

# c-Jun Transcriptional Regulates NPRL2 to Promote the Proliferation Activity of Prostate Cancer Cells via AKT/MDM2/p53 Signaling Pathway

**Daixing Hu**

The First Affiliated Hospital of Chongqing Medical University

**Li Jiang**

The First Affiliated Hospital of Chongqing Medical University

**Shengjun Luo**

The First Affiliated Hospital of Chongqing Medical University

**Xin Zhao**

The First Affiliated Hospital of Chongqing Medical University

**Guozhi Zhao**

The First Affiliated Hospital of Chongqing Medical University

**Xiaoyi Du**

The First Affiliated Hospital of Chongqing Medical University

**Yu Tang**

The First Affiliated Hospital of Chongqing Medical University

**Wei Tang** (✉ [tangwei2060@163.com](mailto:tangwei2060@163.com))

The First Affiliated Hospital of Chongqing Medical University

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## Research

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# Abstract

## Background

High NPRL2 (Nitrogen permease regulator-like 2) expression is a prognostic marker for poor clinical outcomes in prostate cancer (PCa). However, the regulatory mechanisms of NPRL2 in PCa remain unknown.

## Methods

The expression level of NPRL2 in prostate cancer tissues were verified through The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases. The effect of NPRL2 gene in promoting the proliferation of prostate cancer was determined by CCK8 and clone formation assays. The apoptosis rate and cell cycle analysis were tested by flow cytometry. Luciferase reporter gene and ChIP were used to verify the binding relationship between transcription factor c-Jun and the NPRL2 5' region. The effect of NPRL2 and the MK2206 on the AKT/MDM2/p53 signaling pathway was verified by western blotting.

## Results

NPRL2 expression level was significantly increased in prostate cancer tissues than normal tissues base on TCGA and GEO database. We investigated that transcription factor c-Jun can bind to the NPRL2 promoter and regulated NPRL2 transcription directly. Then, we revealed that NPRL2 significantly promotes proliferation and reduces apoptosis in PCa cells. Further mechanistic investigations revealed that NPRL2 mediated proliferation promotion effect is associated with the AKT/MDM2/p53 pathway's participation. Besides, CDK2 might serve as an intermediate effector for NPRL2 to regulate the AKT pathway.

## Conclusion

Taken together, our study identified that c-Jun contributes to transcriptional control of NPRL2 and NPRL2 can promote prostate cancer proliferation by activating AKT/MDM2/p53 signaling.

## Background

Prostate cancer (PCa) is a common male urinary system malignant tumor in western developed countries<sup>1</sup>. The annual number of new cases of PCa were more than 170000, and more than 31000 patient die from prostate cancer each year in 2019 in the USA<sup>2</sup>. There are many treatments strategies for prostate cancer, including surgery, chemotherapy, radiation therapy, and hormone therapy<sup>3</sup>. Androgen-deprivation therapy (ADT) is an effective treatment for locally advanced or metastatic PCa<sup>4</sup>. However, a large number of prostate cancer patients gradually resist ADT treatment and become lethal castration-resistant PCa (CRPC) inevitably, which results in a poor prognosis<sup>5</sup>. Thus, novel diagnostic and

therapeutic approaches need to be developed based on the biological and molecular mechanisms underlying the progression of PCa.

Nitrogen permease regulator-like 2 (NPRL2) is located on chromosome 3p21.3, which was discovered as a tumor suppressor gene by bioinformatics analysis<sup>6,7</sup>. NPRL2 expresses in many normal tissues, a tumor suppressor deregulated in various tumors, including breast cancer<sup>8</sup>, colon cancer<sup>9</sup>, and kidney cancer<sup>10</sup>, and is involved in DNA mismatch repair and cell-cycle checkpoint signaling<sup>11,12</sup>. Our previous studies indicated that the expression level of NPRL2 was significantly higher in prostate cancer tissues and cells than normal tissues and cells, which related to the poor prognosis of prostate cancer patients<sup>13</sup>. Besides, the high expression of NPRL2 promotes the resistance to everolimus by enhancing autophagy in CRPC<sup>14</sup>. Our previous results indicated that silencing NPRL2 gene enhances sensitive to Olaparib treatment in prostate cancer cells, and NPRL2 gene may also be the target of therapy and the key molecule of Olaparib resistance in PCa<sup>15</sup>. These findings suggest NPRL2 can act as an oncogene in prostate cancer.

Here, we verified that the NPRL2 gene is highly expressed in prostate cancer tissues by bioinformatics analysis, which has a molecular role in promoting cell proliferation and reducing apoptosis in prostate cancer cells. Subsequently, we investigated the characterization of the NPRL2 upstream sequence of transcription start, which contains a c-Jun transcription factor binding site. The data showed that the high expression of NPRL2 in PCa cells is partly due to the transcription factor c-Jun, providing a mechanism for NPRL2 to appear as an oncogene in PCa. The results of our study demonstrated that NPRL2 promotes proliferation by regulating AKT/MDM2/p53 pathways. CDK2 might serve as an intermediate effector for NPRL2 to regulate the AKT pathway. Collectively, NPRL2 might act as an oncogene in PCa and promotes prostate cancer cell progression, which may be an important factor for predicting the prognosis and therapeutic target in prostate cancer.

## Methods

### Cell culture

The human PC3 and LNCaP cell were purchased from Shanghai Life Academy of Sciences Cell Library (Shanghai, China). RPMI1640 medium (C11875500BT; Gibco, Beijing, China) and F12K medium (PYG0036, BOSTER; Beijing, China) was used to culture LNCaP and PC3 cells, respectively, with 10% of fetal bovine serum (FBS) (ST190210, ST30-3302p, PAN, Germany) and 1% of penicillin-streptomycin at 37°C, in humidified air containing 5% of CO<sub>2</sub>.

### Bioinformatics mining

RNA-seq data were obtained from the Cancer Genome Atlas (TCGA) database and Gene Expression Omnibus (GEO) datasets (GSE6956 and GSE68882). The sequence of the 2300 bp 5' regulatory region of NPRL2 was downloaded from the National Center for Biotechnology Information. PROMO

([http://alggen.lsi.upc.es/cgi-bin/promo\\_v3](http://alggen.lsi.upc.es/cgi-bin/promo_v3)) software was used to find the binding sites of transcription factors of NPRL2. Interrelationships between protein molecules were analyzed by Gene Mania (<https://genemania.org>).

### **Cell proliferation, apoptosis, and cell cycle assays**

Cell Counting Kit-8 (MedchemExpress, Monmouth Junction, NJ, USA) and colony formation detected cell proliferation activity and cloning capability, respectively. The apoptosis rate and cell cycle analysis were tested by flow cytometry (NovoCyte, ACEA Biosciences, San Diego, CA, USA) and FlowJo performed data analyses.

### **Animal experiments**

All animal studies were approved by the Ethics Committee of Chongqing Medical University and performed in accordance with the guidelines of the National Institutes of Health. Five-week-old male athymic nude mice were subcutaneously injected with  $2 \times 10^6$  PC3 or LNCaP cells in 100  $\mu$ L of a 1:1 Matrigel and PBS mixture in the dorsal region. Tumor volume and body weight of nude mice were measured periodically in the next three weeks after cells injection. Tumor volume was estimated:  $V = A \times B^2/2$  (V, volume; A, length; B, width). After 3-weeks, the mice were sacrificed, and the tumors were dissected and imaged.

### **RNA extraction, reverse transcription, and qRT-PCR**

RNA was isolated from PCa cell lines by the Trizol reagent (TAKARA, Shiga, Japan) and used for reverse transcription and qRT-PCR. CFX Connect Real-time System (BIO-RAD, CA, USA) calculated the relative expression of genes mRNA levels using SYBR Green (TAKARA, Shiga, Japan). Gene expression levels were evaluated with the  $2^{-\Delta\Delta CT}$  method. Specific primers for NPRL2, c-Jun, CDK2, and GAPDH are shown in Supplementary Table 1.

### **Western blot analysis**

Total proteins of cells were extracted by radioimmunoprecipitation assay (RIPA) buffer (Bosterbio) with 1% PMSF. Cell protein lysates were separated by 10% or 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto 0.22- $\mu$ m polyvinylidene fluoride (PVDF) membranes (Sigma). Bovine serum albumin (BSA) was used to block the PVDF membranes for 2 h at room temperature. Primary antibodies, shown in supplementary table 2, incubated overnight. Secondary antibodies (Cell Signaling Technology, Beverly, MA, USA) incubated at room temperature for 30 minutes. Densitometry (Quantity One software; Bio-Rad) was used to quantify the autoradiograms.

### **NPRL2 5' Regulatory Region Reporter Construct Cloning Strategies**

NPRL2 5' regulatory region (-2000 bp tp +300 bp) was generated by PCR amplification of human genomic DNA that was isolated from HEK 293. Raw materials for reaction included dNTP, DMSO, Betaine,

and iProof DNA Polymerase (Bio-Rad, Hercules, CA, USA). The order of PCR cycling was initial denaturation 98°C for 30s, denaturation 98°C for 10s, annealing 55°C–65°C for 30s, extension at 72°C for 75 s for 35 cycles and final extension 72°C for 10 min. The amplified 2300 bp fragment was cloned into the luciferase reporter pGL3 basic vector (Promega). Using the full-length upstream sequence of NPRL2 5' regulatory region, we subsequently constructed deletion sequences of different lengths. All primers used in this step are listed in Supplementary Table 1.

### **Vector construction and cell transfection**

The full-length cDNA of NPRL2 was amplified in 293T cells and then cloned into overexpression vector pcDNA-NPRL2 (Genscript, Jiangsu, China), while the mock vector with no NPRL2 sequence served as a control. siRNA targeting NPRL2 and siRNA-NC were synthesized by Genscript (Jiangsu, China) to knock down NPRL2 expression. The sequence of siRNA-NC and NPRL2 siRNA and the transfection procedure is described in our previous publication<sup>14</sup>. siRNA-NC and NPRL2 siRNA were cloned into the plasmid vector called sh-NPRL2 and sh-NC, respectively. Commercialized si-c-Jun, si-CDK2, pcDNA3.1-c-Jun plasmid, pcDNA3.1-CDK2 plasmid, and negative-control siRNA were purchased from Genscript (Jiangsu, China) (Supplementary Table 1). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used to conduct cell transfections. AKT inhibitor, MK-2206 were purchased from MedchemExpress (Monmouth Junction, NJ, USA).

### **Microarray of mRNA**

Total RNA was extracted from PC3 cells treated with sh-NC and sh-NPRL2. Expression profiling of mRNAs was performed using the MGISEQ-2000 FAST sequencing platform (Sangon Biotech, Shanghai, China). The microarray was used to performed Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis.

