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Original Article

NUCKS1 exacerbates hepatocellular carcinoma cell proliferation and metastasis via the upregulation of Cdc42

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Abstract: Nuclear casein kinase and cyclin-dependent kinases substrate 1 (NUCKS1) is overexpressed in hepatocellular carcinoma (HCC), but its role and regulatory mechanism in the development and progression of HCC remains unknown. Here, we report that the RNA and protein levels of NUCKS1 were significantly increased in HCC tissues. The inhibition of NUCKS1 notably decreased the proliferation and migration of SNU449 and HepG2 cells. However, NUCKS1 overexpression exacerbated cell growth and migration. Additionally, NUCKS1 depletion reduced the sphere formation efficiency and inhibited tumorigenesis in vivo. Mechanistically, depletion of NUCKS1 downregulated the expression of cell division control protein 42 (Cdc42), and NUCKS1 directly bound to the promoter of Cdc42 and transcriptionally upregulated Cdc42, which promoted the development and progression of HCC. Furthermore, the expression of NUCKS1 was positively associated with Cdc42 in HCC tissues. Collectively, our data indicate that the increasing expression of NUCKS1 plays an oncogenic role and promotes progression via transactivation of Cdc42 expression in HCC.

Keywords: NUCKS1, HCC, Cdc42, cell growth

Introduction

Ninety percent of primary liver cancers are hepatocellular carcinomas [1, 2]. Among malignant tumours, hepatocellular carcinoma ranks sixth in incidence and third in mortality [3]. Despite the implementation of various advanced treatments, liver cancer patients have a poor prognosis [4, 5]. Therefore, elucidating the mechanisms underlying liver cancer occurrence and progression is essential. Moreover, the identification of new therapeutic targets is urgently needed for clinical treatment.

Nuclear casein kinase and cyclin-dependent kinase substrate 1 (NUCKS1) is a highly conserved vertebrate-specific protein [6]. The NUCKS1 gene is responsible for encoding a nucleoprotein, and during each cell cycle, NUCKS1 undergoes phosphorylation. Therefore, it is associated with DNA repair and cell growth and proliferation [7]. In vitro studies have shown that NUCKS1 is a second messenger-activated substrate kinase [8]. Increasing evidence indicates that NUCKS1 plays an essential role in cancer. For example, NUCKS1 promotes the progression of colorectal cancer through activation of the PI3K/AKT/mTOR axis [9]. NUCKS1 enhances the aggressiveness of gastric cancer cells via the upregulation of insulin-like growth factor 1 receptor (IGF-1R) [10]. Additionally, NUCKS1 promotes cell proliferation and suppresses autophagy via the mTOR-Beclin1 pathway in gastric cancer [11]. In lung cancer, NUCKS1 promotes cell proliferation and migration by upregulating CDK1 expression [12]. In osteosarcoma, NUCKS1 facilitates tumour progression by increasing asparagine synthesis and suppresses antitumour immunity through activation of CXCL8 expression [13, 14]. Additionally, NUCKS1 is associated with breast cancer development

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and represents a cancer biomarker [15, 16]. Moreover, NUCKS1 has been demonstrated to be overexpressed in skin cancer [17], cervical cancer [18], and ovarian cancer [19]. High expression of NUCKS1 in cervical squamous cell carcinoma is a clinicopathologic variable along with advanced International Federation of Gynaecology and Obstetrics stage, histologic grade, tumour size, lymph node metastasis and recurrence [18]. Recently, NUCKS1 was revealed to be upregulated in HCC tissues, and the expression of NUCKS1 was closely associated with tumour differentiation, tumour node metastasis stage, vascular invasion, and metastasis. HCC patients with high NUCKS1 expression have shorter survival than patients with low NUCKS1 expression [20, 21]. However, the role of NUCKS1 and the relevant mechanism are still unknown. Thus, we investigated NUCKS1 in order to elucidate its function and regulatory mechanism in HCC cells.

Cell division control protein 42 (Cdc42) is a Rho family small GTPase that regulates cell motility and the cell cycle while dysregulating Cdc42 [22]. Cdc42 has been shown to be associated with tumour metastasis [23, 24]. Previous studies have shown that Cdc42 expression in HCC can activate myosin II by downregulating epithelial growth factor receptor (EGFR) expression, which ultimately promotes HCC migration and invasion [25]. Furthermore, Cdc42 facilitates HCC cell invasion into surrounding tissue by inducing filopodium formation [26]. Therefore, Cdc42 is important for promoting tumour biological activity in HCC. The association between Cdc42 and HCC has been preliminarily established, but the regulatory mechanisms of Cdc42 in HCC remain to be further explored.

In this study, we found that NUCKS1 plays an oncogenic role in HCC. NUCKS1 knockdown decreased cell growth and migration *in vitro* and suppressed tumour formation *in vivo*. Further mechanical studies revealed that NUCKS1 bound to the promoter of Cdc42 and facilitated its expression in liver cancer, which led to increased cell proliferation and migration. In summary, we have further clarified the function of NUCKS1 in HCC and identified Cdc42 as a new target gene of NUCKS1.

Materials and methods

Cell culture

The HepG2 cell line was cultured in Dulbecco's modified Eagle medium (DMEM), and RPMI-1640 was used to culture SNU449 cells. Minimum Essential Medium (MEM) was used to culture Hep3B cells. The media contained 10% foetal bovine serum (VivaCell Biosciences, China, Lot: 2307029), 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. We used antibodies against GAPDH (CUSABIO, Wuhan, CHN; CSB-MA000071M0m, 1:1,000), NUCKS1 (Proteintech, 12023-2-AP, 1:1,000), and Cdc42 (Proteintech, 10155-1-AP, 1:1,000).

Lentiviral packaging and infection

The target sequences were cloned and inserted into the pLKO.1-puro vector to generate lentiviral shRNA constructs against human NUCKS1.

The shRNA sequences were as follows: NUCKS1#1: forward: 5-CCGG CATTTCTCTCTCTCTCTCTCTCTTTCTCGAGAAAGAGAGAGAGAAATGTTTTTG-3; reverse: 5-AATTCAAAAACATTTCTCTCTCTCTCTCTTTCTCGAG AAAGAGAGAGAGAGAGAAATG-3; and #2: forward: 5-CCGG GTTGTTGATTACTCACAGTTTCTCGAGAAACTGTGAGTAATCAACAACTTTTTG-3; reverse: 5-AATTCAAAAAGTTGTTGATTACTCACAGTTTCTCGAG AAACTGTGAGTAATCAACAAC-3.

Cdc42: forward: 5-CCGGCAGATGTATTTCTAGTCTGTTCTCGAGAACAGACTAGAAATACATCTGTTTTTG-3, reverse: 5-AATTCAAAAACAGATGTATTTCTAGTCTGTTCTCGAGAACAGACTAGAAATACATCTG-3. The NUCKS1 and Cdc42 lentiviral expression vectors were constructed as pCDH vectors. The primers used were as follows: Cdc42 forward: 5-ATGCAGACAATTAAGTGT-3, reverse: TTAGAATATACAGCACTTCC; NUCKS1 forward: 5-ATGTCGCGGCCTGTCAGA-3, reverse: 5-TTAATCCTCCCCAGAAGG-3. The pLKO.1 vector, pVSVG, pREV and pGAG or pCDH vector, psPax2, and pMD2G were cotransfected into 293T cells to generate lentiviruses. Then, we added the lentivirus to SNU449 or HepG2 cells to create a stable cell line. We subsequently evaluated the efficiency of gene

knockdown or overexpression via western blotting.

Quantitative reverse transcription PCR (qRT-PCR)

Our study used TRIzol (Invitrogen) to isolate total RNA. The PrimeScriptTM RT reagent kit (Takara, RR055A) was used for cDNA synthesis with 1 μg of total RNA as per the instructions. The primers used were as follows: Cdc42 forward: 5-CAGGGCAAGAGGATTATGACAG-3 and reverse: 5-GTTATCTCAGGCACCCACTT-3; GAPDH forward: 5-GCTGAGAACGGGAAGCTTGT-3; and reverse: 5-GCCAGGGGTGCTAAGCAGTT-3; ATXN7 forward: 5-TAAACCTAAACCTCACACCCC-3; reverse: 5-CACTGGATAACCGAGAAGCTG-3; FCGBP forward: 5-CTTCCTCTACAACGGCCAAG-3; reverse: 5-ACCACGACTTTACCCACATG-3. The gene expression levels were normalised with those of GAPDH. The $2^{-\Delta\Delta CT}$ method was used to identify alterations in gene expression.

