Am J Cancer Res 2024;14(11):5338-5350
www.ajcr.us /ISSN:2156-6976/ajcr0158967

Original Article

NCAPG-mediated CDK1 promotes malignant progression of non-small cell lung cancer via ERK signaling activation

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Received July 4, 2024; Accepted November 4, 2024; Epub November 15, 2024; Published November 30, 2024

Abstract: Non-SMC condensing I complex subunit G (NCAPG) has been implicated in tumor progression. However, its role, potential mechanism and prognostic significance in human non-small cell lung cancer (NSCLC) remain elusive. Through the conjoint analysis of the TCGA and The Gene Expression Omnibus (GEO) databases, we confirmed that NCAPG is an upregulated gene. The prognostic value of NCAPG was elucidated through data analysis. The functional roles and mechanistic insights of NCAPG in NSCLC growth and metastasis were evaluated in vitro and in vivo. NCAPG expression was significantly increased in NSCLC. Multivariate Cox regression analysis demonstrated that NCAPG was an independent prognostic factor in patients with NSCLC. The high expression of NCAPG was significantly correlated with lymphatic metastasis. Additionally, the high expression of NCAPG effectively promoted the growth and metastasis of NSCLC in vitro and in vivo. In terms of mechanism, the interaction between NCAPG and Cyclin-dependent kinase 1 (CDK1) promotes the phosphorylation of Extracellular signal-regulated kinase (ERK). Overall, our results reveal the key role of NCAPG in NSCLC and highlight the regulatory function of the NCAPG/CDK1/ERK axis in regulating the progression of NSCLC, providing potential prognosis and therapeutic targets for the treatment of NSCLC.

Keywords: NCAPG, ERK, non-small cell lung cancer, CDK1

Introduction

Although the incidence of non-small cell lung cancer (NSCLC) has decreased globally, its mortality rate remains high [1]. Most patients develop secondary organ metastasis, for which chemotherapy is the primary treatment strategy [2, 3]. However, the targets of chemotherapy drugs are limited; hence, fewer patients benefit from them, and their overall prognosis remains poor [4, 5]. Therefore, further exploring the potential mechanism of NSCLC progression and identifying new treatment targets are of great research significance and clinical value.

NCAPG, a subunit of condensation protein 1, is responsible for chromosomal condensation and stability during meiosis and mitosis [6, 7]. Recent studies have shown that, in common malignant tumors, the upregulation of NCAPG contributes to the proliferation, metastasis, and differentiation of tumor cells and is related to tumor progression [8-10]. Moreover, NCAPG can promote ovarian tumor cell division by regulating the cell cycle [11]. However, the role of NCAPG in NSCLC is unknown. The ERK signaling pathway is a common signaling pathway that plays an important role in regulating cell growth and development. The growth and development of many tumor cells depends on precise ERK signaling pathway regulation [12-14]. The ERK signaling pathway is also an essential way to regulate NSCLC occurrence and development [15, 16].

Here, we confirmed that NCAPG is highly expressed in NSCLC and promotes the G2/M phase transition, proliferation, invasion, and

https://doi.org/10.62347/DNAQ7105

migration of NSCLC cells in vivo and in vitro. We confirmed that NCAPG promotes ERK phosphorylation and upregulates the expression of its downstream molecules, MMP2 and MMP9, through its interaction with CDK1. This study investigated the role of NCAPG in the development of NSCLC to provide further theoretical support for its clinical diagnosis and treatment.

Materials and methods

Public dataset

All RNA sequences and microarray data sets involved in this study are from the GEO (Gene Expression Omnibus) database and TCGA (Cancer Genome Atlas). Among them, 3 datasets were collected from the GEO database (GSE134181, GSE19188, GSE101929), which can be downloaded from (http://www.ncbi.nlm.nih.gov/geo/). And 894 patients with non-small cell lung cancer from the TCGA dataset are available from the Genomic Data Commons (GDC) Data Portal (http://portal.gdc.cancer.gov/). All microarray datasets were log2 converted and normalized using the R software package "limma". RNA sequencing data sets were transformed into log2 (TPM + 1) scale.

Establishment of cell lines

The lentiviruses utilized to construct NCAPG overexpression and knockdown cell lines were purchased from GeneChem Corporation (Shanghai, China). The infection method was strictly in accordance with the manufacturer's instructions. After 3-4 days of infection, the culture medium was changed and an appropriate concentration of puromycin (4 μg/ml, Beyotime) was used to select stable transfected cells for at least one week. The protein and mRNA used in subsequent experiments were collected at the indicated time points.

Cell culture

A549 and H1299 lung cancer cells were obtained from the Cell Line Bank of the Chinese Academy of Sciences. A549 cells were cultured in F12K (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 100 μg/mL penicillin/streptomycin in a humidified incubator under 5% CO_2 at 37°C. H1299 cells were cultured in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum and 100 μg/mL penicillin/streptomycin in a humidified incubator under 5% CO_2 at 37°C. The culture medium was replaced 1-2 days per time.

Western blotting

Cells in the logarithmic growth phase were placed on ice and lysed with lysis buffer (Beyotime, China) for 20 minutes. After centrifugation, the protein concentration was measured with a BCA kit (Thermo Fisher Scientific, USA), and then separated by SDS-PAGE (10%) gel electrophoresis. After that, it was transferred to a PVDF membrane (Millipore, Billerica, MA, USA). Then, the membrane was washed with TBS-T (Beyotime, China) and incubated with 5% skim milk (Beyotime, China) for 1 hour to block the non-specific binding sites, and then incubated overnight with the primary antibody (diluted 1:1000) at 4°C, and finally incubated with the appropriate secondary antibody (diluted 1:5000) for 1 hour. After the final washing, add ECL luminescent substrate and use the ECL detection system for exposure and semi-quantitative analysis. Use the Image J software to quantify and evaluate the strip image.

Cell migration and invasion assay

To evaluate the cell migration ability, Transwell chambers (0.8 μm 24-well plates, Corning, USA) were used for detection. The cells were suspended in serum-free medium and 2×10^4 cells were seeded into the chambers, then 700 μl of medium containing 20% FBS was added to the bottom. For cell invasion, before seeding the cells, Matrigel (Corning, USA) was added to the bottom of the chambers, and the number of seeded cells was 4×10^4. All other procedures were the same. After 24 h of incubation, the chamber was removed, fixed with 4% formaldehyde and stained with 1% crystal violet solution (Beyotime, China). The number of cells was observed under a microscope.