### **Luciferase activity report assays and Chromatin immunoprecipitation (ChIP) analysis**

The LNCaP and PC3 cells were plated into 24-well plates ( $6 \times 10^4$  cells/well). Transfections were performed using prime reagent (Polyplus) with the pGL3 basic-NPRL2 (0.5 µg) luciferase construct together with 100 ng of renilla. Dual-Luciferase Reporter Assay System (Promega) was used to detect luciferase activity 48 h after transfection.

Formaldehyde (final concentration 1%) was used to incubate PC3 and LNCaP cells for 10 min at room temperature, followed by 0.2 M glycine for 5 min. Removed the medium, wash the cells twice with 20 ml ice PBS. After adding ice PBS + PIC (Protease Inhibitor Cocktail), the cells were scraped into the cold buffer. Centrifuge at 2000 x g for 5 min at 4°C. Micrococcal Nuclease was used to digest the DNA to approximately 150-900 bp in length. The lysate was sonicated with several pulses to destroy the nuclear membrane. The chromatin was immunoprecipitated by incubating with antibodies and Magnetic beads (Invitrogen, Waltham, MA, USA). The chromatin was eluted from the antibody/ Magnetic beads and de-crosslinked. Purify DNA using spin columns, followed analyze by the Quantitative PCR. The reaction

mixture (20 $\mu$ L) consisted of 2 $\mu$ L of DNA sample, 1 $\mu$ L of forward and 1 $\mu$ L of reverse primer, 6 $\mu$ L DEPC and 10  $\mu$ L of Syber Green Mastermix (Bio-Rad).

## Statistical analysis

Statistical analyses were performed with the SPSS 25.0 and GraphPad Prism 7. Overall survival and disease-free survival analysis based on TCGA and GEO datasets were performed using the Kaplan–Meier method. All analyses performed were two-sided, and P-values < 0.05 indicated statistical difference. The data are displayed as mean  $\pm$  SD of at least three independent experiments. Throughout this study: \*p < 0.05; \*\*p < 0.01; and \*\*\* p < 0.001.

## Results

### NPRL2 is up-regulated and associated with poor prognosis in PCa based on TCGA and GEO datasets

The expression profile of NPRL2 was depicted by heatmap based on TCGA database (Fig.1a). The expression of NPRL2 in PCa was significantly higher than the adjacent samples (Fig.1b). Among the patients who died, the NPRL2 gene was also significantly higher than that of the surviving patients (Fig.1c). In addition, NPRL2 was significantly higher in tumor tissues than normal prostate tissues based on the GEO datasets (GSE6956 and GSE68882) (Fig.1d, e). Kaplan–Meier curves demonstrated that the high NPRL2 expression group had a worse overall survival (OS) than the low NPRL2 expression group, although there was no statistical difference (Fig.1f). The NPRL2 high expression group was significantly associated with worse disease-free survival (DFS) (Fig.1g). The expression level of NPRL2 was higher in PCa tissues than that in normal tissues in immunohistochemical staining (Fig.1h, i). Hence, we focused our attention on NPRL2 in tumorigenesis and progression of PCa.

### NPRL2 promotes PCa cell proliferation and modulates cell cycle and apoptosis

We first constructed stably overexpression vector and RNAi vector of NPRL2 in PC3 and LNCaP cells (Fig. S1a-d). Overexpression of NPRL2 enhances cell proliferation viability, while silencing NPRL2 inhibited cell proliferation viability in CCK8 assays (Fig. 2a) and colony formation assays in PCa cells (Fig. 2b, c). Knockdown of NPRL2 gene led to higher percentages of PCa cells in the G0/G1 phase in cell cycle analysis (Fig. 2d, e). Meanwhile, the upregulation of NPRL2 led to lower percentages of PC3 and LNCaP cells in the G0/G1 phase, and more cells in the S phase compared with the vector group. Flow cytometry analysis showed that knockdown of NPRL2 gene increases prostate cancer cell apoptosis rate (Fig. 2f, g). The apoptosis rate of NPRL2 overexpression PC3 and LNCaP cells was slightly lower than that of the control group. Furthermore, western blot was performed to detect apoptosis-related protein levels. The results found that the downregulation of NPRL2 in PC3 and LNCaP cells had higher levels of BAX and cleaved caspase-3 and lower levels of BCL2 compared with the NC group (Fig. 2h, i). The levels of BAX and cleaved caspase-3 were lower and BCL2 was higher in NPRL2 overexpression PC3 and LNCaP cells than the control group. To determine the effects of NPRL2 on tumor growth in vivo, PC3 and LNCaP cells were stably transfected with knocked down or sh-NC vector and then injected into male nude mice. the

tumors of NPRL2 knockdown PC3 and LNCaP cells had smaller sizes and lower weights than those of the control group (Fig. 2j-m).

### **NPRL2 expression levels in PCa and detection of NPRL2 gene promoter activity**

As shown in figure 3a, b, LNCaP cell showed a lower protein level of NPRL2 than the PC3 cell line. The difference of NPRL2 mRNA expression level between PC3 and LNCaP cells is almost the same as the protein level (Fig. 3c). These pieces of evidence demonstrated that NPRL2 expression levels are controlled at the transcriptional level in prostate cancer cell lines. We constructed the 2300bp sequence from -2000bp to +300 of the 5' regulatory regions of NPRL2 gene and then cloned into the vector to create pGL3 basic-NPRL2 and a series of 5' deletion constructs (Fig. 3d). Luciferase reporter detection found the most active region of NPRL2 promoter may be located in the sequence from -1100 bp to -1500 bp (Fig. 3e, f).

### **The transcription factor c-Jun regulates the expression of NPRL2 gene**

Promo software analyzed the 5' region from -1100 to -1500bp of the transcription start site of NPRL2, suggesting that c-Jun may be a transcription factor regulating NPRL2. Silencing c-Jun significantly down-regulated the mRNA and protein expression of NPRL2 in PC3 (Fig. 4a, b) and LNCaP cell lines (Fig. S3a, b). Silencing c-Jun also significantly reduced the luciferin activity of the NPRL2 upstream promoter in PC3 (Fig. 4c) and LNCaP cell lines (Fig. S3c). Furthermore, the chip assay confirmed that the transcription factor c-Jun directly bind to the upstream sequence of the NPRL2 gene in PC3 and LNCaP cell lines (Fig. 4d), with an IgG-precipitated sample used as a negative control. Collectively, the transcription factor c-Jun regulates the expression of NPRL2 gene at the transcriptional level, thereby affecting its biological function. The expression of c-Jun was significantly higher in PCa tissues than in normal prostate tissues based on the GSE6956 dataset (Fig. 4e). Besides, the expression levels of NPRL2 was significantly and positively correlated with c-Jun (Fig. 4f). According to the expression median value of the NPRL2 and c-Jun, the high expression level of NPRL2 and c-Jun group were significantly associated with worse DFS and OS (Fig. 4g, h).

### **Silencing and overexpression of c-Jun impacts NPRL2-dependent PCa cell proliferation**

The overexpression vector of c-Jun and the siRNA against c-Jun were constructed. c-Jun overexpression and silence in PC3 and LNCaP cells were verified by western blot and qRT-PCR (Fig. S2a-d). Simultaneous up-regulation of NPRL2 and c-Jun significantly increased the proliferation viability of prostate cancer cells by CCK8 assays, whereas the upregulation of NPRL2 and the downregulation of c-Jun inhibited cell growth (Fig. 5a). Additionally, downregulation of NPRL2 and c-Jun at the same time significantly inhibited the proliferation viability of PC3 and LNCaP cells, whereas downregulation of NPRL2 and upregulation of c-Jun enhanced cell growth. Furthermore, the upregulation of NPRL2 and c-Jun group had lower apoptotic cells than the group upregulation of NPRL2 and downregulation of c-Jun in PC3 and LNCaP cells. Meanwhile, the downregulation of NPRL2 and c-Jun group had significantly higher apoptotic cells than the group downregulation of NPRL2 and upregulation of c-Jun (Fig. 5b). Western

blotting results showed that the upregulation of NPRL2 and c-Jun suppressed the expression level of cleaved caspase-3 and BAX and induced the expression of BCL2. On the contrary, simultaneous down-regulation of NPRL2 and c-Jun significantly increased the expression level of cleaved caspase-3 and BAX, while a reducing the expression of BCL2 (Fig. 5c).

### **NPRL2 promotes proliferation via activating AKT/MDM2/p53 axis**

Western blot analysis found that NPRL2 overexpression enhanced the expression of p-AKT and p-MDM2 and reduced the expression of p53, while the knockdown of NPRL2 had an opposite effect in the LNCaP cell. Overexpression of NPRL2 resulted in the increased expression of the p-AKT and p-MDM2 protein levels in the PC3 cell (Fig. 6a). However, the expression level of p53 was not changed significantly in knockdown or overexpression of NPRL2 in the p53 null PC3 cell. To examine the regulation of AKT/MDM2/p53 pathways mediated by NPRL2, PC3 and LNCaP cells were treated with a p-AKT specific inhibitor (MK2206). We tested the inhibitory effect of MK2206 at 5  $\mu$ M concentrations for 24 h by western blotting. As a result, MK2206 block the expression of p-AKT and p-MDM2 and increase the expression of p53 in the LNCaP cell. However, the expression level of p53 was not changed significantly treated with MK2206 in the p53 null PC3 cell (Fig. 6b). Western blot experiment found that MK2206 significantly up-regulated the expression of cleaved caspase-3 and BAX, while down-regulating the expression of BCL2 even if the NPRL2 gene was overexpressed (Fig. 6c, d). We found that MK2206 significantly suppressed the viability of PC3 and LNCaP cells even if the NPRL2 gene was overexpressed (Fig. 6e).

### **CDK2 serves as an intermediate effector for NPRL2 to regulate the AKT pathway**

CDK2 overexpression and silence in PC3 and LNCaP cells were verified by western blot and qRT-PCR (Fig. S5a-d). The expression levels of NPRL2 was significantly and positively correlated with CDK2 in GSE68882 (Fig. 7a). According to the expression of the NPRL2 and CDK2, the patients were divided into high and low groups, the patients with high expression of NPRL2 and CDK2 was significantly associated with worse DFS and OS in Kaplan–Meier curves (Fig. 7b, c). NPRL2 overexpression upregulated CDK2 mRNA expression, and NPRL2 knockdown suppressed CDK2 mRNA expression in PC3 (Fig. 7d, e) and LNCaP cell line (Fig. S5e, f). CDK2 was significantly higher in tumor tissues than normal prostate tissues based on GSE6956 and GSE68882 (Fig. 7f, g). Bioinformatics prediction indicates that there may be four key molecules between NPRL2 and CDK2, including CDKN1A, CCNB2, CKS1B, and CDKN1B (Fig. S5g). The silencing or overexpression of NPRL2 may significantly affect the expression of p-AKT, but this effect will be reversed by the overexpression and silence of CDK2, respectively (Fig. 7h). Therefore, CDK2 might act as a key intermediate effector for NPRL2 to regulate the AKT pathway.