Colony formation and cell migration assays

For the colony formation assay, SNU449 cells were inoculated into 6-well plates (5,000 cells/well) and incubated in 5% CO_2 at 37°C for 1 week, after which the growth media was replenished every 48 h for 1 week. The cell colonies were subsequently fixed with methanol for 15 min. After being washed with PBS, the cells were subjected to staining with 0.1% crystal violet for 15 min. Bio-Rad ChemiDoc XRS+ equipment was used to capture and quantify images of the cell colonies via ImageJ software.

For the cell migration assay, 20,000 cells were plated onto 24-well Transwell plates containing 8 mm polyethylene terephthalate membrane filters (Corning, 3422), suspended in 200 μL of serum-free medium, and transferred to the upper chamber. Then, 650 μL of complete medium containing 10% FBS was added to the lower chamber and incubated in 5% CO_2 at 37°C. The cells were treated with a 4% formaldehyde solution for 30 min to permeabilize the membrane and subjected to staining with 0.1% crystal violet for 20 min. A cotton swab was subsequently used to remove the upper layer of nonmigrating or noninvasive cells, and the cells were quantified via light microscopy.

Immunohistochemistry (IHC)

The HCC tissue microarrays with normal, adjacent, and cancerous tissues were purchased from Shanghai Otto Biotechnology Co. and contained 30 pairs of cancer tumours together with matched adjacent tissue and normal tissue (product code: HLivH090PG01). The clinical information of the patients can be downloaded from the website (https://www.superchip.com.cn/biology/tissue.html).

Sphere formation assay

The HepG2 and SNU449 cells with NUCKS1 knocked out or enriched for spheroids were cultured in serum-free DMEM-F12 (Gibco) containing B27 (1:50, 20 ng/mL EGF and bFGF). The use of untreated tissue culture flasks allowed us to decrease cell adhesion and facilitate the formation of undifferentiated tumour spheres. Then, we quantified the number of spheres with a diameter above 100 mm in each well following a two-week incubation period.

Chromatin immunoprecipitation (ChIP) test

The ChIP assay was conducted with an anti-NUCKS1 antibody and a kit according to the manufacturer's protocol (EZ-ChIP, 17-409 Millipore). Our study used qRT-PCR with anti-rabbit IgG as a control to elute and amplify the bound DNA fragments. The primers used for P2 were as follows: forward: 5-GTTCGAGACCAGCCTGGG-3; reverse: 5-GCGGGGTCTCCTCGGC-3.

RNA sequencing analysis

SNU449 cells with or without NUCKS1 knockdown were collected and subjected to BioMaker analysis. In brief, 1.0 μg of total RNA per sample was used as input material. RNA integrity was assessed via the RNA Nano 6000 Assay Kit (Agilent) on an Agilent 2100 Bioanalyzer. The ribosomal RNA was removed via the Ribo-Zero™ Gold Kit (Epicentre, USA), and sequencing libraries were generated from the rRNA-depleted RNA via the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB, USA). The libraries were sequenced on an Illumina NovaSeq platform to generate 150 bp paired-end reads according to the manufacturer's instructions. The raw reads

were further processed with BMKCloud (www.biocloud.net).

Gene expression levels were estimated by the number of fragments per kilobase of transcript per million fragments mapped. The formula is as follows: FPKM = cDNA Fragments Mapped Fragments (Millions) * Transcript Length (kb). Differential expression analysis of two samples was performed via edge R. An FDR < 0.01 and a fold change ≥ 2 were set as the thresholds for significantly differential expression. Gene set enrichment was performed via MSigDB with specific gene set collections (hallmarks, cancer modules, and GO).

Promoter reporter and dual luciferase assays

To insert the Cdc42 promoter, a pGL3-basic vector was used. The following primers were used: P1 forward: 5-gcGGTACC CCCAGCTACTTGGGAG-3; reverse: 5-gc GTCGAC GCGGGGTCTCCTCGGC-3; P2 forward: 5-gc GGTACC GTTCGAGACCAGCCTGGG-3; reverse: 5-gc GTCGAC GCGGGGTCTCCTCGGC-3; P3 forward: 5-gcGGTACC CCCAGCTACTTGGGAG-3; reverse: 5-gc GTCGAC CCGTGACCTCAAGTAAG-3. After transfection, the luciferase activity was measured in a 1.5 mL Eppendorf tube with a Promega Dual-Luciferases Reporter Assay Kit (Promega E1980). The relative activity of Renilla luciferase was set to match that of firefly luciferase.

In vivo tumorigenesis test

The animal experiments were approved by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University, Ethics Approval No. YJSDW2023-227, and the animal studies were conducted according to the Animal Research: Reporting of In Vivo Experiments. The NOD SCID female mice (4-6 weeks, 18-20 g) acquired from Beijing Vital River Laboratory Animal Technology Co. Ltd. were randomly divided into the indicated groups (sex had no effect on the outcome). Subcutaneous injections of 5×10^6 of the indicated HepG2 cells, which were resuspended in 100 μL of phosphate-buffered saline (PBS), were administered to NOD SCID female mice. Twenty-five or thirty days following injection, all the animals were sacrificed by cervical dislocation, and the tumours that had been transplanted were collected, weighed, and divided in half for subsequent analysis.

Bioinformatics analysis

The NUCKS1 RNA from the RNA-Seq data of normal samples from noncancer patients and primary tumours or paired tumour and adjacent tissues was analysed via TNMplot (https://tnmplot.com). The NUCKS1 RNA and protein levels were also analysed via the UALCAN database. The association between NUCKS1 expression and Cdc42 in human HCC tissues was also analysed. The TCGA database (https://portal.gdc.com) was accessed to acquire RNA-seq data and related clinical data on liver cancer tumours. P < 0.05 indicated statistical significance.

Statistics and data analysis

All the statistical analyses were conducted with GraphPad Prism 5, and the data are expressed as the means ± SDs. Dunnett's least significant difference (LSD) test was used for multiple comparisons between the treatment groups and the control groups. P < 0.05 indicated statistical significance.

Results

NUCKS1 is highly expressed in HCC tissues

To investigate the role of NUCKS1 in HCC, we first used the TNM plot and UALCAN databases to analyse NUCKS1 expression levels in tumour and normal tissues. Compared with that in normal tissues, NUCKS1 expression levels were significantly increased in HCC tissues (**Figure 1A-D**). Next, we analysed the correlation between NUCKS1 expression and clinicopathologic variables in patients with HCC. The results revealed that high expression of NUCKS1 was related to sex and tumour grade (**Figure 1E**, **1F**). To further confirm this finding, we investigated NUCKS1 expression via immunohistochemistry (IHC) using serial sections of tissue microarrays. Consistently, NUCKS1 was highly expressed in HCC tissues (**Figure 1G**, **1H**).

NUCKS1 promotes cell proliferation and migration in vitro and in vivo

To assess the effects of NUCKS1 on the growth and migration of HCC cells, we first downregu-

