly increased the luciferase activity of KEAP1 wild type reporter, while it had no effect on the mutated reporter. Moreover, the increase by the circKEAP1 could be abolished by miR-141-3p overexpression through miR-141-3p mimic (Fig. 6A). Subsequently, the protein level of KEAP1 was evaluated in cells with overexpressing *circKEAP1*, and resulting to decrease the NRF2 and HDAC4 expression, which was reported as the downstream genes of KEAP1 pathway [29], but it was rescued by *miR-141-3p* overexpression (Fig. 6B-C). Additionally, we also found the protein level of KEAP1 was significantly upregulated in the tumor of circKEAP1 overexpression group, which in turn downregulated the protein level of NRF2 and HDAC4 (Fig. S2B).

Several studies had been confirmed KEAP1 could suppress the expression of NRF2, and reduce its downstream gene HDAC4 expression, result to increase the tumor suppressor miRNAs miR-1/206 level to regulates glucose metabolism in cancer cells [29]. In our study, we found *circKEAP1* could significantly

increase the expression of miR-1 and miR-206 both *in vitro* (Fig. 7A) and *in vivo* (Fig. S2C), however this increase could be attenuated by miR-141-3p overexpression. Additionally, in accordance with the results before, the *circKEAP1* significantly repressed the cancer cells proliferation (Fig. 7B-D), and the suppression could be rescued by miR-141-3p overexpression using miR-141-3p mimic (Fig. 7B-D).

Taken together, our results found circKEAP1 could server as a sponge for miR-141-3p to regulate KEAP1 and activate the KEAP1/NRF2/HDAC4 signal pathway via the ceRNA mechanism to repress tumor growth (Fig. 8).

Discussion

Recently, an increasing number of studies have confirmed the dysregulation of circRNAs play critical roles in modulating tumor development and progression [6]. Several circRNA has been revealed to function as oncogenes or tumor suppressors in lung cancer and other types of cancer [6, 11, 14]. However, only a few circRNAs have been well characterized. In this study, the profiling of circRNAs in LUAD was obtained by the Agilent-069978 Arraystar Human CircRNA microarray V1. We found circKEAP1 significantly downregulated in LUAD cancer tissues, and could suppress the tumor growth. CircKEAP1 exerted its function as a ceRNA that competitively bound to miR-141-3p, then abolished the repression of miR-141-3p for its original gene KEAP1. Elevated KEAP1 could inhibit the expression of NRF2 and HDAC4, and then abolish the suppression of HDAC4 for the tumor suppressor miRNAs miR-1 and miR-206, which result to repress the cell proliferation (Fig. 8). Our results suggested circKEAP1 could inhibit LUAD cell growth via the ceRNA mechanism.

Heterogeneous genetic or epigenetic modifications have been revealed could modify the oncogenes and tumor suppressor genes expression. The dysregulation of these genes exert their effects on multiple cellular processes in which transient modifications of redox balance might occur, such as cell proliferation. These transient cellular changes are mainly coordinated by KEAP1/NRF2 signaling pathway [22]. NRF2 is a transcription factor and could function as a master modulator of cellular defense against toxic and oxidative damage, mitochondrial physiology, differentiation, and stem cell maintenance [30]. NRF2 is tightly regulated by KEAP1 [22, 30]. In normal cell conditions, KEAP1 forms an ubiquitin ligase complex and targets NRF2 for proteolysis. Upon stress exposure, the KEAP1 releases NRF2 which translocates into the nucleus to form a heterodimeric complex which could recognizes the enhancer sequences of antioxidant response elements (AREs) and activates their transcription [22, 30]. Since KEAP1 and NRF2 could modulate cell proliferation, it's considered a hallmark in cancer cells of the deregulation of the KEAP1/NRF2 axis. Previous studies have indicated that abnormal states of the KEAP1-NRF2 pathway exist in lung cancer [30, 31]. Downregulated KEAP1 has been frequently identified in lung cancer, however the mechanism still remains unknown [31]. In this study, we also found KEAP1 was significantly downregulated in LUAD cancer tissues and knockdown of KEAP1 enhance the NRF2 expression. More interestingly, we revealed KEAP1 could encode a circRNA by alternative splicing to sponge miR-141-3p to relive the inhibitor of the miRNA for itself mRNA. In tumor condition, the decrease of the circKEAP1

resulted to the evaluated of miR-141-3p, which in turn suppress the protein level of KEAP1 and increase the NRF2 level. It's reported KEAP1 could modulate the NRF2 to repress HDAC4 methylated the promoter of tumor suppressor miRNA miR-1 and miR-206 [29]. In our study, we found circKEAP1 could upregulate miR-1 and miR-206 by upregulating KEAP1, which could decrease NRF2 expression, and result to suppress the HDAC4 and in turn inhibit the methylation of the promoter of these two miRNAs by HDAC4 (Fig. 8).

The most significant characteristic of cancer cells is uncontrolled cell proliferation. Previous studies have demonstrated that miR-141–3p was upregulated in multiple human cancers, and high expression of miR-141–3p promoted cancer cell proliferation via varying mechanisms [32]. For example, Li et al reported microRNA-141-3p fosters the growth of cervical cancer cells by targeting FOXA2 [33]. Xu et al found p53 is directly targeted by miR-141-3p, and miR-141-3p promotes tumor growth through inhibition of p53 pathways [34]. Li et al reported miR-141-3p could bind with the 3' untranslated region of DAPK1, and repress the expression of DAPK1 which in turn promote the proliferation [35]. MTM et al confirmed miR-141-3p could repress the KEAP1 expression and activate the KEAP1 downstream pathway to modulate cisplatin sensitivity [23]. In this study, we found circKEAP1 could inhibit cell proliferation by acting as a sponge for the miR-141-3p to relieve microRNA repression for target gene KEAP1.