## **Discussion**

In our previous reports, NPRL2 was studied and identified as an oncogene in PCa<sup>14</sup>. Here, we validated the expression level of NPRL2 in TCGA and GEO datasets. NPRL2 was upregulated in PCa tissues and significantly correlated with poor DFS and OS. The NPRL2 gene promote the proliferation activity of

prostate cancer cells in vivo and in vitro, while silencing NPRL2 significantly reduced the proliferation activity and increased apoptosis rate. Previous researches have shown that NPRL2 is closely related to cell cycle<sup>16</sup>. We found silencing NPRL2 caused a large number of PC3 and LNCaP cells to be arrested in the G1 stage.

Much evidence has shown that NPRL2 acts as a tumor suppressor in colon cancer, glioma, lung cancer, and breast cancer<sup>17-20</sup>. The high expression of the NPRL2 gene in prostate cancer and the oncogenic characteristics has aroused great interest in our team. We hope to explore the reasons for its high expression different from other cancers. Protein expression is regulated by transcription and post-transcriptional processes. This study focused on the transcription level of the NPRL2 gene and found that c-Jun can directly bind to the NPRL2 promoter and regulate NPRL2 transcription.

c-Jun is an important member of the AP-1 transcription factor family, which acts as a cancer-promoting transcription factor contributed in differentiation, cell cycle, and apoptosis<sup>21</sup>. In addition, c-Jun also participates in stress response, and the function is conserved in yeast and mammals<sup>22</sup>. Thakur et al. demonstrated that c-Jun is activated by TGF- $\beta$ , leading to invasion of prostate cancer cells, which has high expression levels in aggressive prostate cancer tissues with poor prognosis<sup>23,24</sup>. The transcription regulation of the NPRL2 by c-Jun may be one of the reasons that NPRL2 gene appears as an oncogene in PCa.

KEGG pathway analysis suggested that the PI3K/AKT pathway is a hub differential pathway between NPRL2 knockdown group and NC group (Fig. S4a, b). PI3K/AKT is an important pathway that participates in the multiple biological activities, including cell proliferation, cell repair, cell growth, cell migration, and angiogenesis, and is constitutively activated in nearly all cancer types<sup>25</sup>. AKT is a major effector in the PI3K/AKT signaling axis, one of the essential kinase signaling molecules in prostate cancer development and treatment<sup>26</sup>. Increased murine double minute 2 (MDM2) expression is associated with increased the risk for recurrence, metastasis, and cancer-specific mortality with PCa treated with radiotherapy<sup>27</sup>. p53 is a classic tumor suppressor gene and also an important downstream effector of AKT. Quinn et al. reported that p53 aberrations in radical prostatectomy tissue could predict clinically relevant endpoints of metastatic relapse and prostate cancer-specific mortality<sup>28</sup>. We observed that NPRL2 regulated the AKT/MDM2/p53 signaling in PCa, which serves as an AKT coactivator to stimulate the AKT/MDM2/p53 pathway mediating the proliferation promoting effect.

Gene Ontology (GO) enrichment analysis revealed that the top five enrichment of NPRL2 involved are protein kinase activity, GTPase activator activity, lysosomal membrane, cellular response to nitrogen starvation, and positive regulation of autophagy (Fig. S4c). Liu et al. reported that cyclin-dependent kinase 2 (CDK2), an important protein kinase, promotes AKT phosphorylation activation through facilitating or functionally compensating<sup>29</sup>. CDK2 was significantly upregulated in PCa tissues and was positively correlated with the expression level of NPRL2 in the GEO dataset. Therefore, we inferred that there might be a connection between NPRL2 and CDK2. We found that NPRL2 overexpression

upregulated CDK2 mRNA expression and NPRL2 knockdown suppressed CDK2 mRNA expression in PC3 and LNCaP cell lines. Furthermore, western blot demonstrated that the p-AKT levels caused by overexpressing or silencing NPRL2 could be reversed by CDK2 silencing or overexpressing, respectively. In summary, these data suggested that CDK2 might serve as a downstream molecule and regulated by NPRL2, thereby affecting the phosphorylation of AKT, which acts as an intermediate effector for NPRL2 to regulate the AKT/MDM2/p53 pathway.

## Conclusion

In this study, we provided a novel mechanism to address how NPRL2 promotes tumor proliferation in PCa. Our data showed that the NPRL2 acts as an oncogene in PCa, which is positively regulated by c-Jun to coregulate the AKT/MDM2/p53 signaling. In addition, the effect of NPRL2 gene on the AKT/MDM2/p53 pathway may be mediated through CDK2. Accordingly, NPRL2 may be a novel molecular therapeutic target for developing a new treatment approach for prostate cancer.

## Abbreviations

NPRL2: Nitrogen permease regulator-like 2; PCa: prostate cancer; ADT: Androgen-deprivation therapy; TCGA: The Cancer Genome Atlas; GEO: Gene Expression Omnibus; KEGG: Kyoto Encyclopedia of Genes and Genomes; ChIP: Chromatin immunoprecipitation; OS: overall survival; DFS: disease-free survival.

## Declarations

### Ethics approval

All studies were approved by the Ethics Committee of Chongqing Medical University.

### Consent for publication

Not applicable.

### Availability of data and materials

The datasets used and analyzed in the present study are available from the corresponding author on reasonable request.

### Conflict of interest

The authors declare no potential conflicts of interest.

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## Author contributions

D.H., L.J., and W.T. conceived and designed the study; D.H., X.Z., Y.T., G.Z., and X.D. conducted experiments and collected data; D.H., X.Z., Y.T., and L.J. analyzed and interpreted data; D.H. and X.Z. drafted the paper; L.J. and S.L. provided technical or material support; W.T. provided valuable comments and suggestions to improve the paper and data presentation; L.J. and W.T. supervised the whole study; all authors read and approved the final version of the paper.

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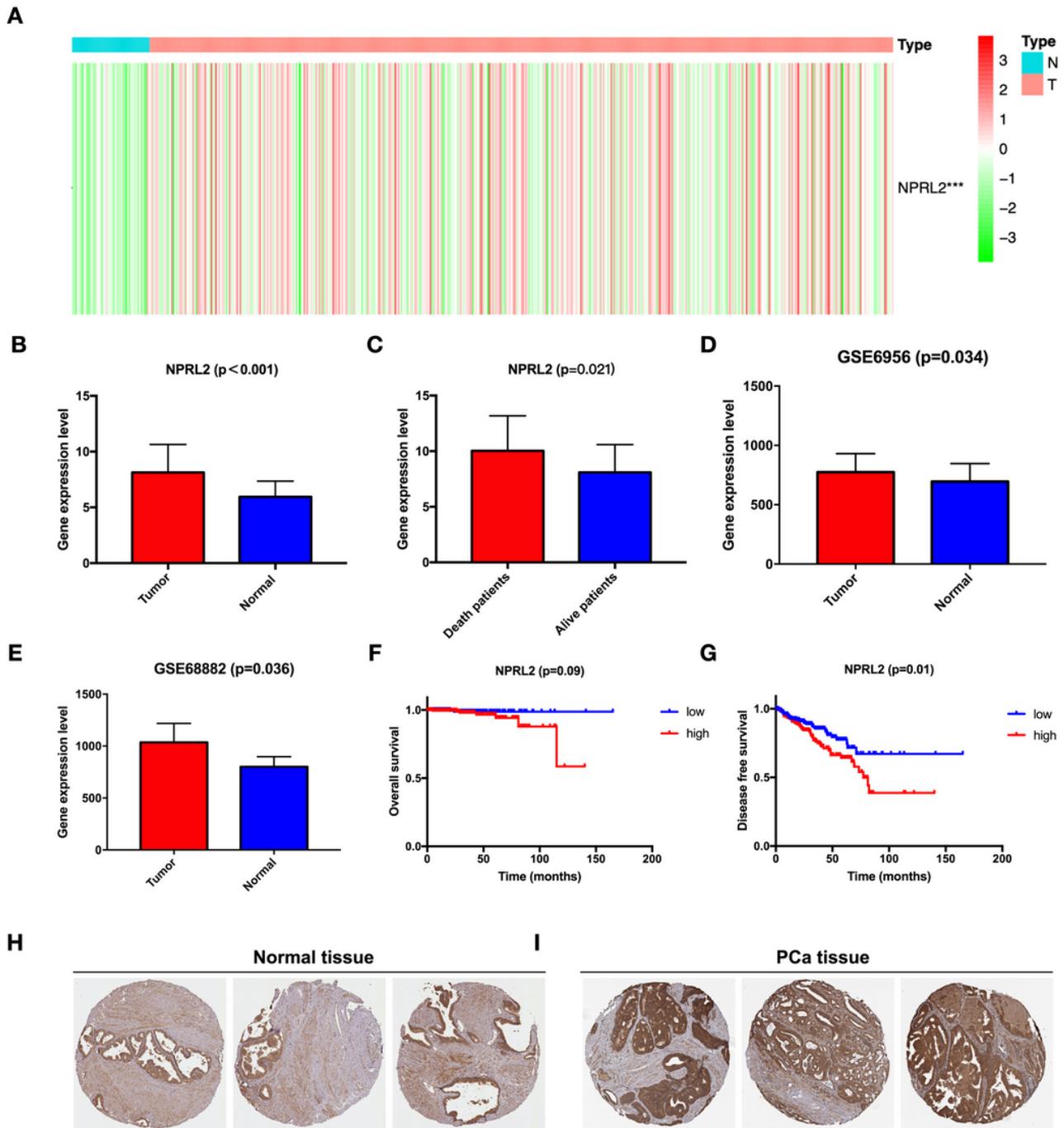
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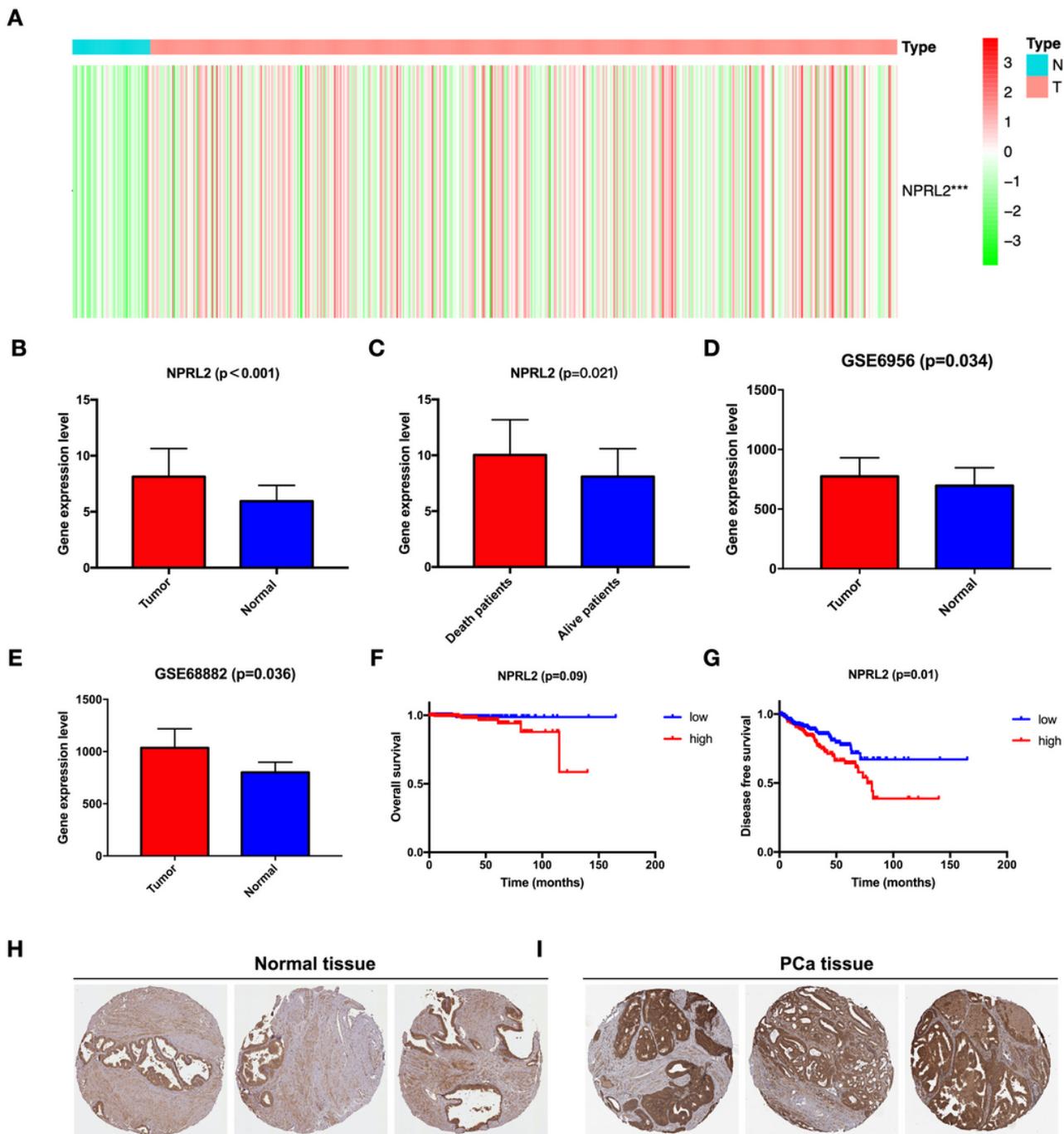
## Figures