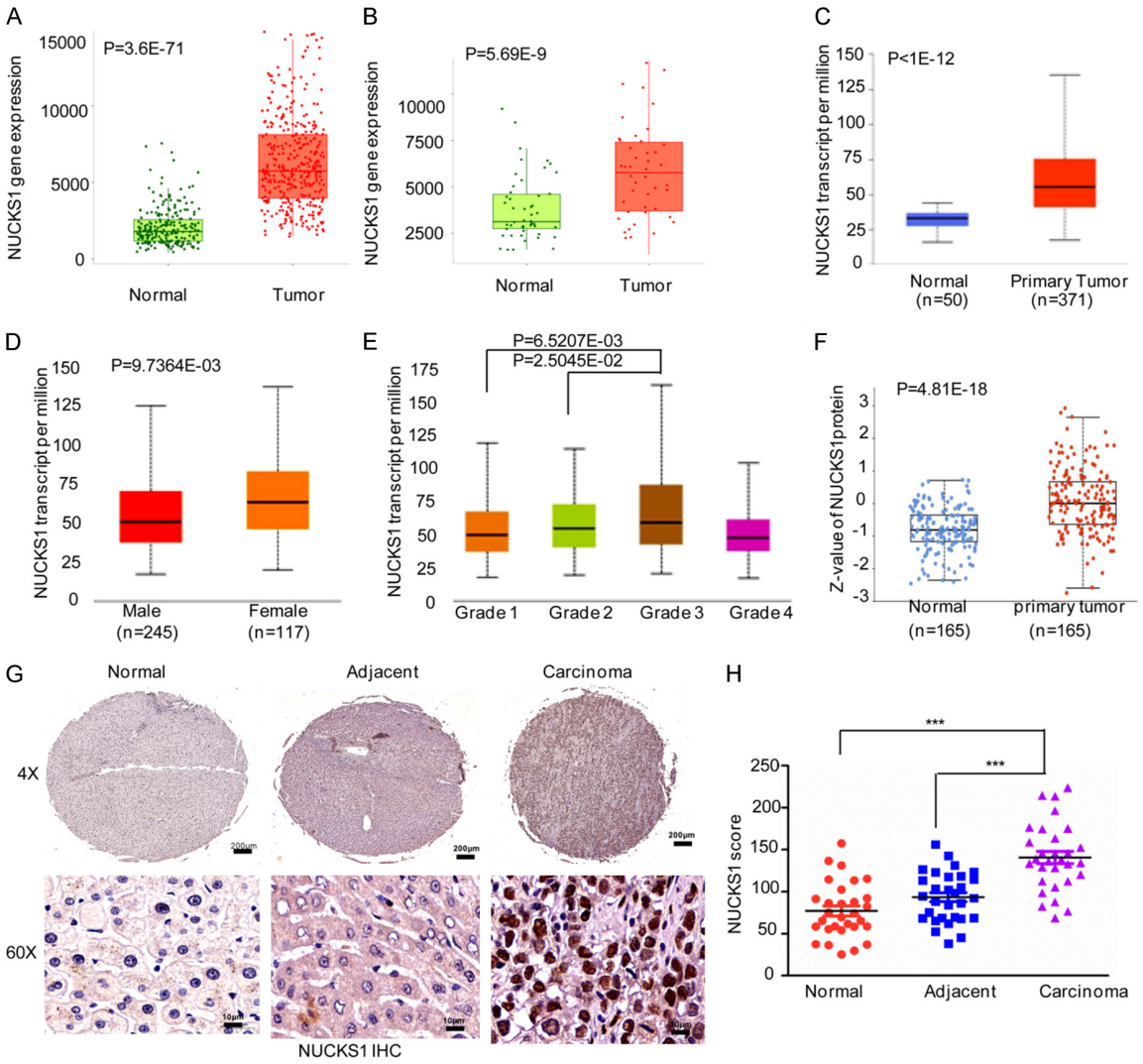
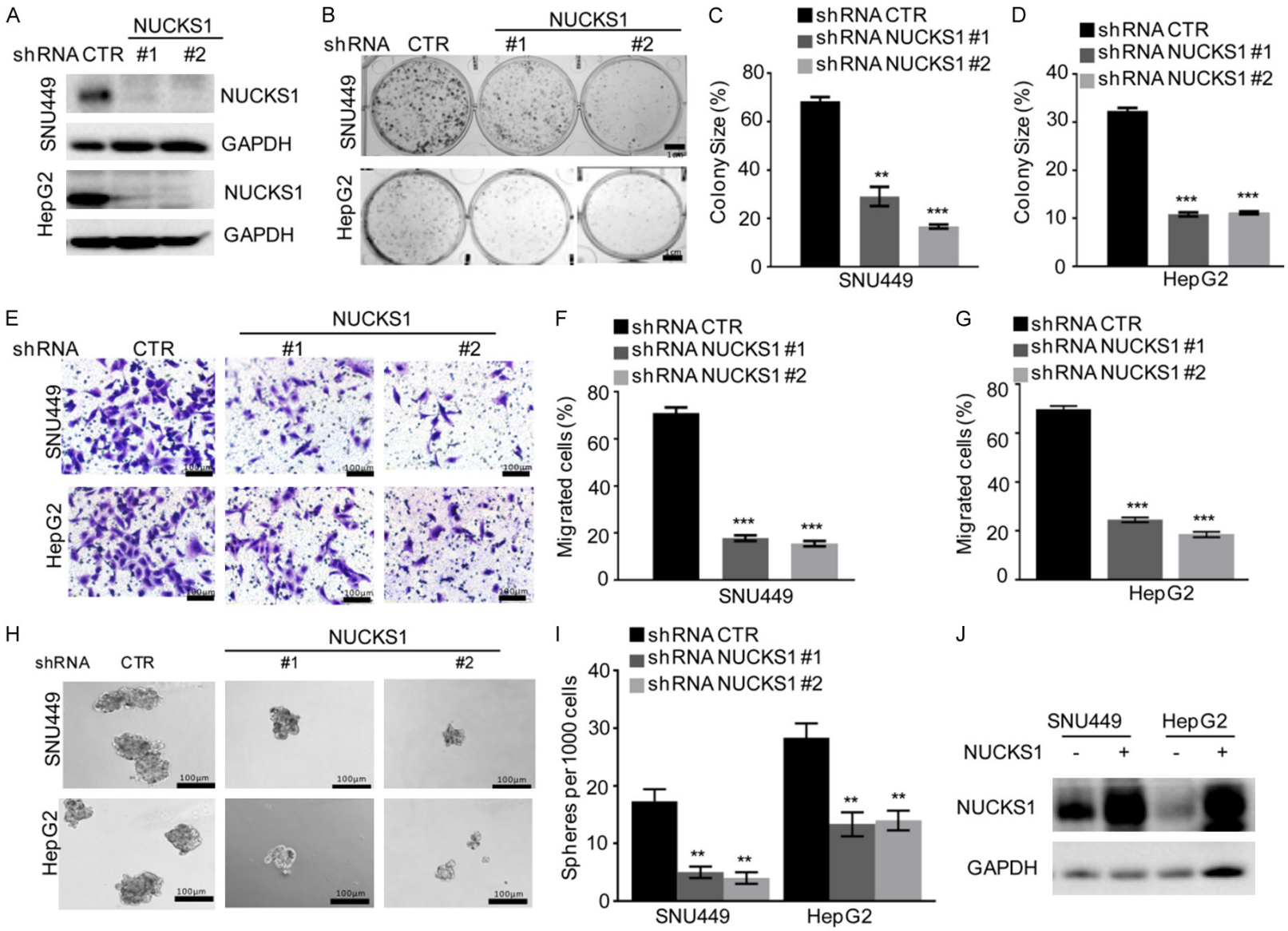


Figure 1. NUCKS1 is highly expressed in HCC tissues. A. Analysis of NUCKS1 expression in HCC (n = 371) and normal tissues (n = 225) from the TNM plot database. B. NUCKS1 expression in paired tumour and adjacent normal tissues (n = 50) was analysed via the TNM plot database. C. NUCKS1 expression levels in HCC (n = 371) and normal tissues (n = 50) were analysed via the UALCAN database. D. NUCKS1 protein expression levels in HCC (n = 165) and normal tissues (n = 165) were analysed via the UALCAN database. E, F. Analysis of the relationships among NUCKS1 expression, patient sex, and tumour grade from the UALCAN database. G, H. Representative NUCKS1 IHC images of normal (n = 30), adjacent (n = 30), and HCC tissue (n = 30) sections. The data were analysed via Student's t test. ***P < 0.001.