Tumor formation and metastasis assays

Collect cells in the logarithmic growth phase and prepare cell suspension with serum-free medium. 3×10^6 cells were injected into the right armpit of each mouse to construct a subcutaneous xenograft model. The tumor size was measured and recorded every 3 days. After

3-4 weeks, the mice were anesthetized with isoflurane. Under anesthesia, the mice were sacrificed by cervical dislocation, and then the tumors were removed and weighed. In the lung metastasis model, the cell suspension was prepared in the same way, and 4×10^6 stably transfected A549 cells were injected into the tail vein of nude mice. After 40 days, the mice were anesthetized and killed in the same way, and the lungs were harvested to examine the metastasis sites.

All animal experiments were performed in accordance with the Animal Protection Committee of Fujian Medical University (Fuzhou, China) and approved by the Ethics Committee of Fujian Medical University/Laboratory Animal Center (Fuzhou, China) (No. FJMU IACUC 2021-0283). All methods involving animals were performed in compliance with the ARRIVE guidelines.

Immunofluorescence

The cells cultured in the confocal dish (Biosharp, USA) were fixed with 4% formaldehyde for 15 min. Then, they were permeated in 0.1% Triton X-100 for 15-20 min and blocked with 5% bovine serum albumin for 1 hour. Cells were incubated with NCAPG (Abcam) and CDK1 (Abcam) primary antibodies at 4°C overnight, and then incubated with secondary antibodies (Abcam) for 1 h. Cell nuclei were visualized with DAPI (Beyotime, China) in the dark for 15 min. Then, anti-fluorescence quenching agent was added, and the images were observed by fluorescence microscope.

Co-immunoprecipitation assay (Co-IP)

NCAPG and CDK1 immunoprecipitation was performed using a Protein A/G Co-immunoprecipitation Kit (EpiZyme, China) in accordance with the manufacturer's protocol, and NCAPG, CDK1 and IgG antibodies were purchased from Abcam.

Statistical analysis

The data in this study are expressed as mean ± SD and analyzed by t-test or analysis of variance. Chi-square test was used to test the correlation between NCAPG expression and clinicopathological parameters. Kaplan-Meier was used to analyze the survival distribution of the two groups. SPSS 20.0 and Prism 8 software were used for statistical analysis. $P < 0.05$ was considered statistically significant.

Results

Increased NCAPG expression correlates with a poor prognosis of patients with NSCLC

To identify dysfunctional genes associated with the poor prognosis of NSCLC, we analyzed clinical public data (TCGA and GEO databases) (**Figure 1A**). Firstly, we screened out blue modules closely related to the incidence by Weighted gene co-expression network analysis (WGCNA) (Figure S1A, S1E, S1F), and 86 hub genes were selected (Figure S1C, S1D). Then, through the difference analysis of the GEO databases (Figure S1B), 203 up-regulated genes were obtained. Surprisingly, 23 genes were simultaneously involved in both results, and NCAPG was significantly correlated with NSCLC patient survival in the GEO and TCGA databases (**Figure 1B**, **1C**).

We further investigated the relationship between NCAPG and NSCLC. We analyzed the cancer tissues and adjacent tissues of 99 patients in the TCGA database and found that NCAPG is highly expressed in NSCLC (**Figure 1D**). Subsequently, we analyzed 333 patients with lymph node metastasis and 620 patients without lymph node metastasis and found that NCAPG is highly expressed in patients with metastasis (**Figure 1E**). Next, we verified NCAPG expression in NSCLC cell lines. The results indicated that NCAPG was overexpressed in NSCLC cell lines compared to normal lung epithelial cell lines BEAS-2B (**Figure 1F**).

NCAPG promotes NSCLC growth in vitro and in vivo

To examine the function of NCAPG in NSCLC, we established A549 cells with stable NCAPG overexpression and H1299 cells with NCAPG knockdown (**Figure 2A**). Experimental results indicated that the stable knockdown by shNCAPG in H1299 significantly reduced the proliferation (**Figure 2B**), while stable overexpression of NCAPG in A599 promoted the proliferation (**Figure 2C**). Flow cytometry was utilized to analyze the cell cycle and the results revealed that the proportion of cells in the G2 phase decreased significantly in NCAPG overex-

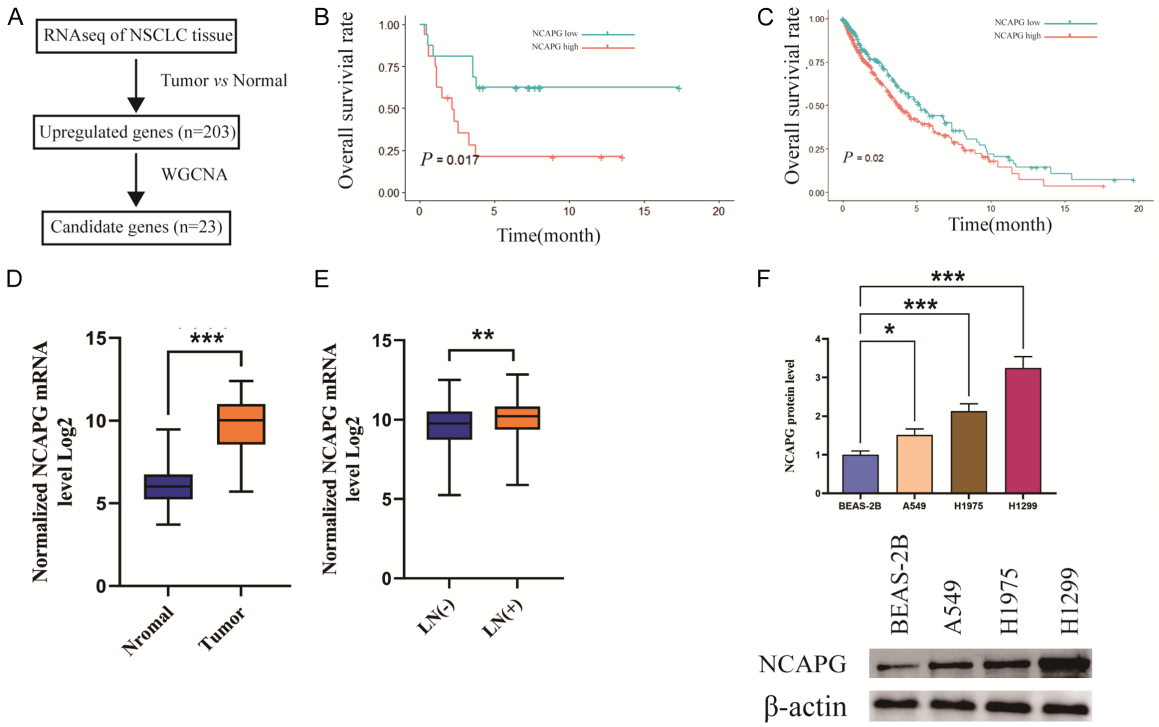


Figure 1. The prognostic value and expression of NCAPG in NSCLC. A. Flowchart of the screening process of candidate genes. B, C. Kaplan-Meier analyses of the correlations between NCAPG expression and overall survival. D. The mRNA levels of NCAPG in tumor and adjacent tissues (***P < 0.001). E. The mRNA levels of NCAPG in no lymph node metastasis and lymph node metastasis (**P < 0.01). F. The level of protein expression of NCAPG in normal lung epithelial cells (BEAS-2B) and NSCLC cell lines (A549, H1975 and H1299) was detected by western blotting and quantified (*P < 0.05; ***P < 0.001). NCAPG, non-SMC condensing I complex subunit G; NSCLC, non-small cell lung cancer.