**Figure 1**

Expression profiles and prognostic characteristics of NPRL2 in PCa. a,b Heatmap and histogram displayed the expression levels of NPRL2 in PCa and non-tumor samples from the TCGA database. c NPRL2 mRNA expression in alive prostate cancer patients and dead prostate cancer patients from TCGA databases. d, e NPRL2 expression levels of prostate cancer tissues and adjacent tissues from GEO (GSE6956 and GSE68882) databases. f, g Association between DFS and OS of PCa patients and NPRL2

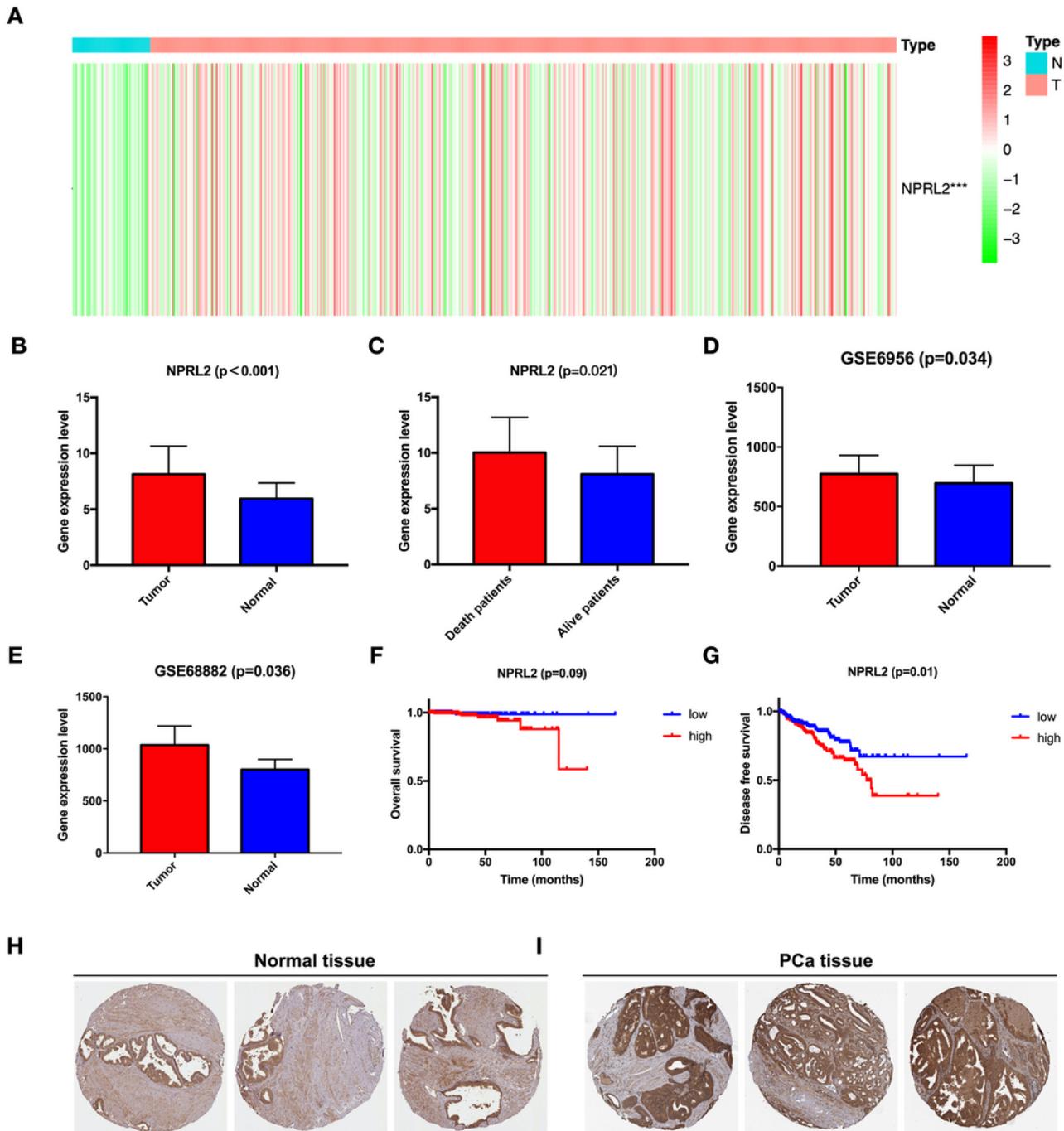
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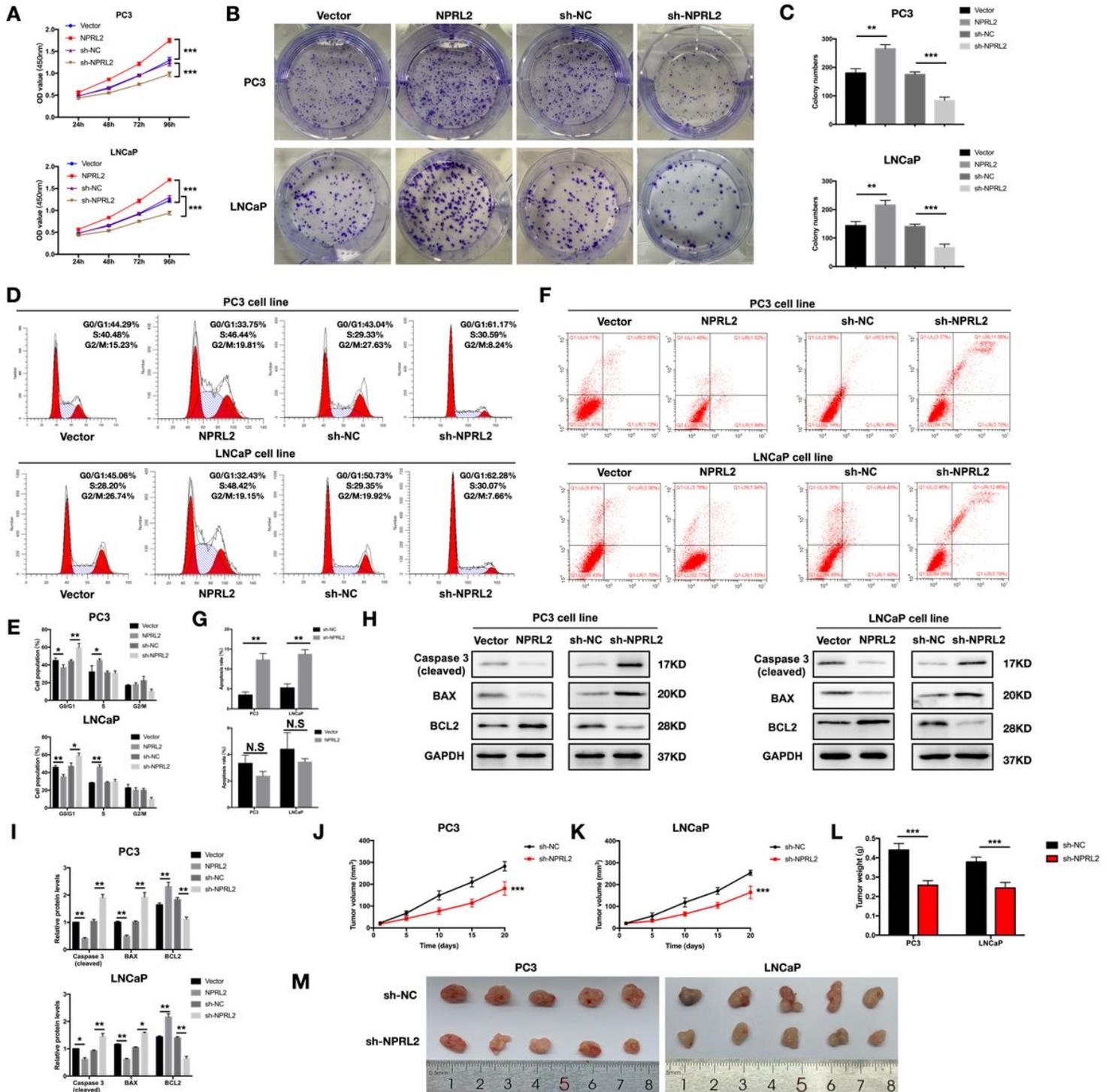
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**Figure 2**