lated NUCKS1 expression in SNU449 and HepG2 cells via the use of two independent shRNAs. As shown in **Figure 2A**, the NUCKS1 expression level was significantly lower in the NUCKS1 shRNA group than that in the control group. The colony formation assay results revealed that NUCKS1 depletion dramatically decreased cell proliferation (**Figure 2B-D**). Similar results were obtained in HepG3B cells (Supplementary Figure 1A-C). The subsequent Transwell cell migration assay revealed that NUCKS1-knockdown cells migrated to a significantly lower extent than the control cells (**Figure 2E-G**). Further sphere-formation assays also revealed that NUCKS1 knockdown significantly decreased the sphere-forming efficiency of SNU449 and HepG2 cells (**Figure 2H**, **2I**). On the other hand, NUCKS1 overexpression promoted cell growth and migration (**Figure 2J-N**). In addition, we used a nude mouse model to implant HepG2 cells depleted of NUCKS1 or not. Compared with those in the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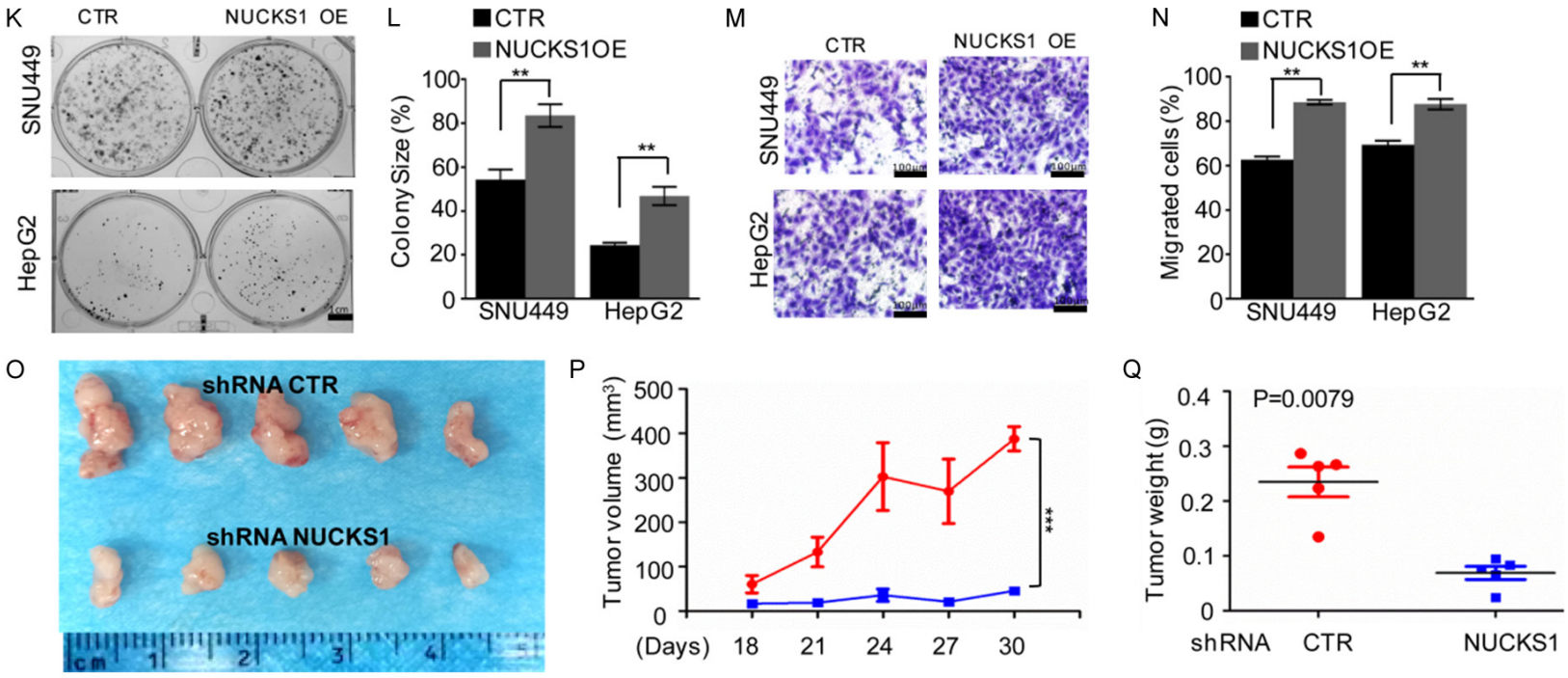


Figure 2. NUCKS1 knockdown inhibits HCC cell proliferation and migration *in vitro* and *in vivo*. (A) NUCKS1 protein levels were verified by western blotting in SNU499 and HepG2 cells with or without NUCKS1 knockdown. The shRNA CTR represented the control group and the shRNA NUCKS1#1, #2 represented two NUCKS1 depleting groups. (B-D) Effects of NUCKS1 knockdown on SNU449 and HepG2 cell growth were detected through a colony formation assay. (E-G) Detection of the effects of NUCKS1 knockdown on SNU449 and HepG2 cell migration via a Transwell assay. (H, I) Analysis of the sphere-forming capacities of SNU449 and HepG2 cells with or without NUCKS1 knockdown. (J) NUCKS1 protein levels were verified by western blotting in SNU499 and HepG2 cells with or without NUCKS1 overexpression. (K-N) Effects of NUCKS1 overexpression on cell growth and migration. (O-Q) Analysis of the tumour volume and weight of HepG2 cells with and without NUCKS1 knockdown. The data in (C, D, F, G, I, L) and N represent three independent experiments and were analysed via Student's t test, **P < 0.01, ***P < 0.001.

control group, HepG2 xenograft tumour growth was significantly suppressed by NUCKS1 depletion, as evidenced by decreased tumour weight and size (**Figure 2O-Q**). The expression levels of NUCKS1 in these tumors were detected by western blot (Supplementary Figure 1D). Collectively, our data show that NUCKS1 plays an oncogenic role in HCC and facilitates the malignancy of cancer cells.

NUCKS1 upregulates Cdc42 expression in SNU449 and HepG2 cells

To elucidate the potential molecular mechanism by which NUCKS1 enhances SNU449 and HepG2 cell proliferation and metastasis, RNA sequencing was performed to identify genes with altered expression between NUCKS1-proficient and NUCKS1-deficient SNU449 cells. Under the screening criteria of an absolute log2-fold change > 1 or < -1 and a q value < 0.05, 126 genes were downregulated, and 385 genes were upregulated in NUCKS1-knockdown cells (**Figure 3A**, **3B**). Among the 126 downregulated genes, we screened the downstream genes of NUCKS1 under the criteria of log2-fold change < -2 which excluded the new genes and long non-coding RNAs. As shown in **Figure 3C**, we obtained 11 significantly downregulated genes. Then, we analyzed the correlation between the 11 downregulated genes and NUCKS1 using the GEPIA database. We found that the downregulated genes ATXN7, FCGBP, and Cdc42 were positively associated with NUCKS1 in HCC (Supplementary Figure 2A-K). Furthermore, we investigated the expression levels of ATXN7, FCGBP and Cdc42 in HCC using the UALCAN databases. We obtained that the RNA and protein levels of FCGBP and Cdc42 were significantly upregulated in HCC tissues. However, the RNA level and protein level of ATXN7 was not consistent. ATXN7 protein level was not increased in HCC tissue (Supplementary Figure 3A-F). Meanwhile, the qRT-PCR results showed that Cdc42 not ATXN7 and FCGBP was significantly downregulated when NUCKS1 was knockdown in SNU449 and HepG2 cells (**Figure 3D**). Based on these data, we thus chose the Cdc42 as the downstream gene of NUCKS1.

To confirm the relationship between NUCKS1 and Cdc42, we first assessed the protein levels of Cdc42 in SNU449 and HepG2 cell lines with or without NUCKS1 knockdown and found that depletion of NUCKS1 decreased the protein levels of Cdc42 (**Figure 3E**). To further examine this effect, we also elevated NUCKS1 in SNU449 and HepG2 cells and found that NUCKS1 overexpression increased Cdc42 expression (**Figure 3F**, **3G**). Taken together, our results indicate that NUCKS1 promotes Cdc42 expression in SNU449 and HepG2 cells.

NUCKS1 binds to the Cdc42 promoter in SNU449 and HepG2 cells

To further investigate whether NUCKS1 transcriptionally upregulated Cdc42, the upstream promoter sequence of Cdc42 was cloned and inserted into a luciferase reporter plasmid named P1 (**Figure 4A**). After that, P1 was transfected into SNU449 and HepG2 cells with or without NUCKS1 knockdown, and the luciferase activity of the Cdc42 promoter was subsequently measured. As shown in **Figure 4B**, **4C**, NUCKS1 depletion reduced Cdc42 promoter (P1) luciferase activity. To identify the key region for the upregulation of Cdc42 expression by NUCKS1, two truncations of the Cdc42 promoter were inserted into the pGL3 basic vectors P2 and P3 (**Figure 4D**). Then, P2 and P3 were introduced into SNU449 cells with or without NUCKS1 depletion, and the luciferase activity was measured. We found that NUCKS1 inhibition reduced the luciferase activity of P2 but had no effect on P3, indicating that NUCKS1-mediated transactivation of Cdc42 expression relies on the P2 region (**Figure 4E**). Additionally, ChIP assays demonstrated that the number of chromatin fragments harbouring P2 exhibited a distinct increase in anti-NUCKS1 immunoprecipitation. Moreover, the ability of NUCKS1 to bind to the Cdc42 promoter was diminished in NUCKS1-depleted SNU449 cells (**Figure 4F**). Taken together, these results indicate that NUCKS1 binds to the Cdc42 promoter and transactivates its expression in SNU449 and HepG2 cells.