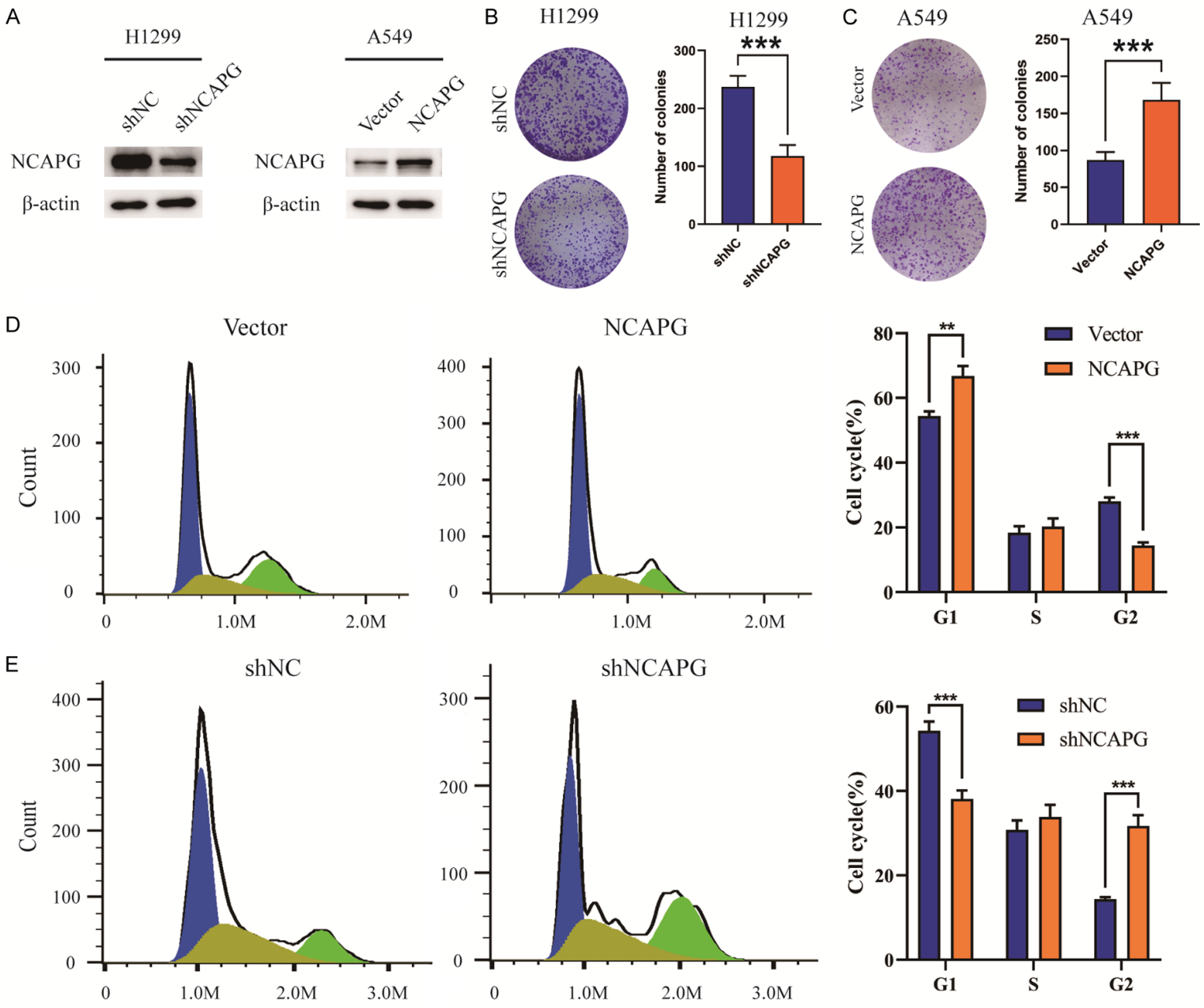
pression A549, whereas the NCAPG Knockdown cell lines showed the opposite trend (**Figure 2D**, **2E**). In order to evaluate the effect of NCAPG in vivo, we constructed the xenograft animal models. The effect of NCAPG on NSCLC in vivo is consistent with the in vitro results. Compared with the control group, the growth of the xenograft with NCAPG overexpression is much faster, while the growth of the xenograft with I shNCAPG Knockdown is much slower (**Figure 2F**, **2G**). The above results proved that NCAPG may promote NSCLC by regulating the cell cycle.

NCAPG promotes NSCLC metastasis in vitro and in vivo

In order to clarify the role of NCAPG in NSCLC metastasis, we first carried out migration and invasion assays in vitro. The experimental results showed that when NCAPG was knocked down by shRNA, the migration and invasion ability of NSCLC cells were significantly inhibited. Conversely, when NCAPG was overexpressed, the migration and invasion ability of NSCLC cells were significantly improved (**Figure 3A**, **3B**). In order to assess whether the effect of NCAPG in vivo was consistent with that in vitro, we injected NCAPG overexpression cells and corresponding control cells into nude mice via the tail vein to construct a lung metastasis model. After 4 weeks, we investigated the lungs. The metastatic foci in the NCAPG overexpression group were significantly more than those in the control group. Through H&E staining of the dissected lungs, it was confirmed that the overexpression group had more metastatic nodules than the control group (**Figure 3C**, **3D**). These results suggest that NCAPG plays a critical role in promoting the metastasis of NSCLC.