In conclusion, our study reveals that circKEAP1 competitively binds miR-141-3p to abolish the suppressive effect of miR-141-3p on KEAP1, which result to suppress the NRF2/HDAC4 signal pathway and inhibit the cell proliferation. Our findings revealed an insight into understanding the KEAP1-NRF2 signal pathway during the development and progression of LUAD, and provide a potential therapeutic approach for LUAD.

Abbreviations

CircRNAs: Circular RNAs; LUAD: lung adenocarcinoma; miRNAs: microRNAs; EMT: Epithelial–mesenchymal transition; RT-PCR: Reverse transcription polymerase chain reaction; qRT-PCR: Quantitative Reverse transcription polymerase chain reaction; RIP: RNA immunoprecipitation; cDNA: reverse-transcribed RNA; gDNA: genomic DNA; EDU: 5-ethynyl-2'-deoxyuridine; CCK-8: Cell Counting Kit-8; 3'UTR: Three prime untranslated region

Declarations

Ethical Approval and Consent to participate

This study was approved by the Ethics Committee of Harbin Medical University Cancer Hospital. All experiments were performed in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article. Raw and processed data are stored in the laboratory of GB and are available upon request.

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Competing interests

The authors declare no potential conflicts of interest.

Authors' contributions

JunFeng Wang designed the experiments. Yanbo Wang, Fenghai Ren, Dawei Sun, Jing Liu, BenKun Liu, Sainan Pang, BoWen Shi, FuCheng Zhou, Lei Yao and YaoGuo Lang performed the experiments and analyzed results. ShiDong Xu and JunFeng Wang wrote the manuscript.