NUCKS1 promotes cell proliferation and metastasis by upregulating Cdc42 expression

To evaluate whether NUCKS1 enhances cell proliferation and metastasis by regulating Cdc42 expression, we first overexpressed Cdc42 in NUCKS1-depleted HCC cells and found that the overexpression of Cdc42 reversed the decrease in cell growth and migra-

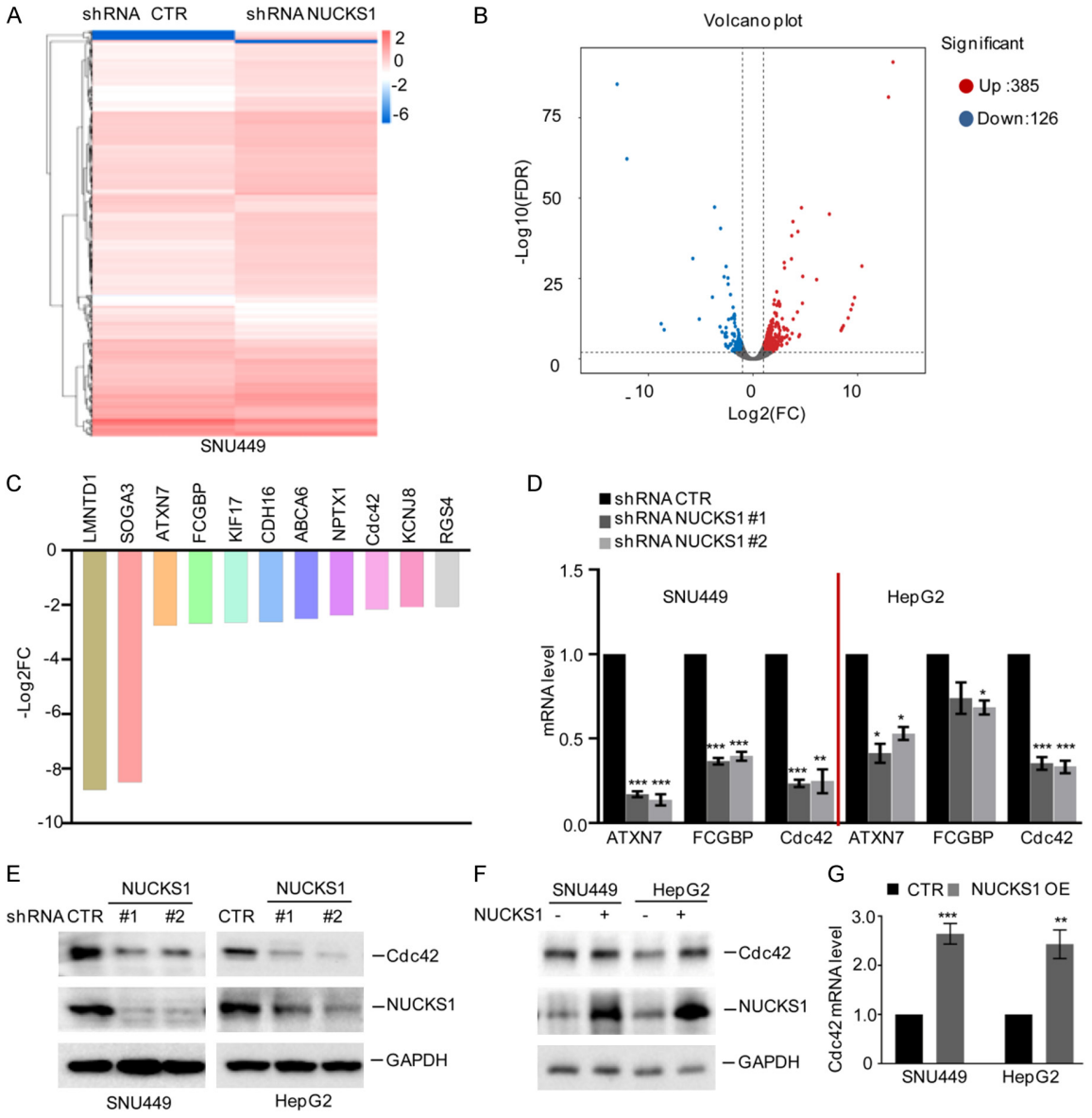


Figure 3. NUCKS1 transcription upregulates Cdc42. (A, B) RNA sequencing analysis of differentially expressed genes in NUCKS1-knockdown SNU449 cells. (C) The downregulated candidate genes in NUCKS1 depleting SNU449 cells were listed (log2FC < -2, P < 0.05). (D) The RNA levels of ATXN7, FCGBP and Cdc42 were assessed by qRT-PCR in NUCKS1 depleting SNU449 and HepG2 cells. (E) Western blotting was used to detect Cdc42 protein expression in SNU449 and HepG2 cells with NUCKS1 knockdown via shRNAs. (F, G) NUCKS1 was overexpressed in SNU449 and HepG2 cells. NUCKS1 and Cdc42 expression levels were detected via western blotting and qRT-PCR. The data in (D) and (G) represent three independent experiments and were analysed by Student's t test, *P < 0.05, **P < 0.01, ***P < 0.001.

tion induced by NUCKS1 knockdown (**Figure 5A-E**). To confirm this observation, we knocked down Cdc42 expression in SNU449 and HepG2 cells without NUCKS1 overexpression. The NUCKS1 and Cdc42 expression levels were analysed via western blotting (**Figure 5F**), and changes in cell proliferative and metastatic capacity were detected via colony formation and Transwell assays. We found that the knockdown of Cdc42 weakened NUCKS1 function and abolished NUCKS1-promoted proliferation and migration (**Figure 5G-J**). Consistently, *in vivo* tumour formation assays revealed that Cdc42 knockdown impaired NUCKS1 overexpression-induced HepG2 xenograft tumour growth (**Figure 5K-M**). The expression levels

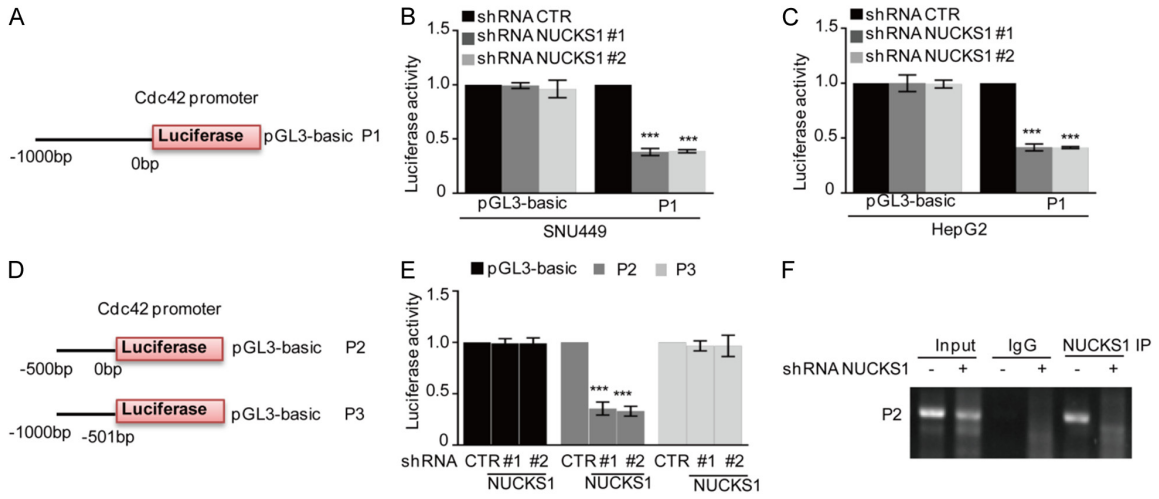


Figure 4. NUCKS1 transcriptionally upregulates Cdc42 expression. (A) A luciferase assay was used to determine the transcriptional activity of Cdc42 using pGL3-based reporter constructs. (B, C) Luciferase activity was measured in SNU449 and HepG2 cells with or without NUCKS1 depletion after the transfection of the Cdc42 promoter P1 and the pGL3 vector. (D) A luciferase assay was used to determine the transcriptional activity of Cdc42 via the use of pGL3-based reporter constructs named P2 and P3. (E) Luciferase activity was measured after the transfection of the pGL3 vector and the Cdc42 promoter P2/3 into SNU449 cells with or without NUCKS1 knockdown. (F) In SNU449 cells, the NUCKS1 antibody bound to the Cdc42 promoter, as shown by ChIP analysis. The negative control was an isotype-matched IgG. The data in (B-E) represent three independent experiments and were analysed by Student's t test, ***P < 0.001.

of NUCKS1 and Cdc42 in HepG2 xenograft tumour were also analyzed by western blot (Supplementary Figure 4). Overall, NUCKS1 promoted cell proliferation and metastasis through increasing Cdc42 expression.