NPRL2 promotes PCa cell proliferation in vitro and in vivo. a CCK8 assays evaluated the proliferation of cells with different transfected vectors. b, c Colony formation were performed to detect the proliferation

activities. d-g The cell cycle and apoptosis rate were analyzed by flow cytometry. h, i The apoptosis-related proteins were detected by western blot. j, k Tumor volume in nude mice. l Weight of xenograft tumors when they were harvested. m Photographs of xenograft tumors. Significance: \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, N.S, nonsignificant

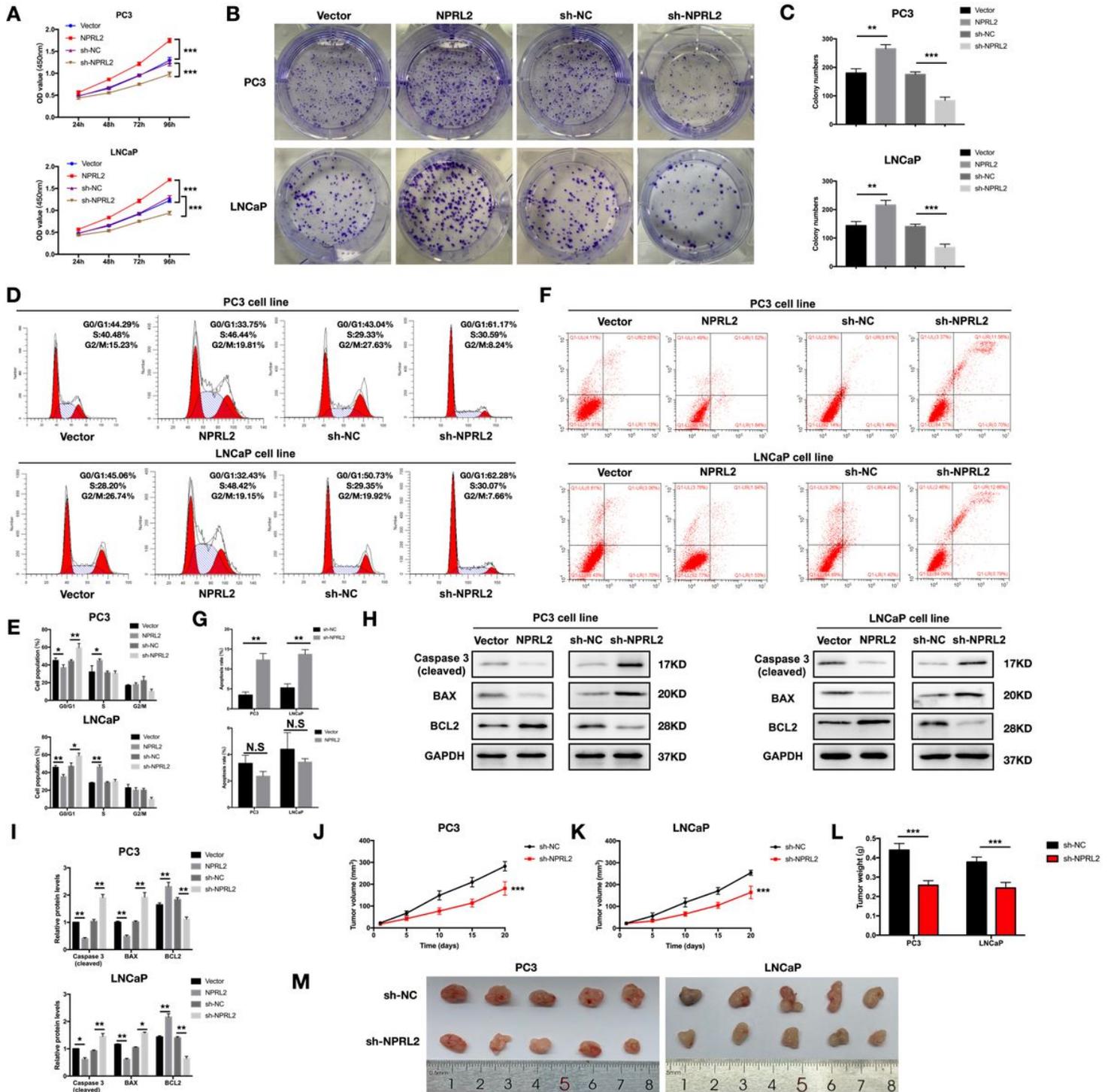
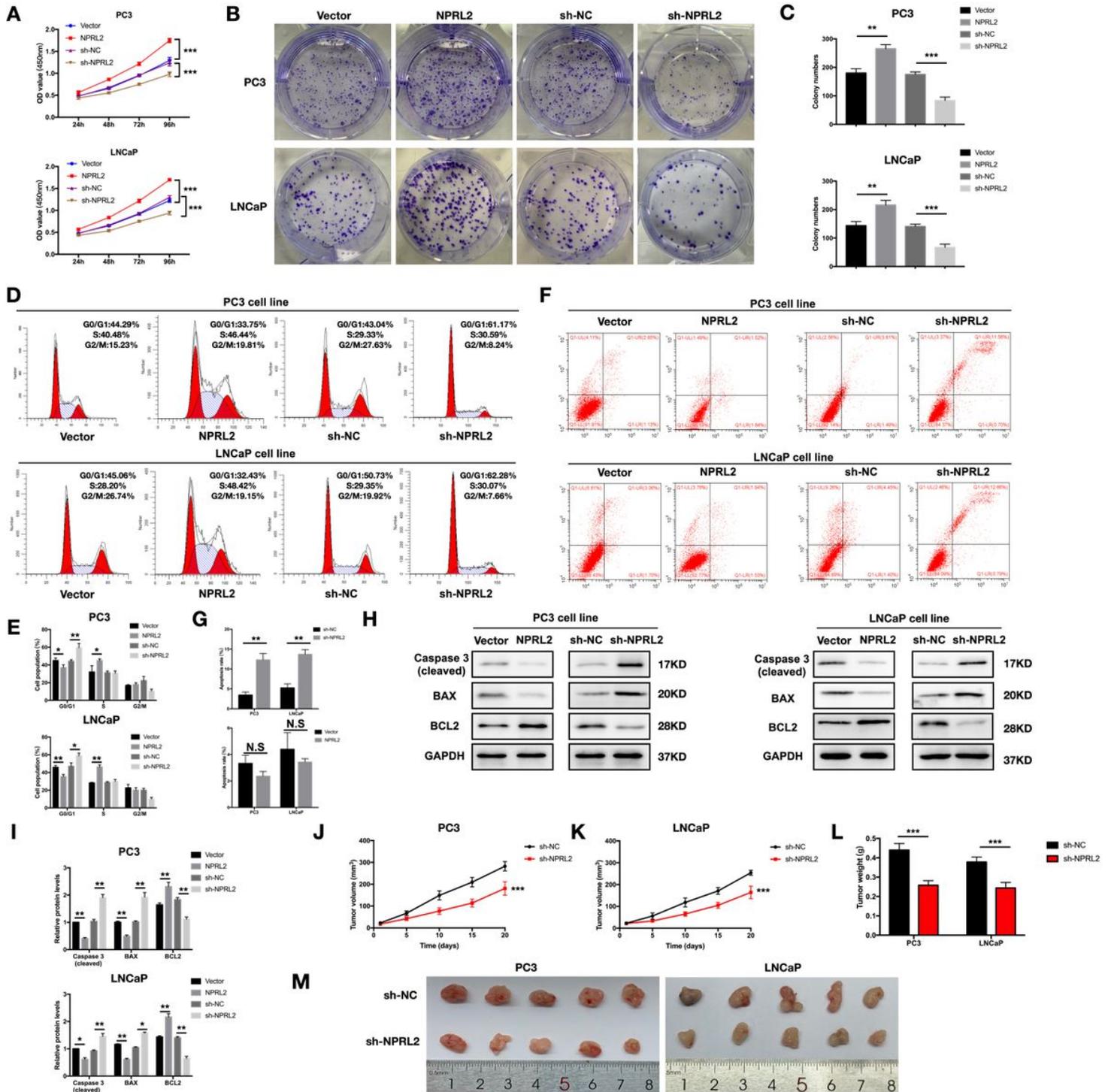


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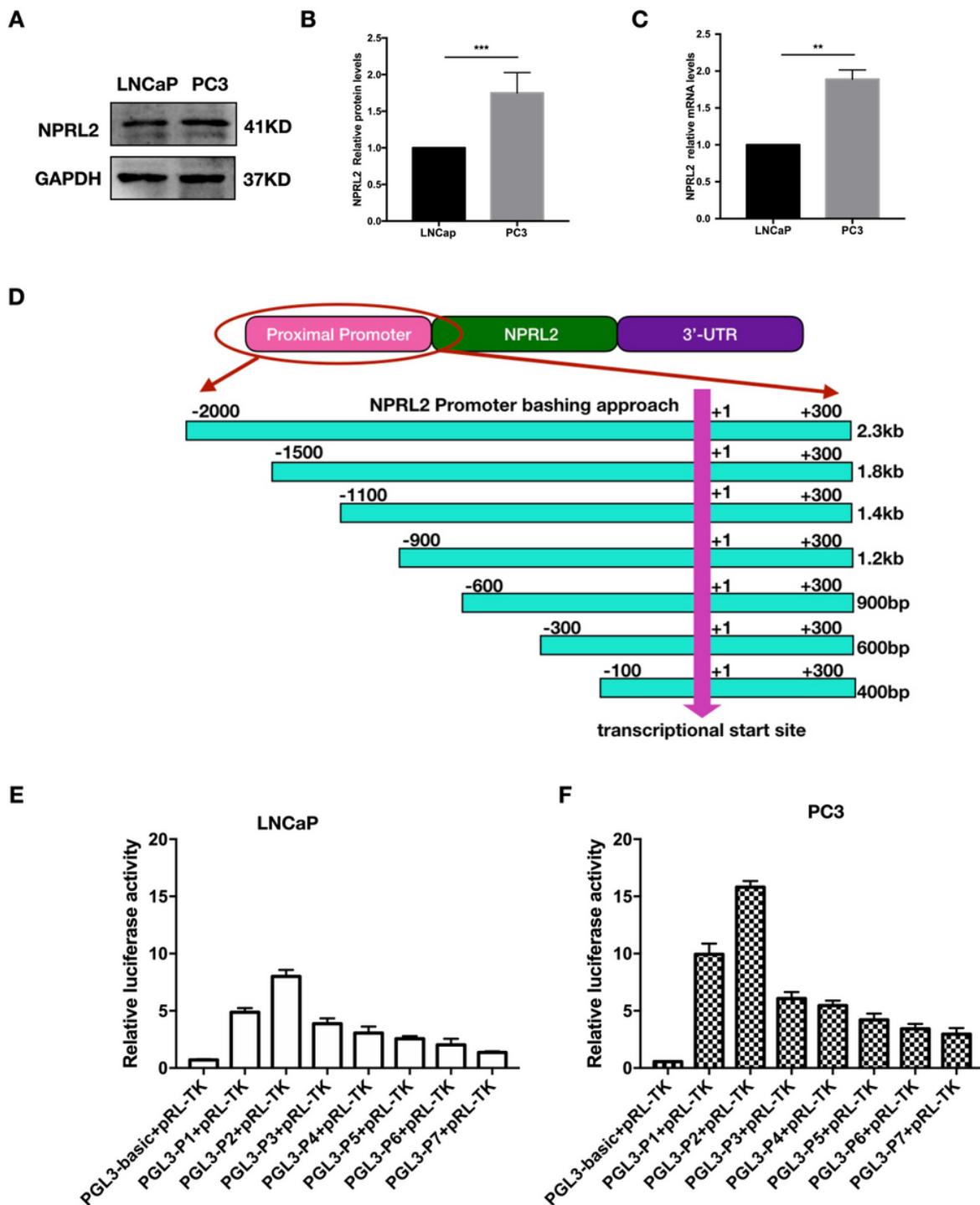
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**Figure 3**