NCAPG promotes NSCLC via ERK signaling

Next, we further explored the potential molecular mechanism of NCAPG promoting NSCLC. Through Gene Set Enrichment Analysis (GSEA)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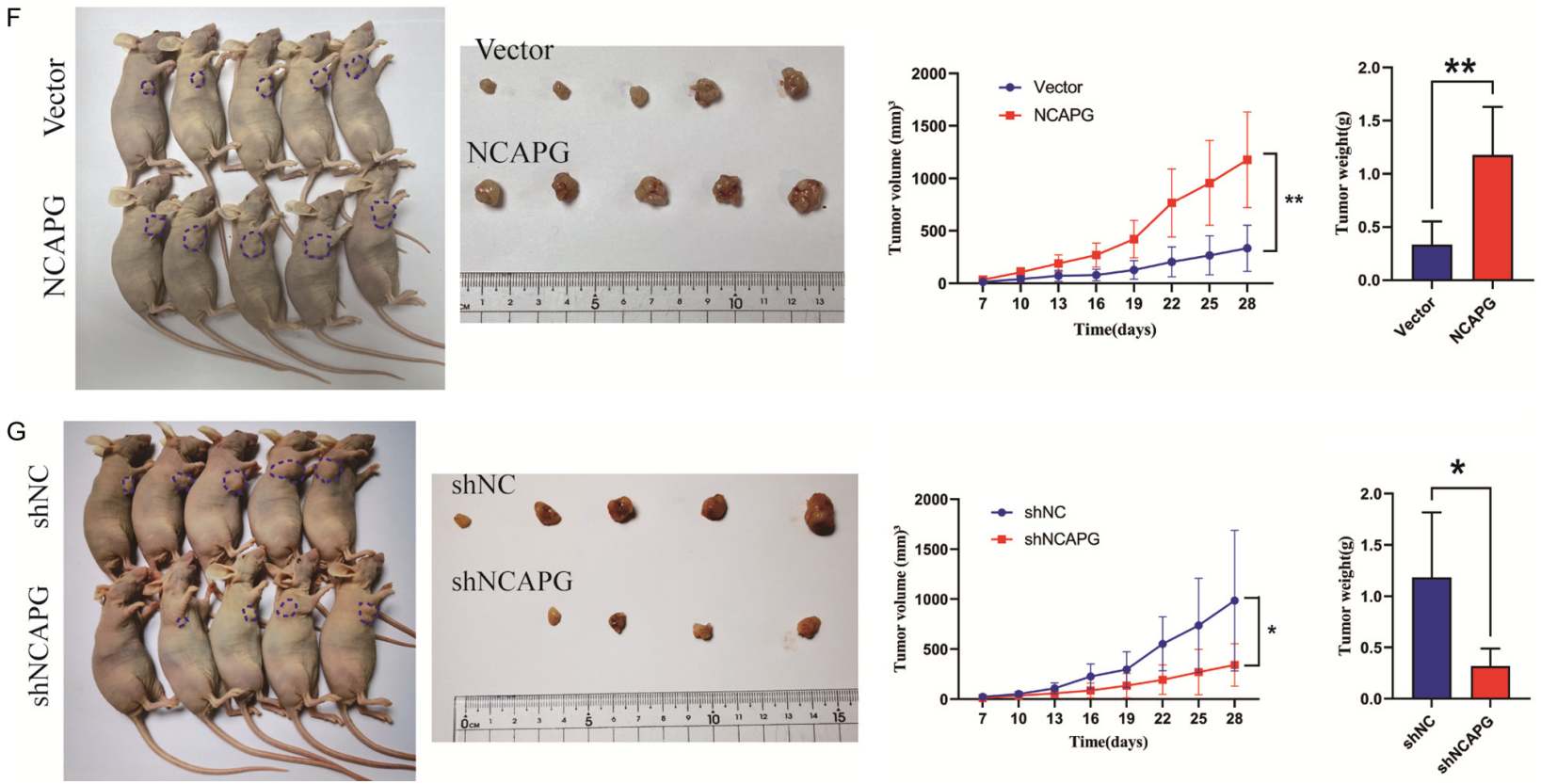

Figure 2. NCAPG promotes NSCLC in vitro and in vivo. A. A549 cells with stable NCAPG overexpression and H1299 cells with NCAPG Knockdown were created. The NCAPG expression was confirmed by western blotting. B, C. The proliferation ability of stably transfected A549 or H1299 cells was studied by colony formation assay. Representative images and quantization results are given (***P < 0.001). D, E. The cell cycle of stably transfected A549 or H1299 cells was analyzed via flow cytometry and representative images and quantization results are given (**P < 0.01; ***P < 0.001). F. Overexpression of NCAPG promotes NSCLC growth in a subcutaneous xenograft model. Tumor size was measured at regular time points. After the mice were sacrificed, the tumors were extracted and weighed (**P < 0.01). G. NCAPG Knockdown inhibits NSCLC growth in a subcutaneous xenograft model. Tumor size was measured at regular time points. After the mice were sacrificed, the tumors were extracted and weighed (*P < 0.05). NCAPG, non-SMC condensing I complex subunit G; NSCLC, non-small cell lung cancer.