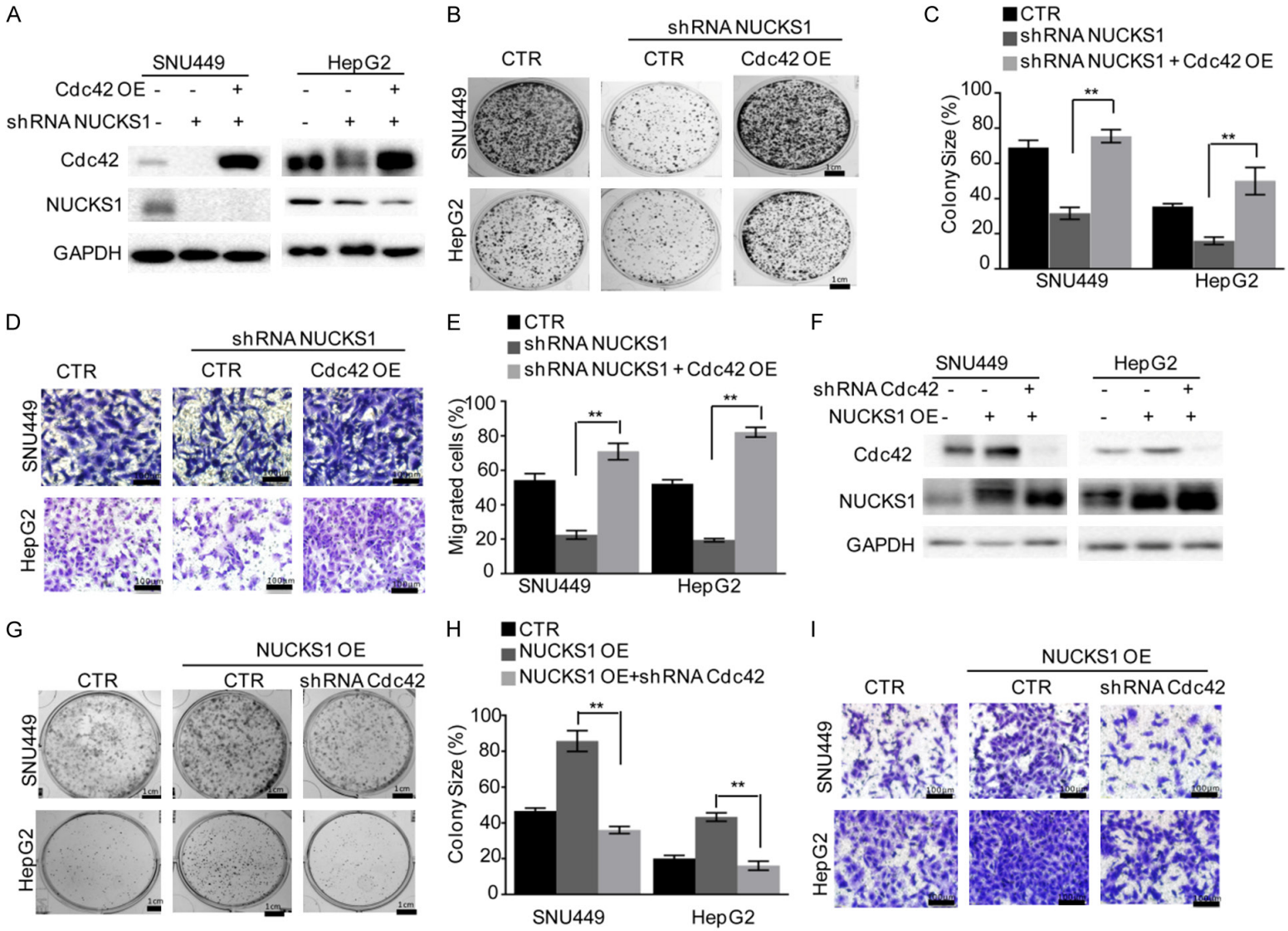
High expression of Cdc42 is positively correlated with NUCKS1 upregulation in HCC tissues

To further verify the role of the NUCKS1/Cdc42 axis in HCC, we first investigated NUCKS1 expression via IHC using serial sections of tissue microarrays. The IHC results revealed that Cdc42 was highly expressed in HCC tissues compared with normal and adjacent tissues (**Figure 6A**, **6B**). The correlation between NUCKS1 and Cdc42 was subsequently verified by IHC staining. The results revealed that Cdc42 was positively correlated with NUCKS1 in HCC (**Figure 6C**, **6D**).

Discussion

HCC is a common malignancy and one of the leading causes of cancer-related death. The development of HCC is complex, and multiple factors are reportedly involved in this progression [27]. Copy number aberrations are particularly important in HCC, where amplification of oncogenes and/or deletion of tumour suppressor genes are involved in the formation of HCC [28, 29]. Increasing evidence indicates that the amplification of chromosome 1q can be found in 58-78% of patients with HCC [30, 31]. NUCKS1, a high-mobility protein family member, is located on chromosome 1q32.1, and the copy number of NUCKS1 in HCC is increased, which leads to the upregulation of NUCKS1 expression. High expression of NUCKS1 is associated with poor prognosis [21, 32, 33]. However, its role and relevant mechanism are still unclear. Our study is the first to reveal the mechanism by which NUCKS1 promotes proliferation and metastasis in HCC cells, revealing that NUCKS1 expression is increased in HCC tissues and that elevated NUCKS1 exacerbates HCC metastasis and proliferation by upregulating the expression of Cdc42.

NUCKS1 is widely expressed in all mammalian tissues [33]. The functions of NUCKS1 are related to the regulation of chromatin structure and transcription, DNA repair, and cell cycle regulation [6]. Higher NUCKS1 expression has been detected in tumours than in adjacent normal tissues, such as breast cancer [15]. In addition, elevated NUCKS1 expression in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B
shRNA NUCKS1
CTR
CTR
Cdc42 OE
SNU449
HepG2
C
CTR
shRNA NUCKS1
shRNA NUCKS1 + Cdc42 OE
Colony Size (%)
100
80
60
40
20
0
**
**
SNU449
HepG2
D
shRNA NUCKS1
CTR
CTR
Cdc42 OE
SNU449
HepG2
E
CTR
shRNA NUCKS1
shRNA NUCKS1 + Cdc42 OE
Migrated cells (%)
100
80
60
40
20
0
**
**
SNU449
HepG2
F
SNU449
HepG2
shRNA Cdc42 - - + - - +
NUCKS1 OE - + + - + +
Cdc42
NUCKS1
GAPDH
G
NUCKS1 OE
CTR
CTR
shRNA Cdc42
SNU449
HepG2
H
CTR
NUCKS1 OE
NUCKS1 OE+shRNA Cdc42
Colony Size (%)
100
80
60
40
20
0
**
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SNU449
HepG2
I
NUCKS1 OE
CTR
CTR
shRNA Cdc42
SNU449
HepG2

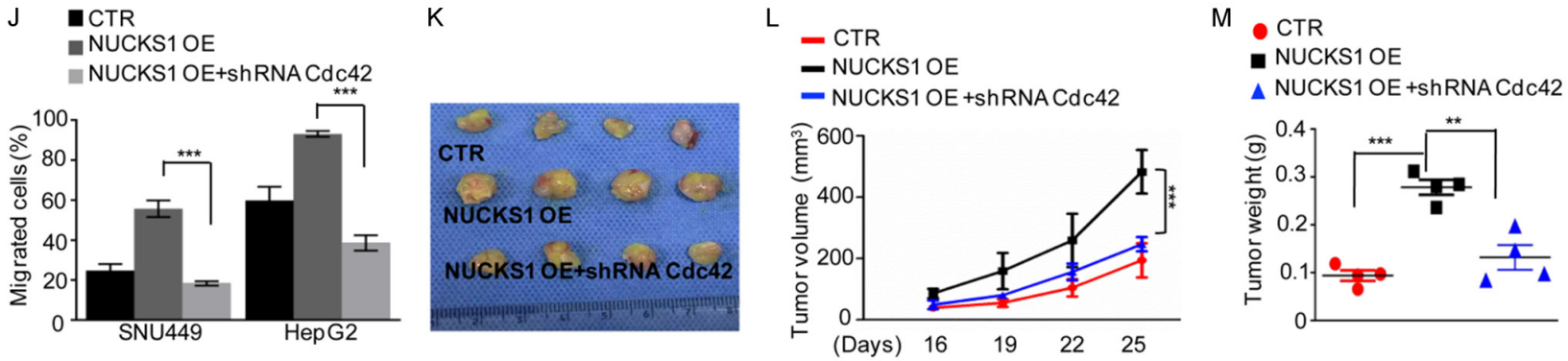

Figure 5. NUCKS1 promotes cell proliferation and metastasis by upregulating Cdc42 expression. (A) Western blotting was used to analyse Cdc42 and NUCKS1 expression. (B-E) Colony formation and Transwell assays were used to detect changes in the capacity of the cells to proliferate and migrate. (F) Western blotting was used to analyse Cdc42 and NUCKS1 expression. (G-J) Colony formation and Transwell assays were used to detect changes in the capacity of the cells to proliferate and migrate. (K-M) The tumour volume and weight of the indicated HepG2 cells were detected. The data in (C, E, H and J) represent three independent experiments and were analysed by Student's t test; *P < 0.05, ***P < 0.001.