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Figures

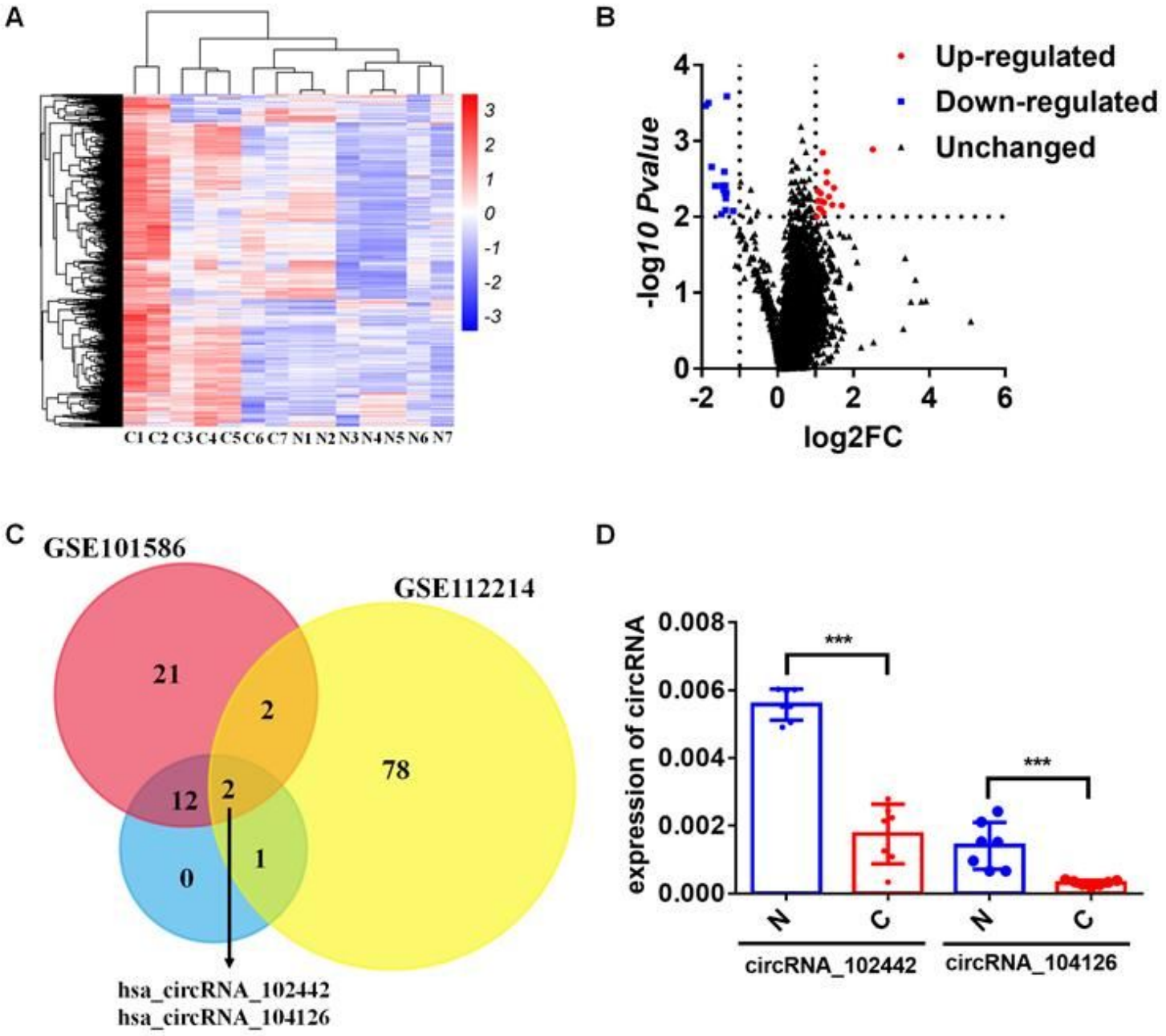

Figure 1

circKEAP1 was significantly downregulated in LUAD by circRNA microarray. (A-B) The heatmap (A) and volcano plot (B) of circRNA profiles in LUAD cancer tissues (C) and adjacent normal tissues (N). (C) Venn diagrams presenting circRNAs downregulated in our circRNA expression profile and circRNA expression profile of GSE112214 and GSE101586. (D) qRT-PCR for the abundance of hsa_circRNA_104126 and hsa_circRNA_102442 in the severn paired samples of LUAD cancer tissues (C) and adjacent normal tissues (N).

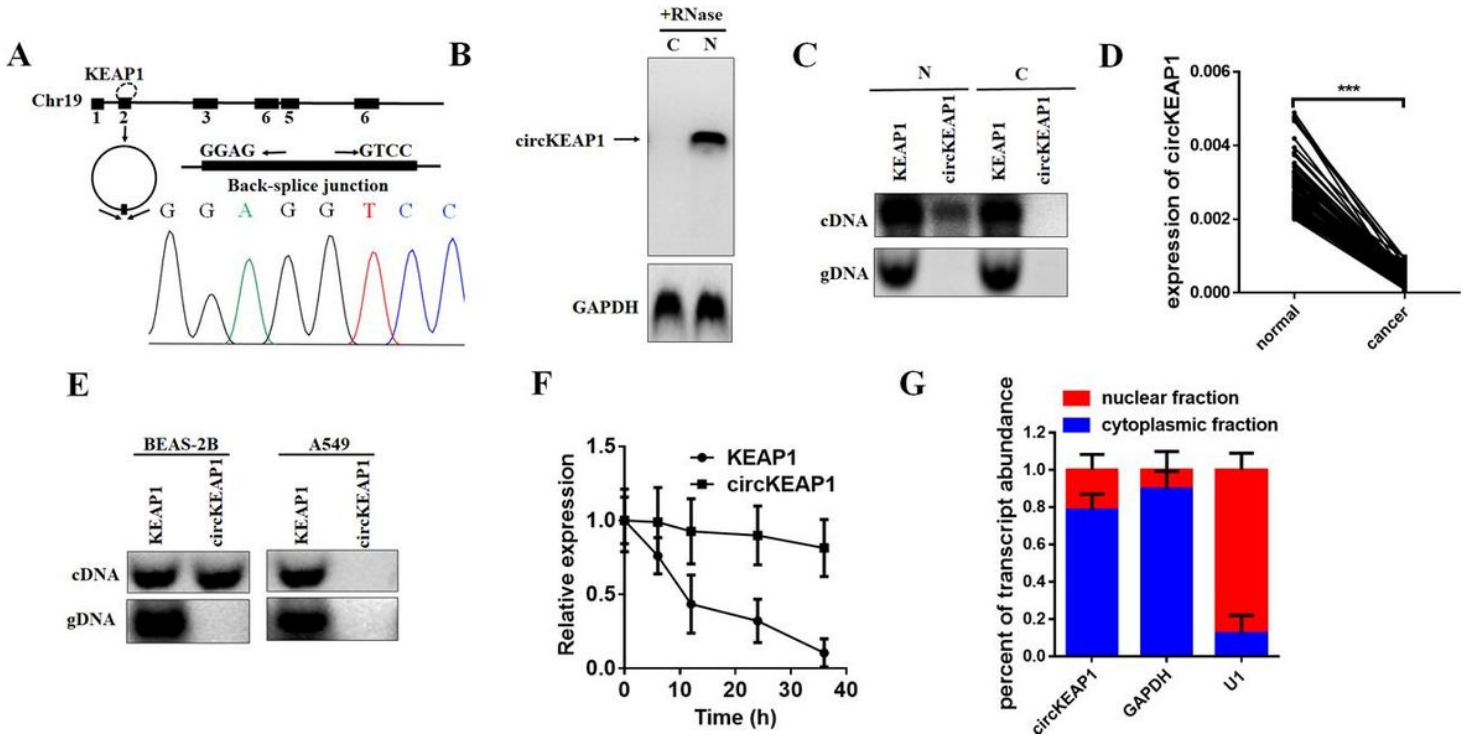

Figure 2

Characterization of circKEAP1 in LUAD. (A) Genomic loci of circKEAP1 gene. CircKEAP1 is produced at the KEAP1 gene locus containing exon 2. The back-splice junction of circKEAP1 was identified by Sanger sequencing. (B) Northern blot analysis showed the abundance of circKEAP1 in one paired sample of LUAD cancer tissue (C) and adjacent normal tissues (N). (C) PCR analysis for circKEAP1 and its linear isoform KEAP1 in cDNA and genomic DNA (gDNA) in one paired sample of LUAD cancer tissue (C) and adjacent normal tissues (N). (D) qRT-PCR for the abundance of circKEAP1 in 105 paired samples of LUAD cancer tissues (C) and adjacent normal tissues (N). (E) PCR analysis for circKEAP1 and its linear isoform KEAP1 in cDNA and genomic DNA (gDNA) in human LUAD cell lines A549 and normal human bronchial epithelial cell line BEAS-2B. (F) qRT-PCR for the abundance of circKEAP1 and KEAP1 in BEAS-2B cells treated with Actinomycin D at the indicated time point. (G) Levels of circKEAP1 in the nuclear and cytoplasmic fractions of BEAS-2B cells. Data are shown as the means ± standard error of the mean (n =3), statistical analysis was performed by two-tailed Student's t-test; *P < 0.05, **P < 0.01, ***P < 0.001.