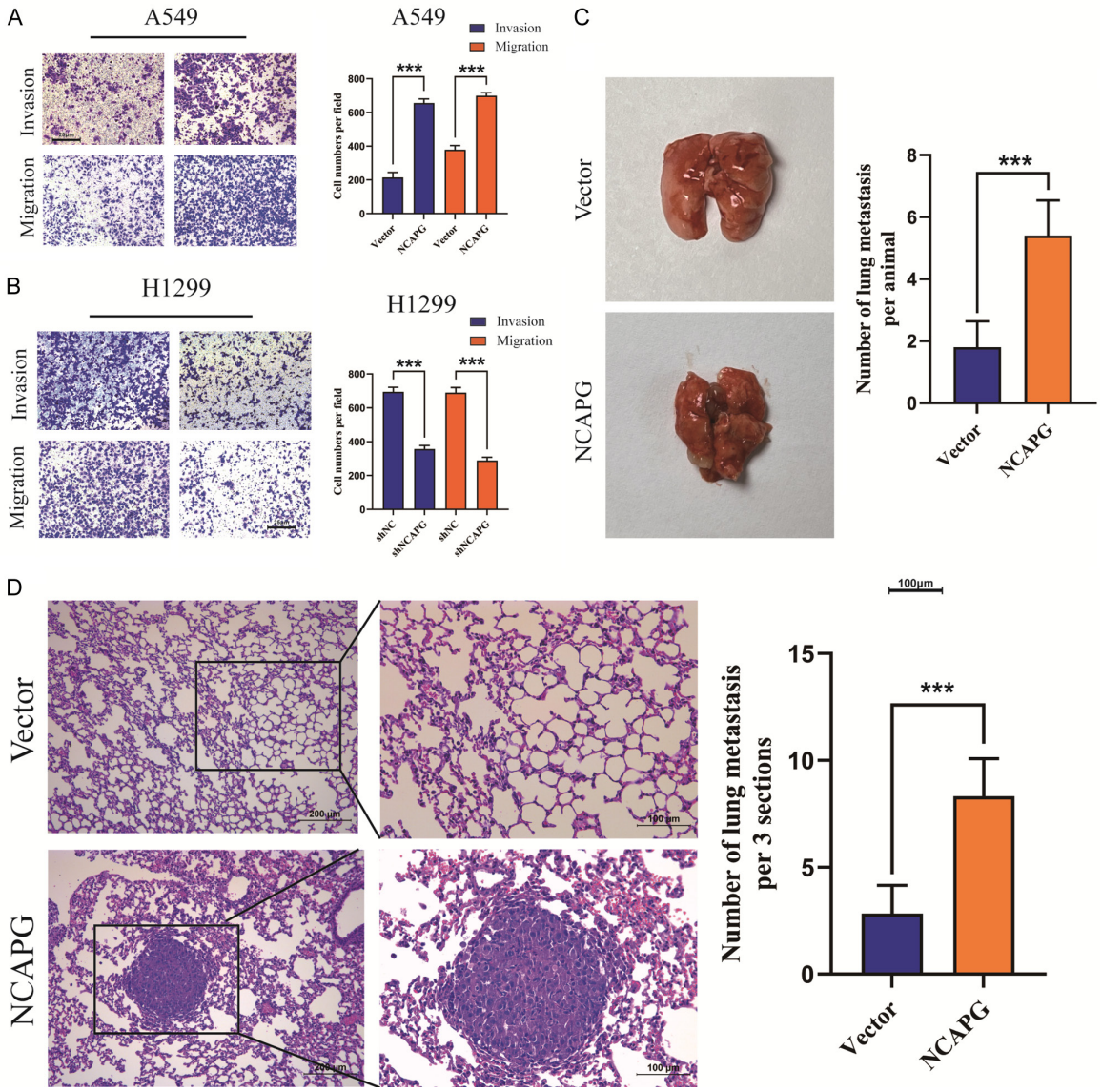

Figure 3. NCAPG promotes NSCLC metastasis in vitro and in vivo. A, B. Transwell assay was performed with stably transfected A549 and H1299 cells. Representative images and quantization results are given (***P < 0.001). C, D. In A549 cells, NCAPG overexpression significantly increased the number of lung metastases nodules. Representative images of lung metastasis and H&E (hematoxylin and eosin) staining are given. Metastatic nodules were counted and recorded under the microscope (***P < 0.001). NCAPG, non-SMC condensing I complex subunit G; NSCLC, non-small cell lung cancer.

on NSCLC patients in the TCGA database, we found that the MAPK signaling pathway was closely related to it (**Figure 4A**). To test this hypothesis, we first conducted a Western blotting experiment. The results showed that NCAPG knockdown significantly blocked the phosphorylation of ERK, JNK, and p38 (**Figure 4B**). On the contrary, NCAPG overexpression promoted the phosphorylation levels of ERK, JNK, and p38. Meanwhile, the key proteins Ras and p-c-Raf in the MAPK pathway showed the same trend. Next, we explored the NCAPG Knockdown cell line H1299 by using TBHQ (An ERK activator). We found that promoting the expression of p-ERK abrogated the inhibition of proliferation, migration, and invasion of the NCAPG stable Knockdown cell line H1299 (**Figure 4E**, **4F**). Furthermore, we analyzed ERK-mediated transcriptional targets MMP2 and MMP9, which confirmed that the expression of MMP2 and MMP9 decreased in NCAPG Knockdown cells (**Figure 4C**). In the stable knockdown cell line H1299, TBHQ increased the expression level of p-ERK and its down-

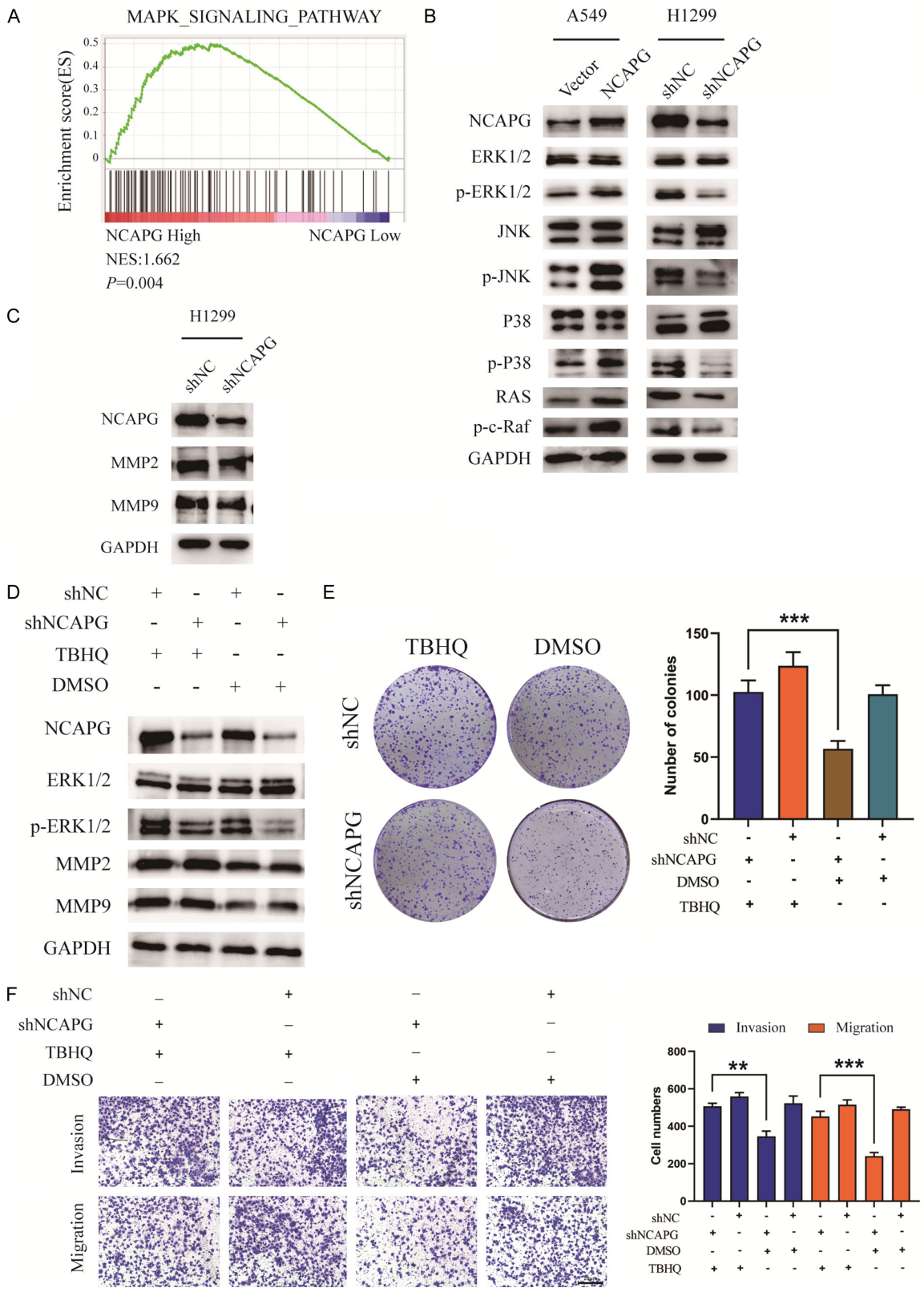

Figure 4. ERK as a target of NCAPG regulating NSCLC. A. GSEA indicated that NCAPG was significantly associated with the MAPK signaling pathway. B. The expression of critical members of the MAPK signaling pathway in stably transfected A549 and H1299 cells was detected via western blotting. C. The expression of ERK downstream targets MMP2 and MMP9 was examined by western blotting in stably transfected H1299 cells. D. After treatment with TBHQ

or DMSO, the protein levels of ERK, p-ERK, MMP2 and MMP9 in stably transfected H1299 cells were detected by western blotting. E, F. After TBHQ treatment, the effects of NCAPG reduction on proliferation, migration and invasion were rescued in H1299 cells stably transfected (**P < 0.01; ***P < 0.001). NCAPG, non-SMC condensing I complex subunit G; NSCLC, non-small cell lung cancer; GSEA, Gene Set Enrichment Analysis; EKR, Extracellular signal-regulated kinase.