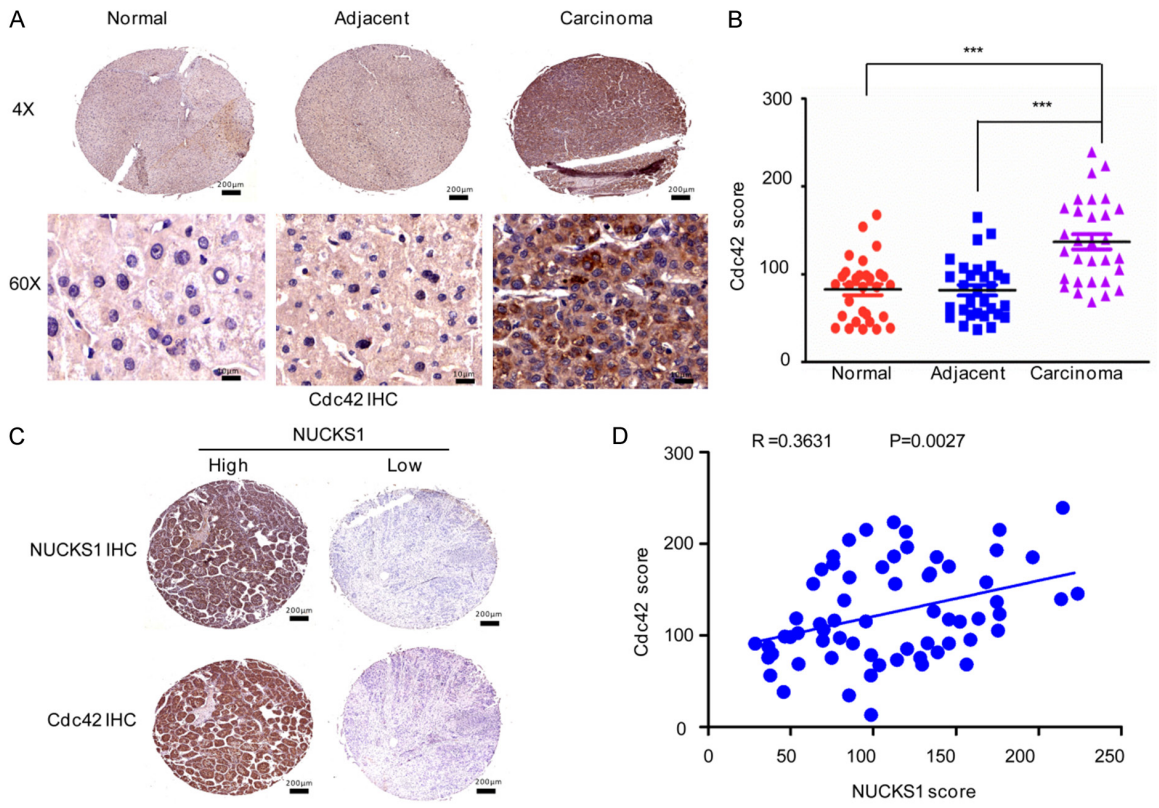

Figure 6. Cdc42 overexpression in HCC tissues is positively correlated with NUCKS1 expression. A, B. Representative Cdc42 IHC images of normal (n = 30), adjacent (n = 30), and HCC tissue (n = 30) sections. C, D. Analysis of the correlation between NUCKS1 and Cdc42 in HCC tissues via IHC staining. The data were analysed via Student's t test. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with the control.

tumour tissues increases tumour cell value [6]. NUCKS1 expression has been shown to be associated with cervical squamous cell carcinoma progression and recurrence [18]. These previous reports are consistent with our results. Previous studies also indicated that the expression levels of NUCKS1 were increased in HCC tissues. In our study, we confirmed that NUCKS1 expression, when reduced, can hinder tumour cell growth and metastasis by knocking down NUCKS1 expression both *in vitro* and *in vivo*. Given that NUCKS1 functions to promote tumour growth by regulating downstream genes, NUCKS1 has been shown to increase gastric cancer cell tumour activity via PI3K/AKT/mTOR pathway activation [10]. NUCKS1 promoted cell proliferation and migration by upregulating CDK1 expression [9]. Recently, NUCKS1 has been reported to transcriptionally upregulate ASNS and CXCL8 in osteosarcoma, which facilitated tumour progression and suppressed antitumour immunity [14]. Moreover, NUCKS1 enhanced SKP2 transcription and controlled S phase entry [34]. To further explore the mechanism by which NUCKS1 enhances HCC cell proliferation and metastasis, we identified the genes downstream of NUCKS1 via RNA sequencing. Our results revealed that NUCKS1 upregulated the protein and mRNA levels of Cdc42 in HCC cells.

The Cdc42 gene belongs to the Rho family of small GTPases that control cell motility, polarity, and cell cycle progression and is overexpressed in several other malignancies [22, 35]. In addition, Cdc42 can promote the metastasis and proliferation of HCC [36]. Decreasing the expression of Cdc42 inhibits the oncologic activity of HCC cells [37]. In our experiments, we first demonstrated that decreased expression of NUCKS1 could directly downregulate Cdc42 expression. In addition, NUCKS1 was observed to bind the Cdc42 promoter, thereby upregulating Cdc42 expression in HCC and promoting HCC cell proliferation. Finally, knocking down Cdc42 impaired NUCKS1 function in

HCC cells and attenuated its promotion of tumour activity in HCC cells, which further suggested that NUCKS1 promoted tumour activity in HCC by upregulating Cdc42.

Increasing evidences suggested that many genes involved in the regulation of NUCKS1 expression. For example, long non-coding RNA CBR3-AS1could elevate the protein levels of NUCKS1 by sponging miR-140-5p and elevated the mRNA stability of NUCKS1 via recruiting DDX54 [38]. Circular RNA circATP9A promoted NUCKS1 expression by elevating its mRNA stability [39]. Recent study indicated that NUCB2 interacted with NUCKS1 and suppressed its degradation [14]. However, the mechanism of NUCKS1upregulation is unknown and need to be investigated in future.

In conclusion, our study shows that NUCKS1 promotes the tumour activity of HCC and is the first to show that Cdc42 is a downstream target gene of NUCKS1 in HCC. NUCKS1 promotes HCC cell proliferation and migration via the upregulation of Cdc42 gene expression. Collectively, our results suggest that NUCKS1 may be a valuable therapeutic target for HCC.

Disclosure of conflict of interest

None.