NPRL2 expression levels, and identification of a 5' regulatory region for NPRL2. a NPRL2 protein expression. b Quantitation of data for (a). c NPRL2 mRNA levels detected by Quantitative PCR. d

Schematic diagram of deletion sequence on NPRL2 5' region. e, f Reporter activity of NPRL2 5' deletion region reporter. Significance: \*\*P < 0.01, \*\*\*P < 0.001

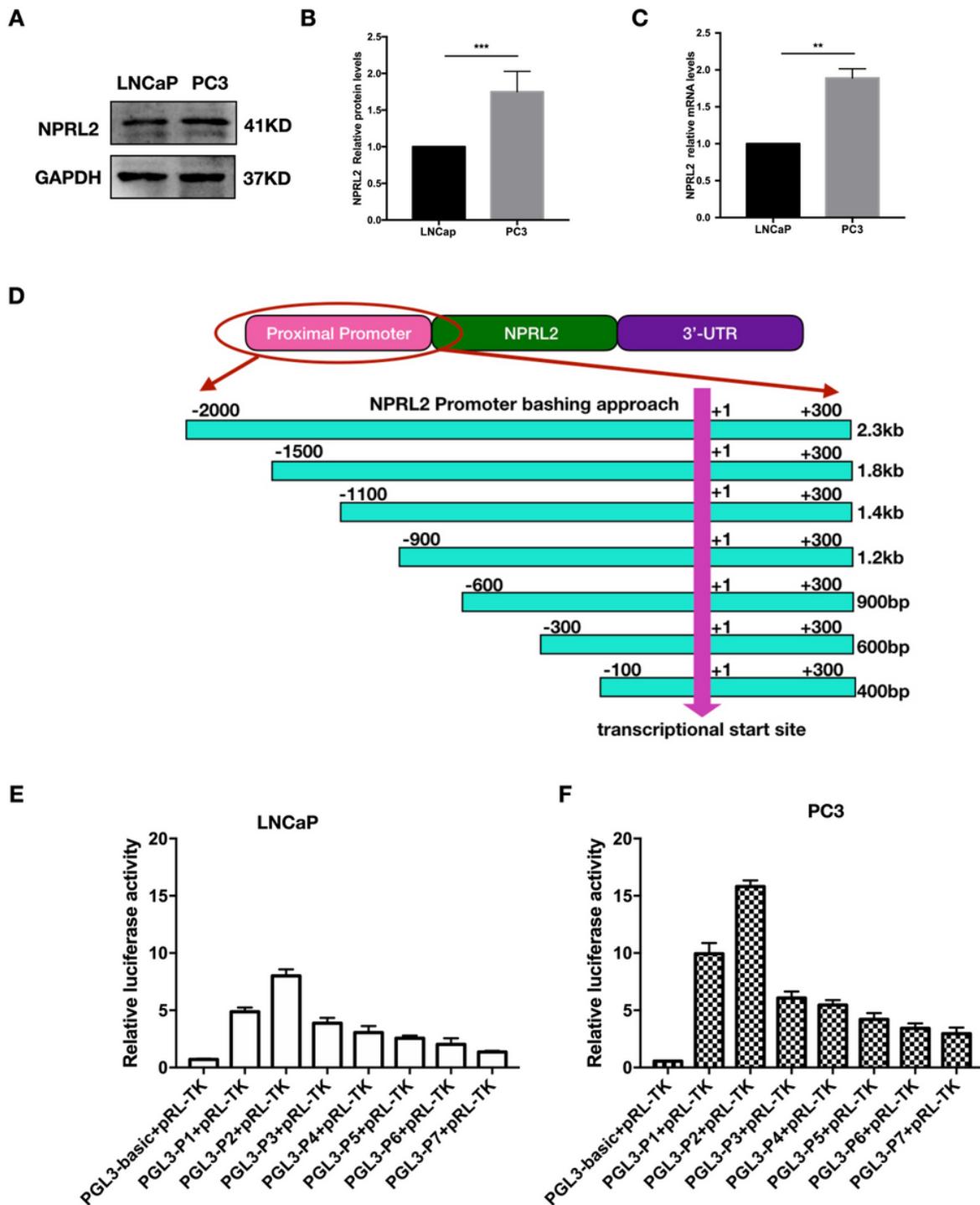
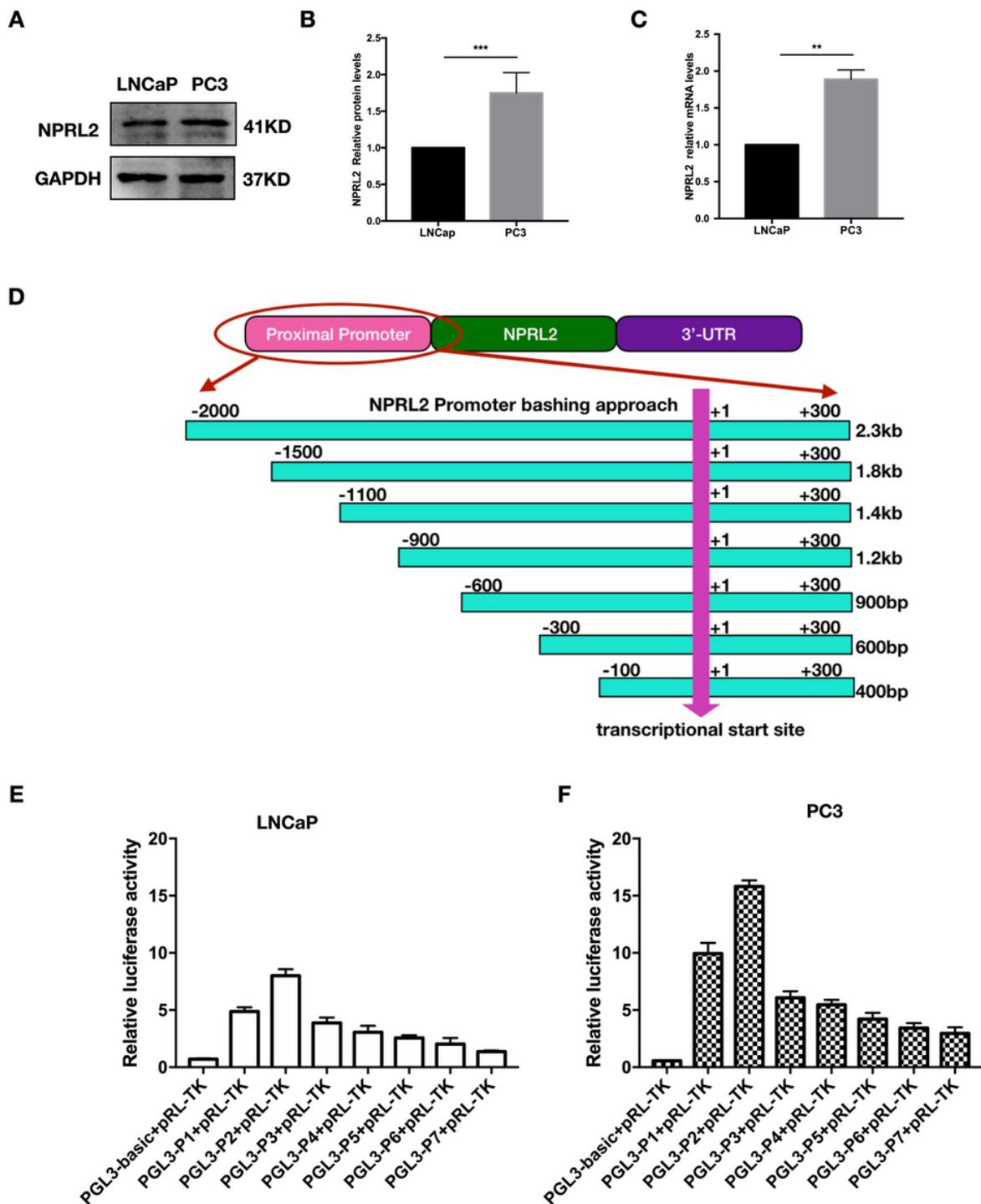