stream targets (**Figure 4D**). These data suggest that NCAPG-mediated phosphorylation of ERK may be a potential mechanism for NCAPG to promote NSCLC.

NCAPG depends on CDK1 to regulate the phosphorylation of ERK

We analyzed the potential proteins interacting with NCAPG in the Search Tool for the Retrieval of Interacting Genes (STRING) database, and we noticed the CDK1 protein (Figure S2). In order to verify our conjecture, we first conducted a Western blotting assay. The results showed that the expression of CDK1 was decreased in the NCAPG Knockdown group and increased in the NCAPG overexpression group (**Figure 5A**). The Co-IP assay showed that NCAPG interacted with the CDK1 protein (**Figure 5B**). Then, we performed an immunofluorescence staining assay and found that NCAPG colocalized with CDK1 in NSCLC cells (**Figure 5C**). The effect of CDK1 on the NCAPG function in NSCLC cells was then explored using Ro-3306 (a CDK1-specific inhibitor). We found that the inhibition of CDK1 expression reduced the expression of p-ERK in the stable overexpressed cell line A549 (**Figure 5D**) and offset the increased proliferation, migration, and invasion of NSCLC cells (**Figure 5E**, **5F**). To eliminate random errors, we constructed the NCAPG knockdown cell line H1975. The Western blotting results indicated that the expression levels of CDK1 and p-ERK in the knockdown cell line were significantly reduced (**Figure 6A**). Then, we utilized Ro-3306 to explore the effect of CDK1 in non-small cell lung cancer cells. We discovered that in the stable knockdown cell line H1975, the inhibition of CDK1 expression decreased the expression level of ERK phosphorylation (**Figure 6B**). Collectively, these results indicated that NCAPG depends on CDK1 to activate the ERK signaling and promote NSCLC (**Figure 6C**).

Discussion

NCAPG is a subunit of the condensin complex, which is required to transform interphase chromatin into mitotic-like condensed chromosomes. Recent discoveries have shown that NCAPG plays a key role in the growth and development of various tumors. However, its role in NSCLC remains unclear. Our study demonstrated that the expression level of NCAPG in NSCLC was higher than that in normal tissues, and the high expression of NCAPG was closely related to a poor prognosis. In addition, we discovered for the first time that NCAPG interacted with CDK1 and promoted the ERK signal to drive the growth and metastasis of NSCLC.

Accumulating evidence has shown that NCAPG is related to cycle regulation, immune cell infiltration and drug resistance [17-19]. According to our study, we found that NCAPG can promote the growth and metastasis of NSCLC in vivo and in vitro. In addition, NCAPG promotes the G2/M phase transition of NSCLC cells, and inhibiting NCAPG can induce NSCLC cells to arrest in the G2/M phase, which can partly explain why NCAPG promotes cell proliferation. Therefore, we confirmed that NCAPG acts as a tumor-promoting factor in NSCLC.

It is reported that cells can regulate cell growth and development through various signal transduction pathways, including Wnt/β-Catenin, PI3K/Akt, ERK, Hippo and NF-κB signaling pathways [20-22], among which the ERK signaling pathway is an important way to regulate proliferation [23, 24], and it is one of the key ways to regulate NSCLC and plays a vital role in promoting the growth and metastasis of NSCLC [25, 26]. One of the potential mechanisms by which ERK induces cell growth and development is by regulating the expression of matrix metalloproteinases (MMPs). MMP2 and MMP9 are members of the matrix metalloproteinase family. When the expression of MMP2 and MMP9 increases, it can promote the degradation of type IV collagen and the extracellular matrix (ECM), thus promoting the occurrence of tumor metastasis [27, 28]. In this study, we revealed that NCAPG promotes the phosphorylation of ERK protein and then upregulates the expression of MMP2 and MMP9, thereby enhancing the proliferation, migration and invasion of NSCLC cells.