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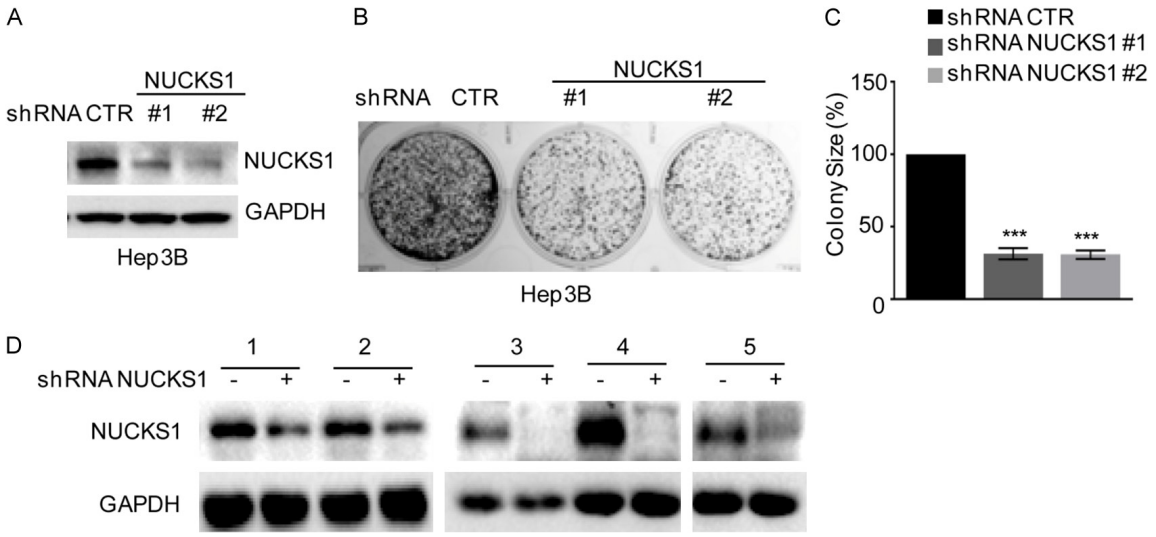
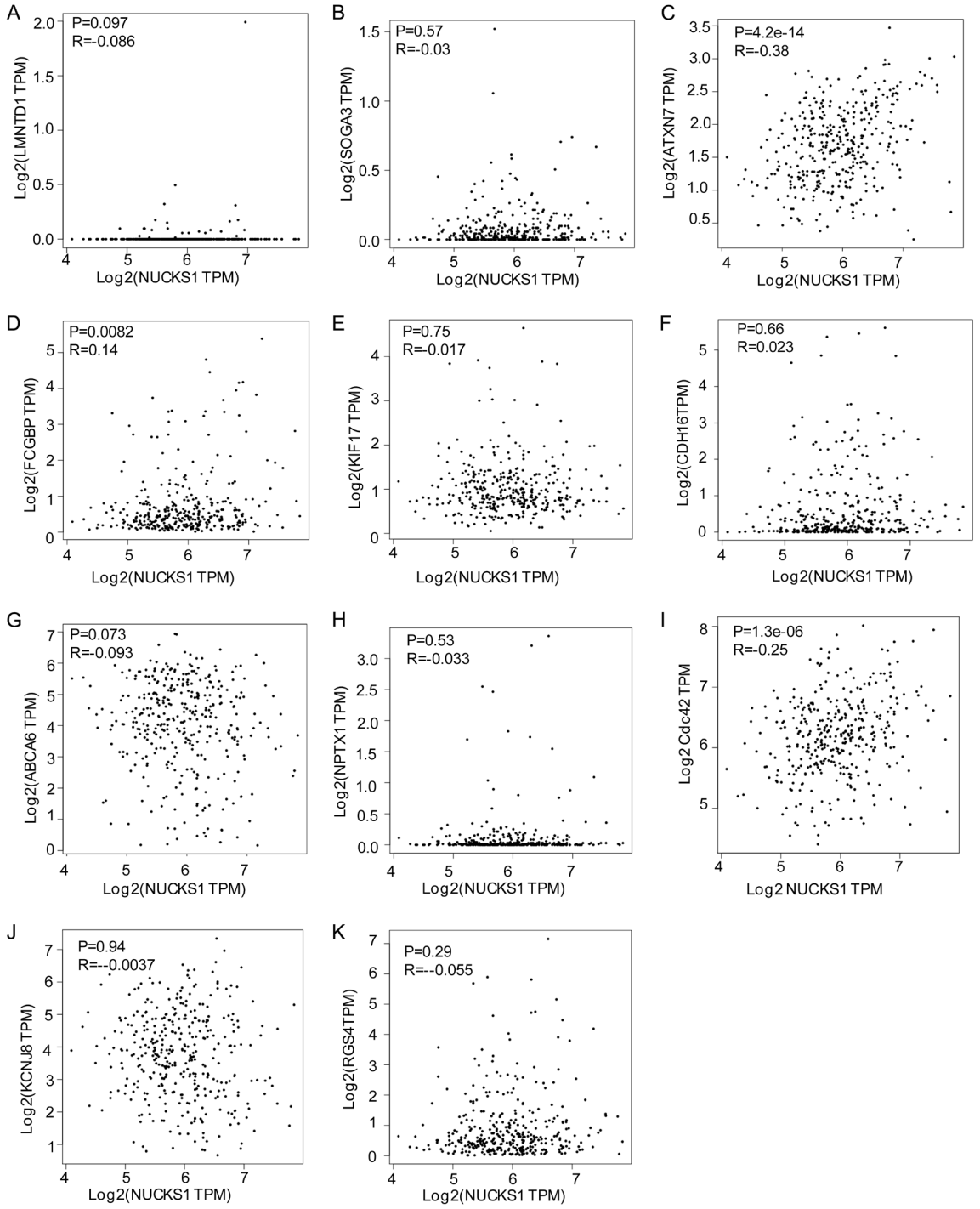
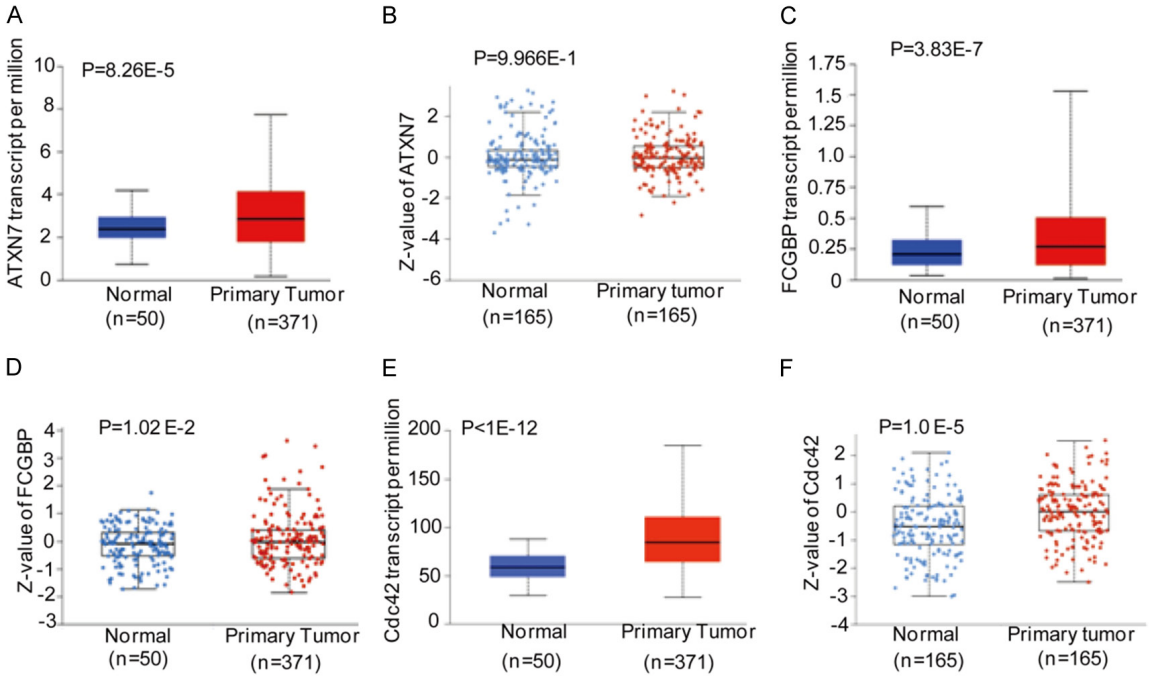


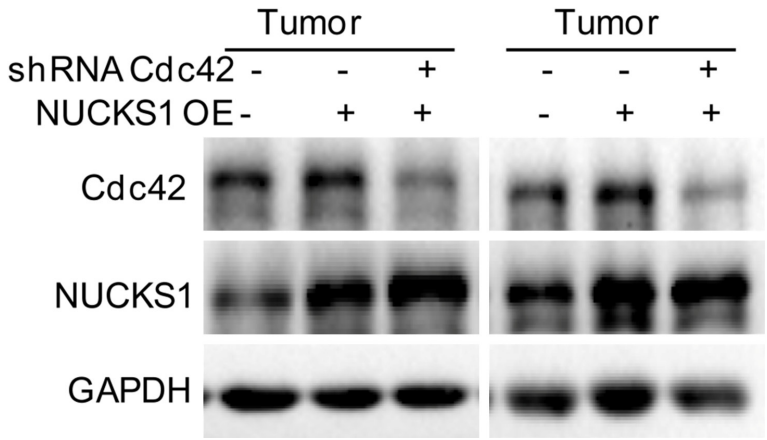
Supplementary Figure 1. NUCKS1 knockdown suppresses cell growth and migration in Hep3B cells. A. NUCKS1 protein levels were verified by western blotting in Hep3B cells with or without NUCKS1 knockdown. B, C. Effects of NUCKS1 knockdown on Hep3B cell growth were detected through a colony formation assay. D. The expression levels of NUCKS1 in HepG2 xenograft tumours were analyzed using western blot.



Supplementary Figure 2. Analysis of the correlation between NUCKS1 and11 downregulated genes. A-K. Analysis of the correlation between NUCKS1 and LMNTD1, SOGA3, ATXN7, FCGBP, KIF17, CDH16, ABCA6, NPTX1, Cdc42, KCNJ8, RGS4 in HCC tissues from the GEPIA database.



Supplementary Figure 3. Analysis of ATXN7, FCGBP and Cdc42 mRNA and protein expression in HCC. A-F. Analysis of ATXN7, FCGBP and Cdc42 mRNA and protein expression in HCC (n = 371) and normal tissues (n = 50) via the UALCAN database.



Supplementary Figure 4. Analysis of the expression NUCKS1 and Cdc42 in HepG2 xenograft tumours. The expression levels of NUCKS1 and Cdc42 in HepG2 xenograft tumours were analyzed using western blot.