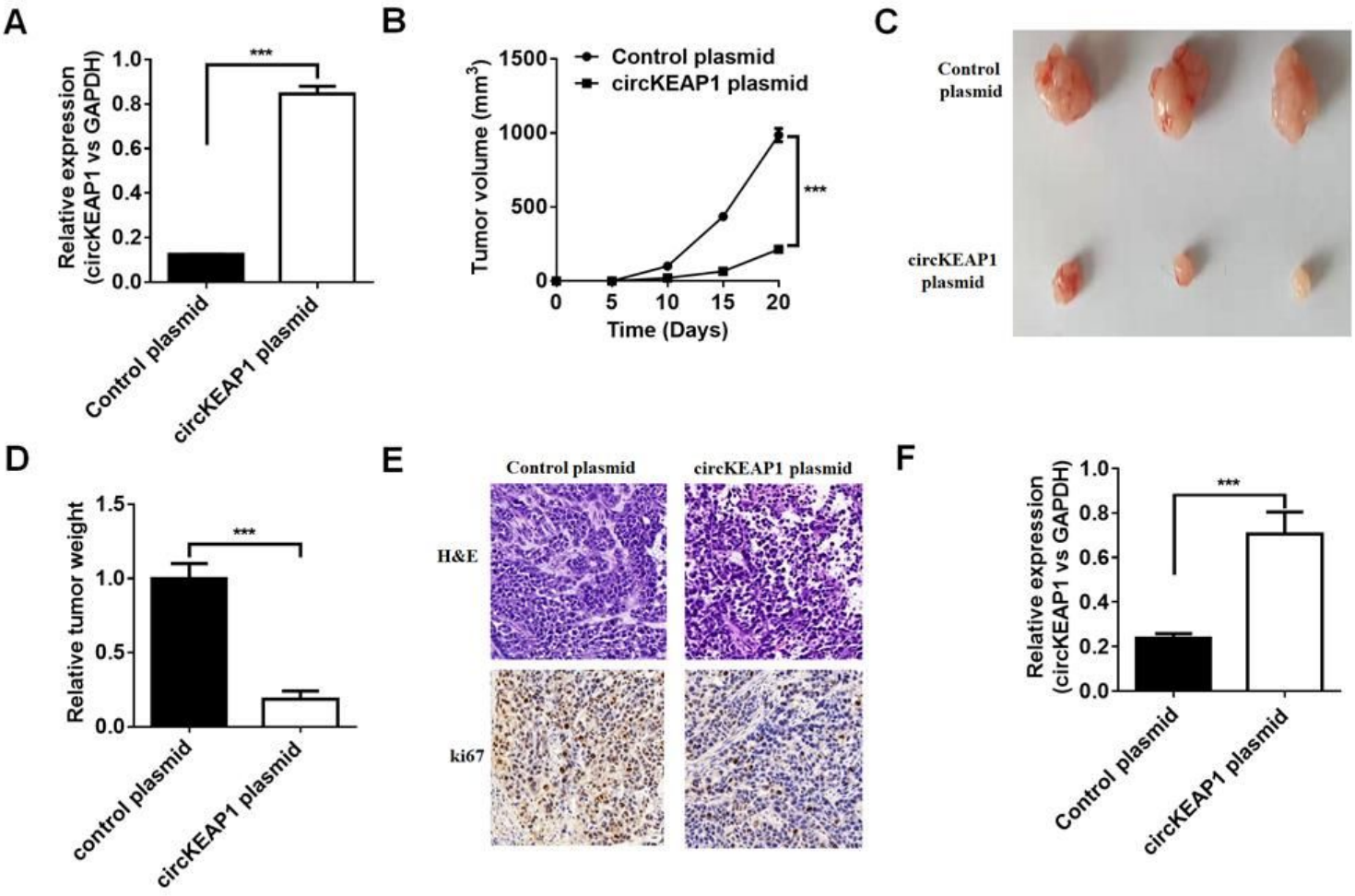

Figure 3

circKEAP1 inhibits tumor growth in vivo and in vivo. (A) The expression level of circKEAP1 in A549 cells transfected with control plasmid or circKEAP1 overexpression plasmid. (B) The volume of subcutaneous xenograft tumors of A549 cells isolated from nude mice. (C-D) The weight of subcutaneous xenograft tumors of A549 cells isolated from nude mice. (E) HE staining and IHC staining for Ki-67 in xenografted tumors. (F) Expression levels of circKEAP1 in xenografted tumors. Data are shown as the means ± standard error of the mean (n =3), statistical analysis was performed by two-tailed Student's t-test; *P < 0.05, **P < 0.01, ***P < 0.001.