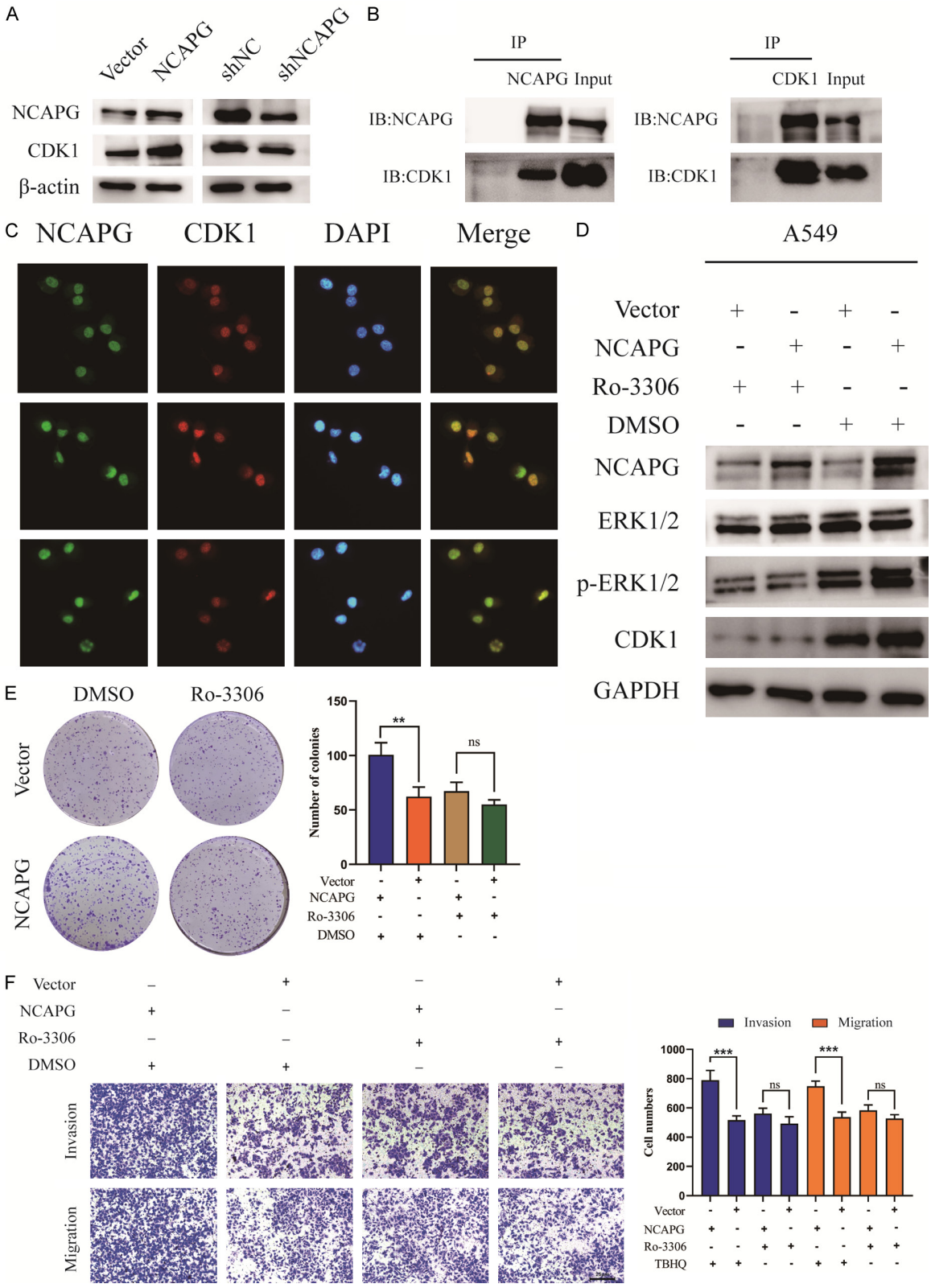

Figure 5. CDK1 plays a critical role in NCAPG-mediated ERK promotion. A. The expression of CDK1 in stably transfected A549 and H1299 cells was detected by western blotting. B. The interaction between NCAPG and CDK1 in H1299 cells was analyzed by Co-IP. C. Immunofluorescence staining showed the localization of NCAPG (green), CDK1 (red), and DAPI (blue) in H1299 cells. D. After treatment with Ro-3306 or DMSO, the protein levels of ERK,

p-ERK, and CDK1 in stably transfected A549 cells were detected by western blotting. E, F. After Ro-3306 treatment, the effects of NCAPG increase on proliferation, migration, and invasion were rescued in A549 cells stably transfected. Cdk1, Cyclin-dependent kinase 1; NCAPG, non-SMC condensing I complex subunit G; EKR, Extracellular signal-regulated kinase.

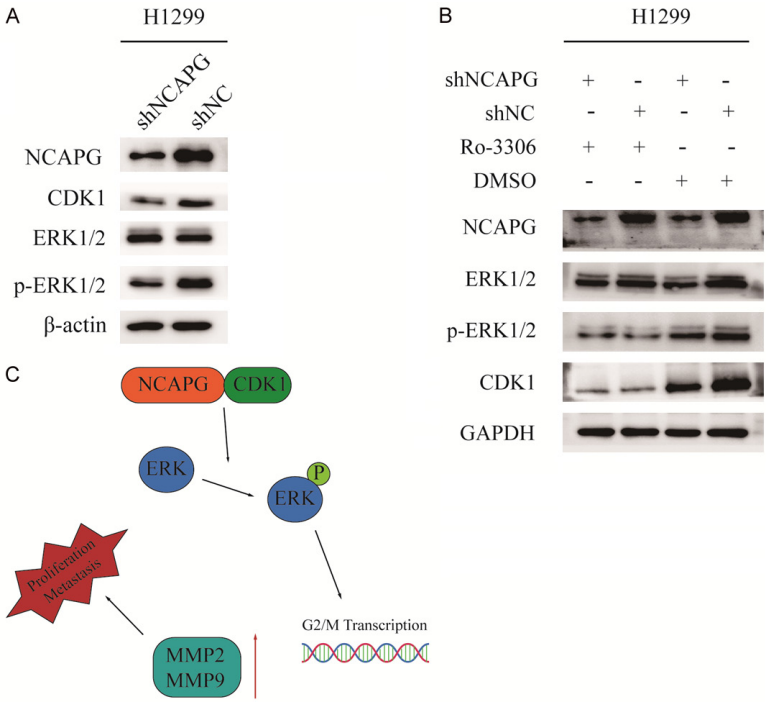

Figure 6. CDK1 is critical for NCAPG-mediated promotion of ERK signaling. A. The expression of CDK1, ERK and p-ERK in stably transfected H1975 cells was detected by western blotting. B. After treatment with Ro-3306 or DMSO, the protein levels of ERK, p-ERK, and CDK1 in stably transfected H1975 cells were detected by western blotting. C. Proposed mechanism scheme of NCAPG in NSCLC. NCAPG promotes ERK phosphorylation in a CDK1-dependent manner, thereby activating the ERK signaling pathway and further promoting NSCLC. NCAPG, non-SMC condensing I complex subunit G; NSCLC, non-small cell lung cancer; EKR, Extracellular signal-regulated kinase.

CDK1 is a key protein in cell cycle regulation and plays an important role in the cell cycle transition [29, 30]. Meanwhile, CDK1 is also a key factor in regulating the growth and metastasis of non-small cell lung cancer [31, 32]. CDK1 can increase the phosphorylation level of ERK, thereby promoting the ERK pathway [33-36]. One of the potential mechanisms is to promote the phosphorylation of ERK by activating the substrate ribosomal S6 kinase of ERK, and then activating the ERK pathway, thereby promoting the growth and development of cells [37, 38]. Our research reveals that there is an interaction between NCAPG and CDK1. When the expression of NCAPG rises, the expression of CDK1 also increases, thereby activating the phosphorylation of ERK and consequently enhancing the proliferation, migration, and invasion capabilities of non-small cell lung cancer cells. However, the specific binding site between CDK1 and ERK and the manner in which CDK1 affects the phosphorylation process of ERK still need to be further investigated.

Conclusion

In summary, our study reveals that NCAPG enhances the proliferation, migration, and invasion of NSCLC. The mechanism is to accelerate the cell cycle period and the degradation of type IV collagen and the ECM through the NCAPG/CDK1/ERK axis, thereby promoting NSCLC. In addition, the high expression of NCAPG is associated with the poor prognosis of NSCLC patients and may be a potential target for the treatment of NSCLC patients.