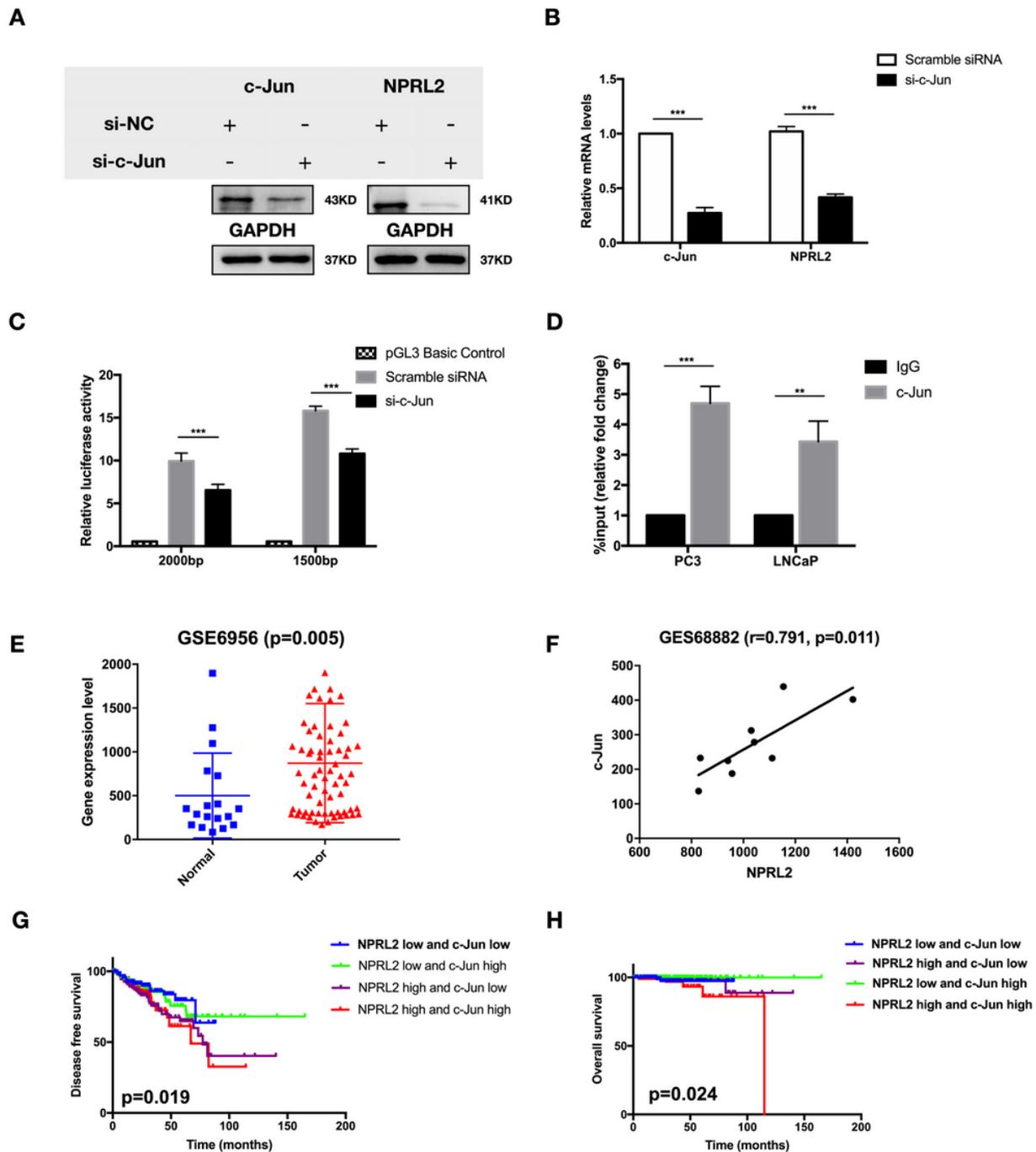
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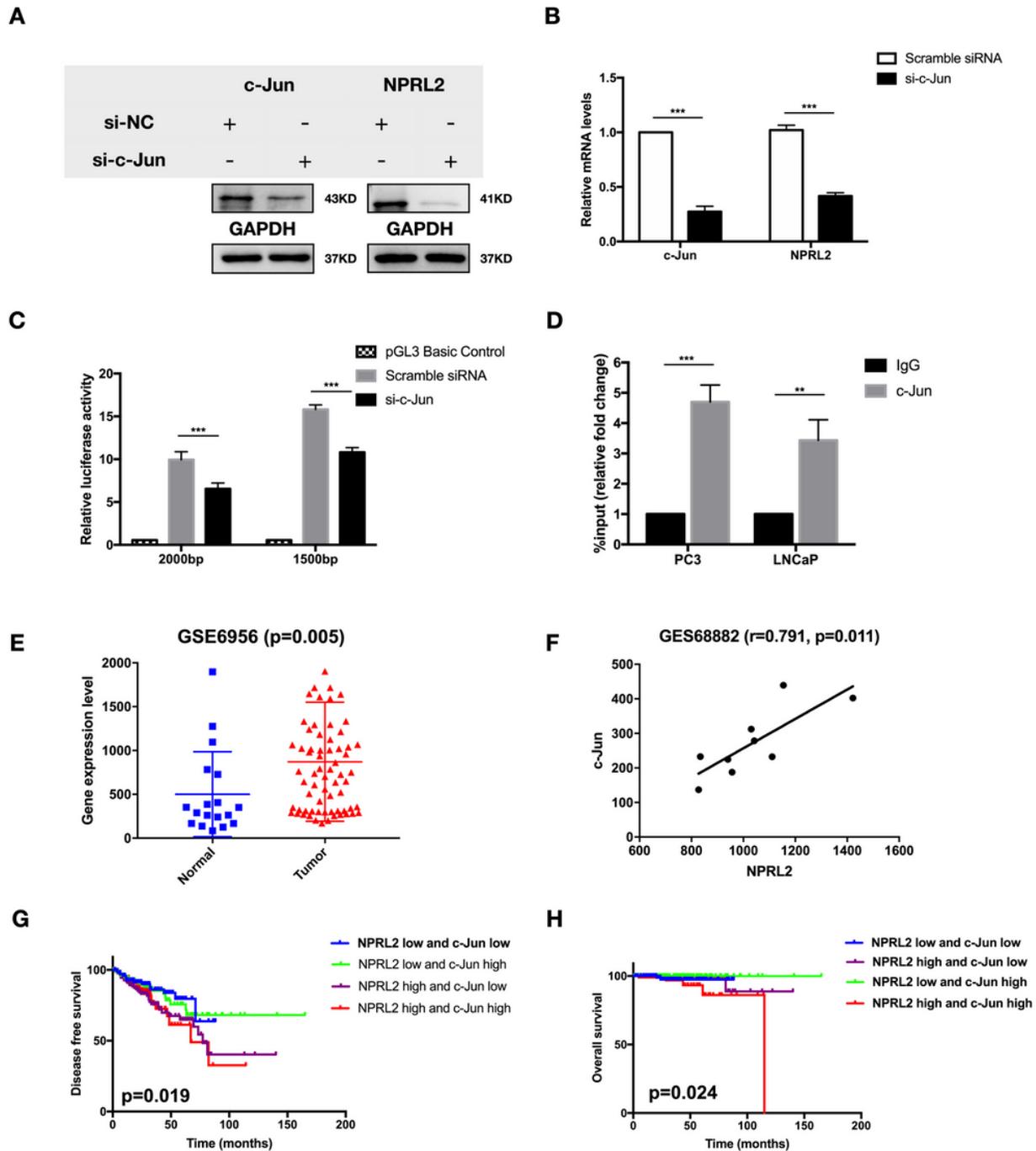
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**Figure 4**

Identification of c-Jun as an important transcription factor regulating the expression of NPRL2. a-c c-Jun knockdown suppresses NPRL2 protein expression, mRNA expression, and 5' upstream region activity in the PC3 cell line. d ChIP assays detected that c-Jun directly bind to the upstream sequence of NPRL2 and quantified by RT-qPCR. e c-Jun mRNA expression was higher in prostate cancer tissues than normal tissues in the GSE6956 database. f NPRL2 and c-Jun expression levels had a positive correlation in the