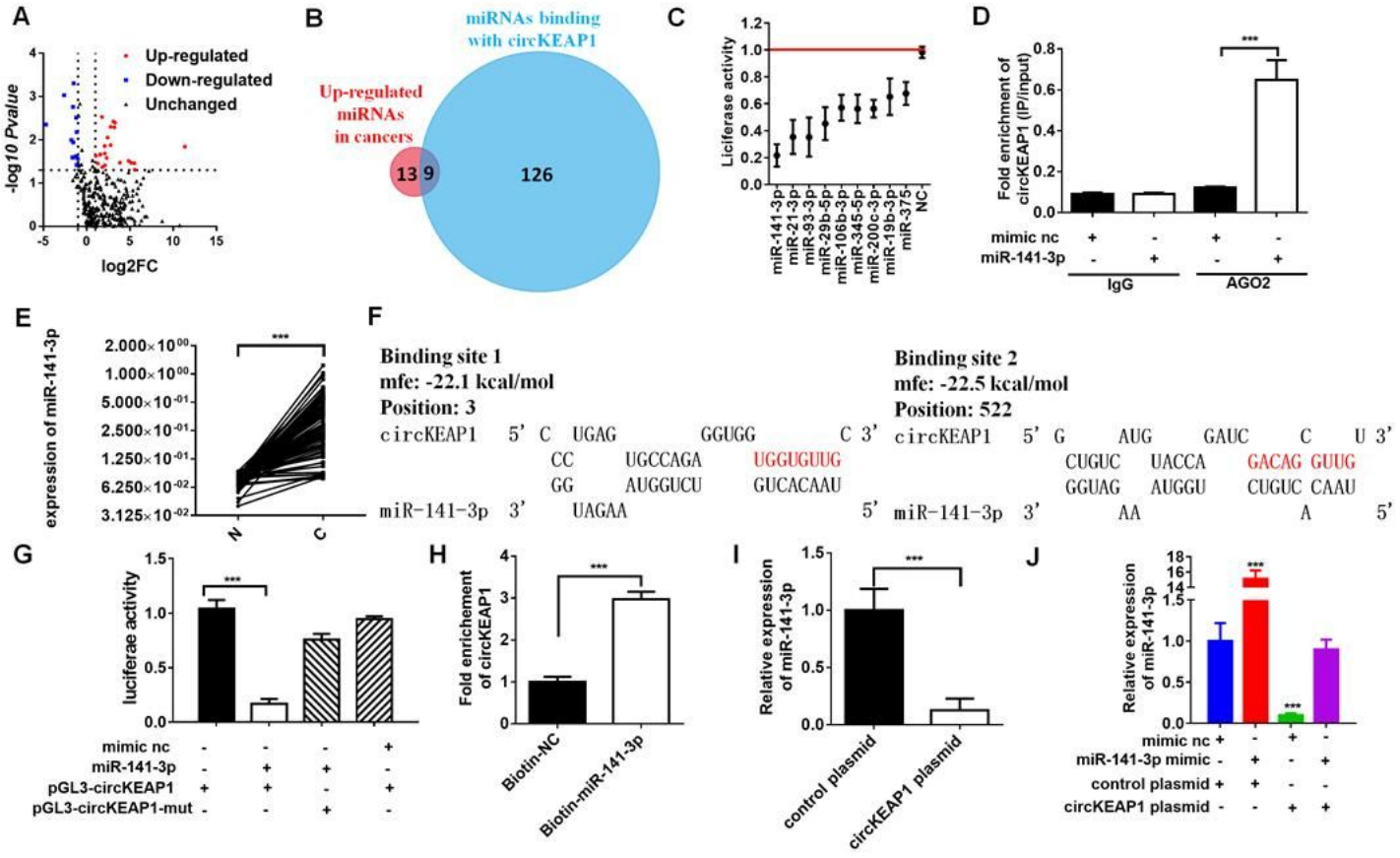

Figure 4

circKEAP1 acts as a sponge for miR-141-3p. (A) The volcano plot of miRNA in seven paired LUAD cancer tissues and adjacent normal tissues. (B) A schematic model shows the putative binding sites of 9 predicted miRNAs on circKEAP1. (C) Luciferase activity of circKEAP1 in A549 cells transfected with miRNA mimics which are putative binding to the circKEAP1 sequence. Luciferase activity was normalized by Renila luciferase activity. (D) RIP was performed using AGO2 antibody in BEAS-2B cells transfected with miR-141-3p mimics or mimics NC, then the enrichment of circKEAP1 was detected. (E) qRT-PCR for the abundance of miR-141-3p in 105 paired LUAD cancer tissues (C) and adjacent normal tissues (N). (F) The binding sites of miR-141-3p with circKEAP1 were predicated via targetScan. (G) Luciferase reporter activity of circKEAP1 in A549 cells co-transfected with miR-141-3p mimics and circKEAP1 luciferase reporter plasmid. (H) circKEAP1 was pulled down and enriched with 3'-end biotinylated miR-141-3p in BEAS-2B cells. (I) Expression levels of miR-141-3p in A549 cells treated with control plasmid and circKEAP1 plasmid. (J) Expression levels of miR-141-3p in A549 cells co-transfected with miR-141-3p mimics and circKEAP1 overexpression plasmid. Data are shown as the means ± standard error of the mean (n =3), statistical analysis was performed by two-tailed Student's t-test; *P < 0.05, **P < 0.01, ***P < 0.001.