Acknowledgements

We thank the Central Laboratory of the Affiliated Union Hospital of Fujian Medical University for its assistance in our experiment. This work was supported by the National Natural Science Foundation of China (No. 82171566), the Fujian Provincial Health Technology Project (No. 2020CXA029), the Science and Technology Innovation Joint Fund Project of Fujian Province (No. 2020Y9086), and the Fujian Provincial Natural Science Foundation (grant number: 2020J011006).

Disclosure of conflict of interest

None.

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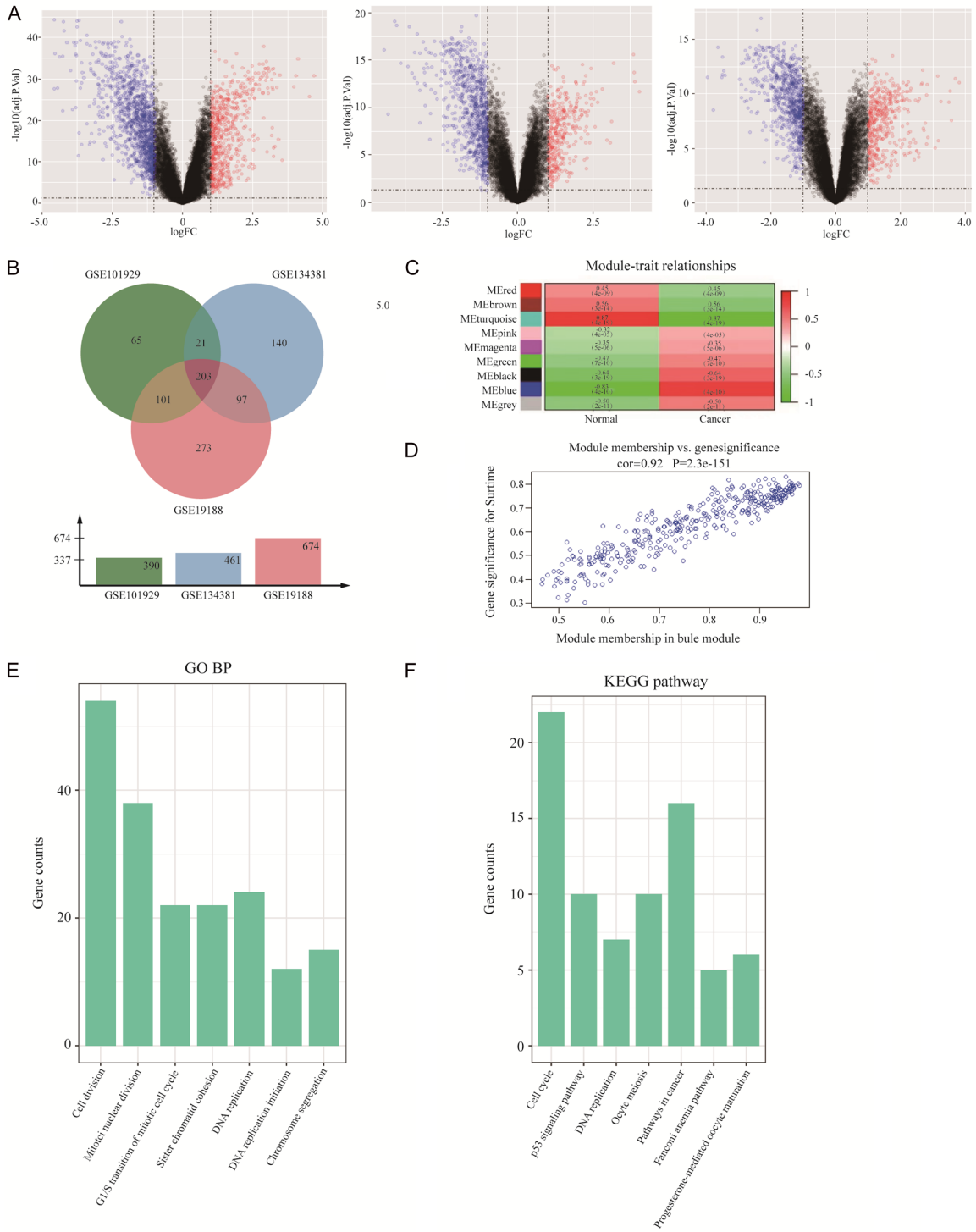

Figure S1. Analysis of the data from the TCGA and GEO databases. A, B. Gene sequencing volcanic maps of NSCLC tumor tissue and adjacent normal tissue (blue indicates down-regulated genes and red indicates up-regulated genes). And three groups of highly expressed genes were combined to create a Venny diagram. C, D. The genes were clustered into different sets by weighted gene co-expression network analysis (WGCNA), and then the blue module was selected and the hub genes were screened. D, F. GO and KEGG analysis revealed that the up-regulated genes were involved in cell cycle, mitosis, DNA replication, and other cycle-related biological processes.

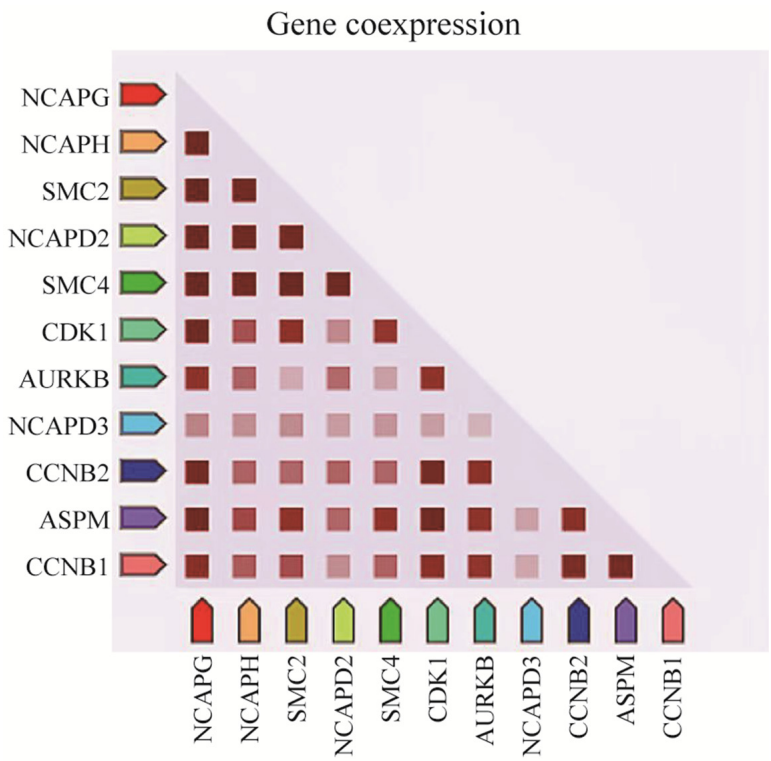

Figure S2. NCAPG may interact with CDK1 as shown in the Search Tool for the Retrieval of Interacting Genes (STRING) database.