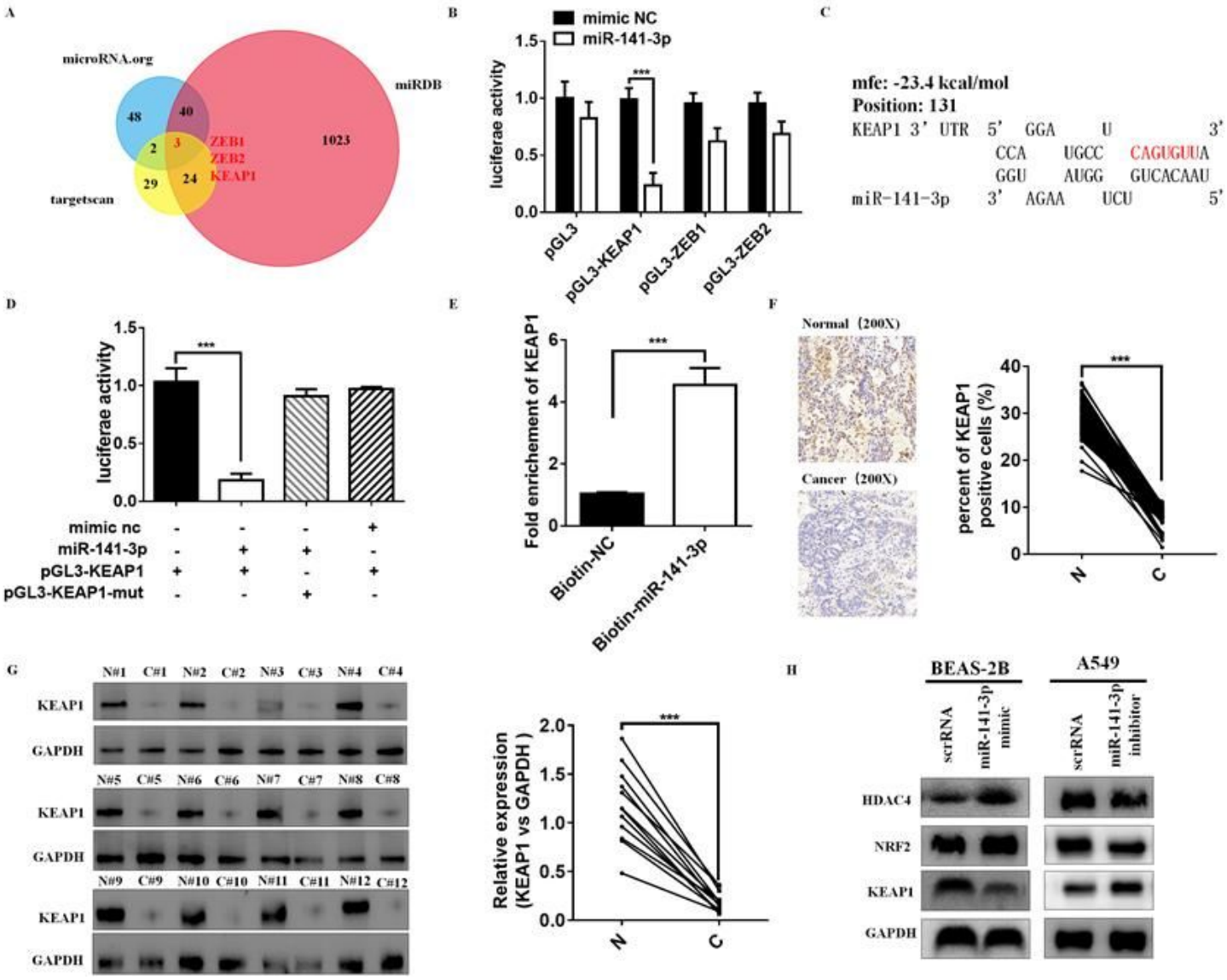

Figure 5

miR-141-3p inhibits KEAP1 expression. (A) Putative target genes of miR-141-3p were predicated by trargetScan, miRDB and miRDana. (B) Luciferase activity of miR-141-3p in A549 cells transfected with luciferase reporter plasmid containing the putative binding of target genes to miR-141-3p. Luciferase activity was normalized by Renila luciferase activity. (C) The binding sites of miR-141-3p with KEAP1 were predicated via targetScan. (D) Luciferase reporter activity of KEAP1 in A549 cells co-transfected with miR-141-3p mimics and KEAP1 luciferase reporter plasmid. (E) KEAP1 was pulled down and enriched with 3'-end biotinylated miR-141-3p in BEAS-2B cells. (F) Expression levels of KEAP1 protein in 105 paired LUAD cancer tissues (C) and adjacent normal tissues (N) by IHC staining. (G) Expression levels of KEAP1 protein in 12 paired LUAD cancer tissues (C) and adjacent normal tissues (N) by western blotting. (H) The protein levels of KEAP1 in BEAS-2B cells transfected with mimics of miR-141-3p and A549 cells transfected with inhibitors of miR-141-3p. Data are shown as the means ± standard error of the mean (n =3), statistical analysis was performed by two-tailed Student's t-test; *P < 0.05, **P < 0.01, ***P < 0.001.

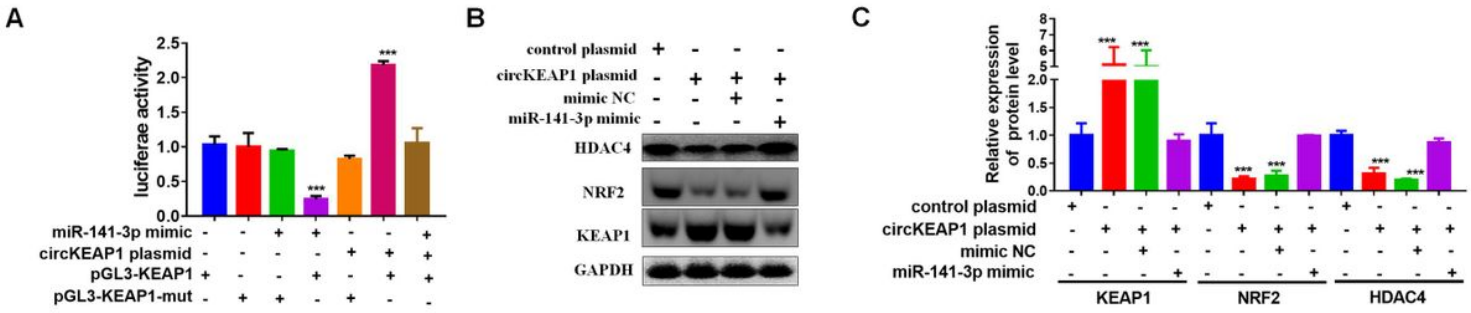

Figure 6

circKEAP1 regulate KEAP1 signaling pathway by sponging miR-141-3p. (A) Luciferase activity in A549 cells co-transfected with miRNA mimics or circKEAP1 overepression plasmid and luciferase reporter plasmid which have putative binding site of KEAP1 to miR-141-3p. Luciferase activity was normalized by Renila luciferase activity. (B-C) Western blot analysis of KEAP1, NRF2 and HDAC4 levels in A549 cells co-transfected with mimic nc or miR-141-3p mimic or circKEAP1 overexpression plasmid. Data are shown as the means ± standard error of the mean (n =3), statistical analysis was performed by two-tailed Student's t-test; *P < 0.05, **P < 0.01, ***P < 0.001.

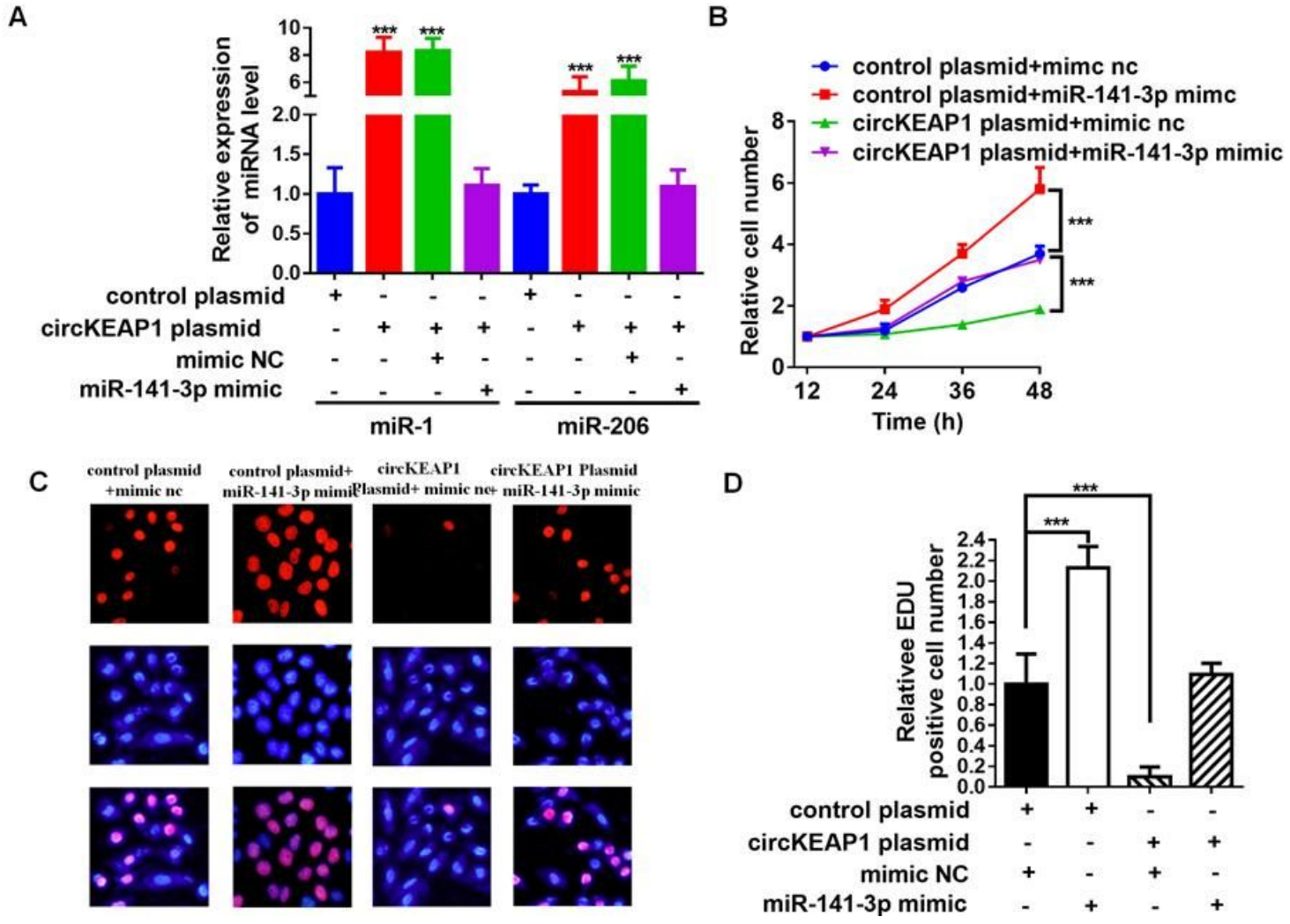

Figure 7

circKEAP1 regulate cell proliferation by sponging miR-141-3p. (A) The expression level of miR-1 and miR-206 in A549 cells co-transfected with mimic nc or miR-141-3p mimic or circKEAP1 overexpression plasmid. (B) Cell proliferation analysis for A549 cells co-transfected with mimic nc or miR-141-3p mimic or circKEAP1 overexpression plasmid by CCK-8 assay. (C-D) Cell proliferation analysis for A549 cells co-transfected with mimic nc or miR-141-3p mimic or circKEAP1 overexpression plasmid. Data are shown as the means ± standard error of the mean (n =3), statistical analysis was performed by two-tailed Student's t-test; *P < 0.05, **P < 0.01, ***P < 0.001.

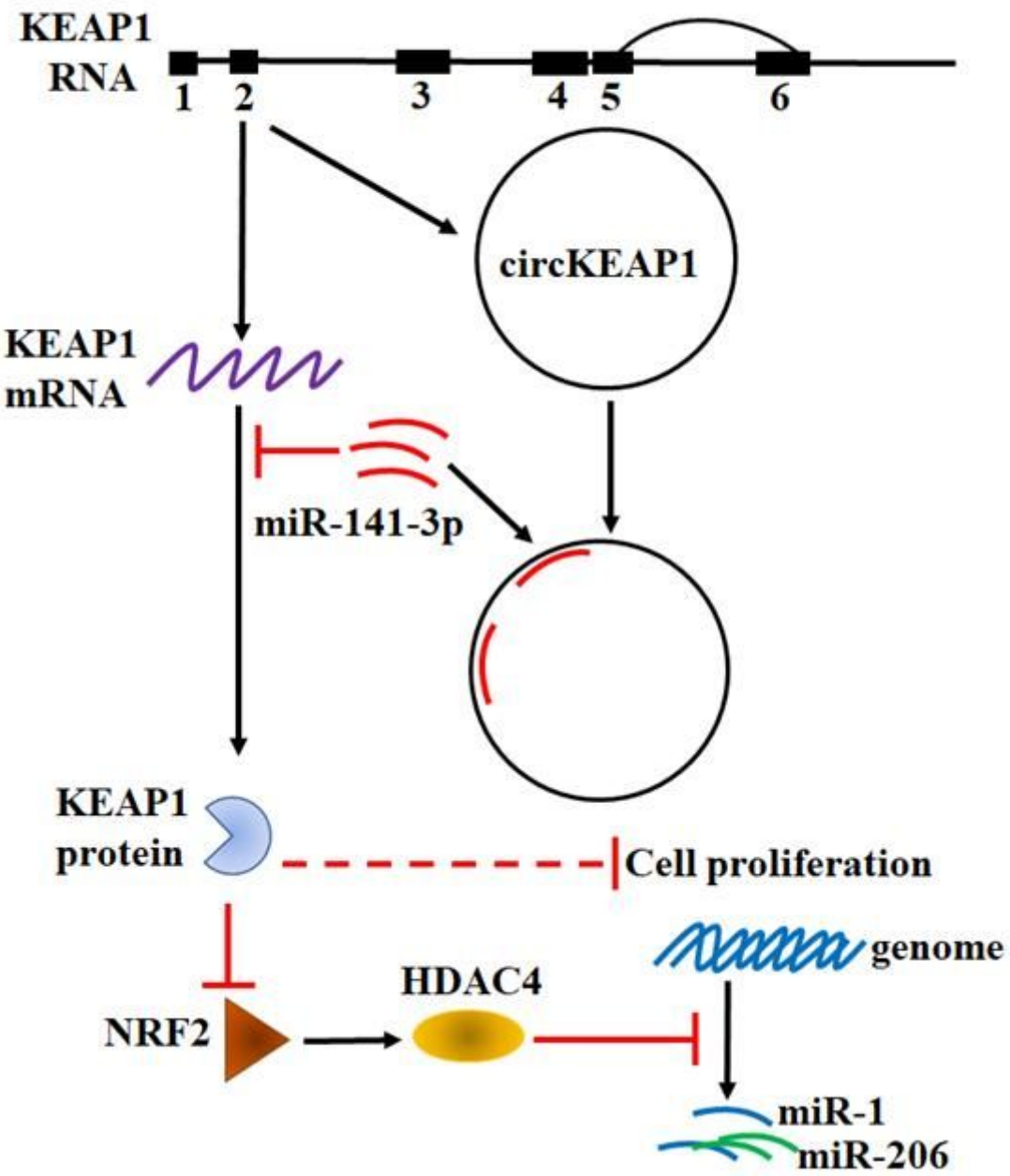

Figure 8

Hypothesis diagram illustrates function and mechanism of circKEAP1 in LUAD progress.

Supplementary Files

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