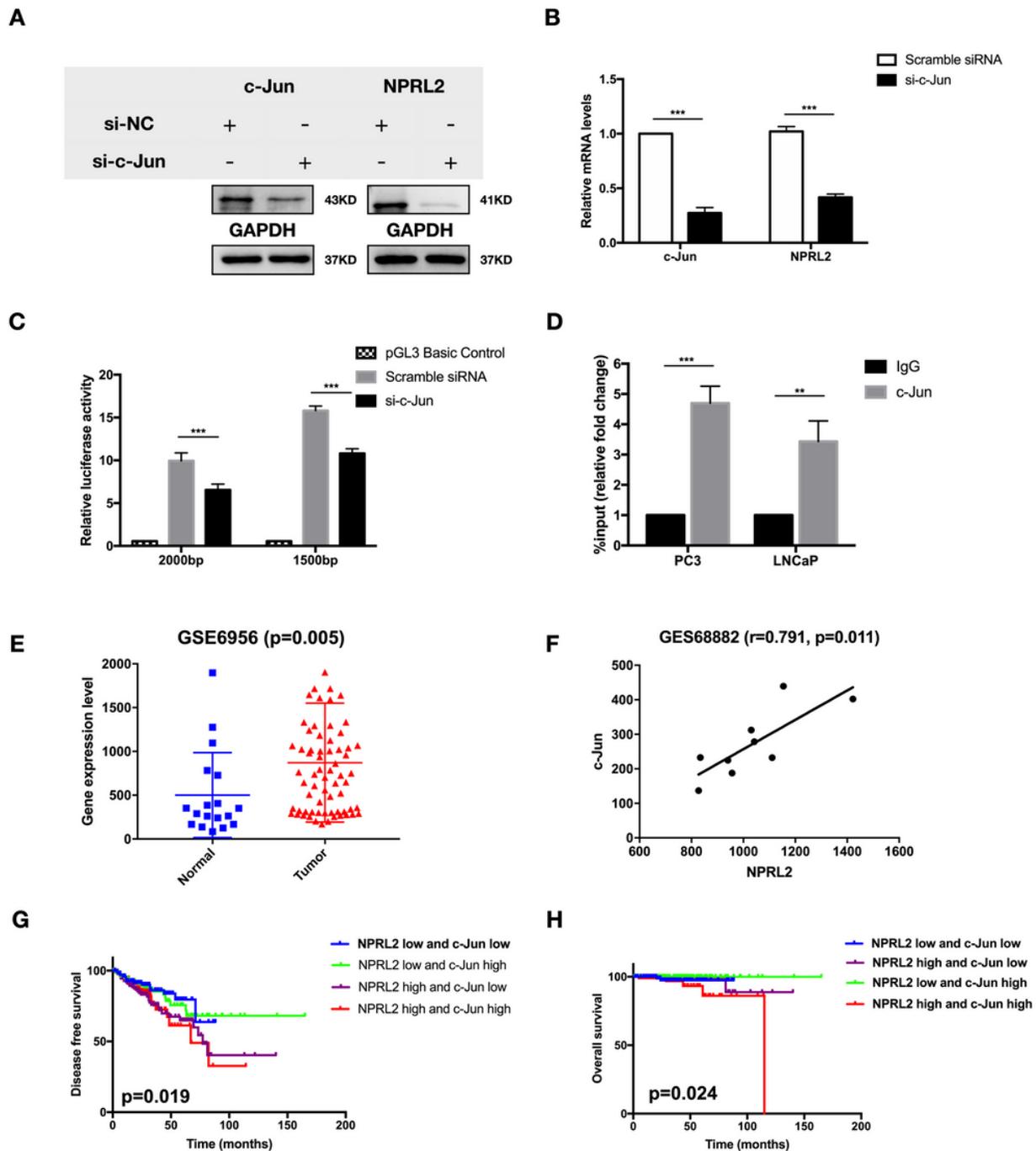
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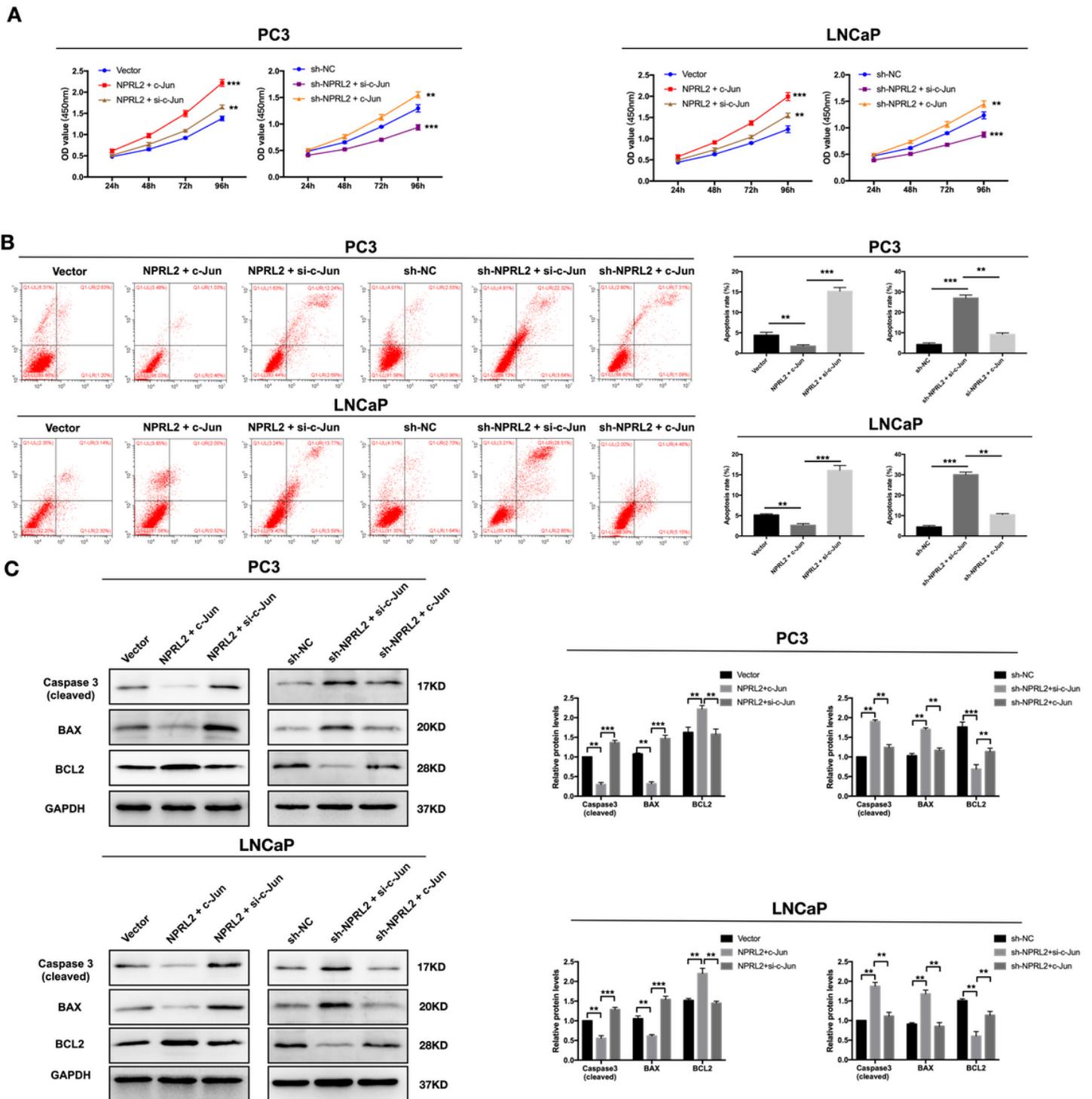
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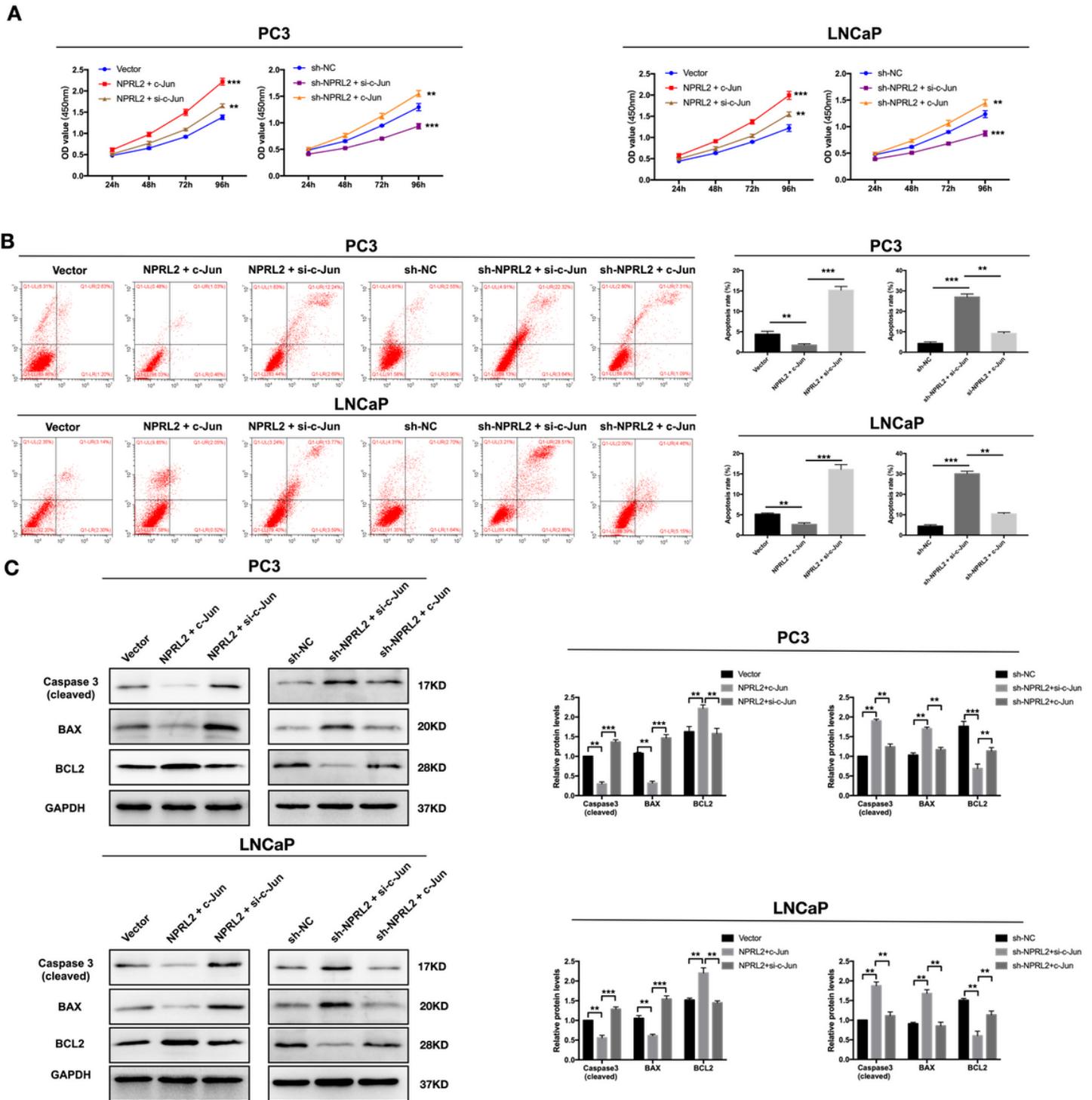
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Silencing and overexpression of c-Jun impact NPRL2-dependent PCa cell proliferation. a Effect of NPRL2 and combined with c-Jun transfected with indicated vectors in proliferation measured by CCK8 assays. b

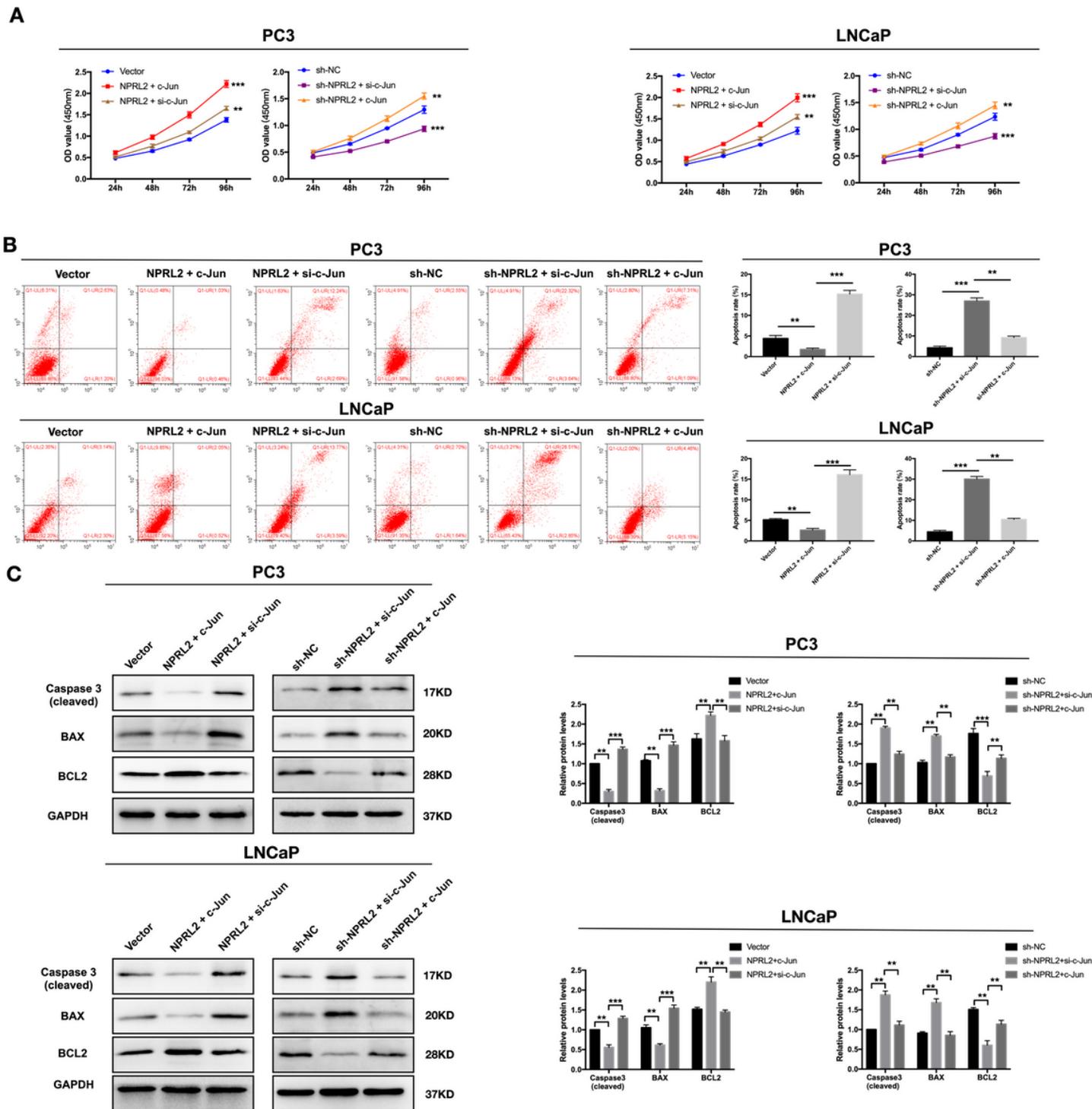
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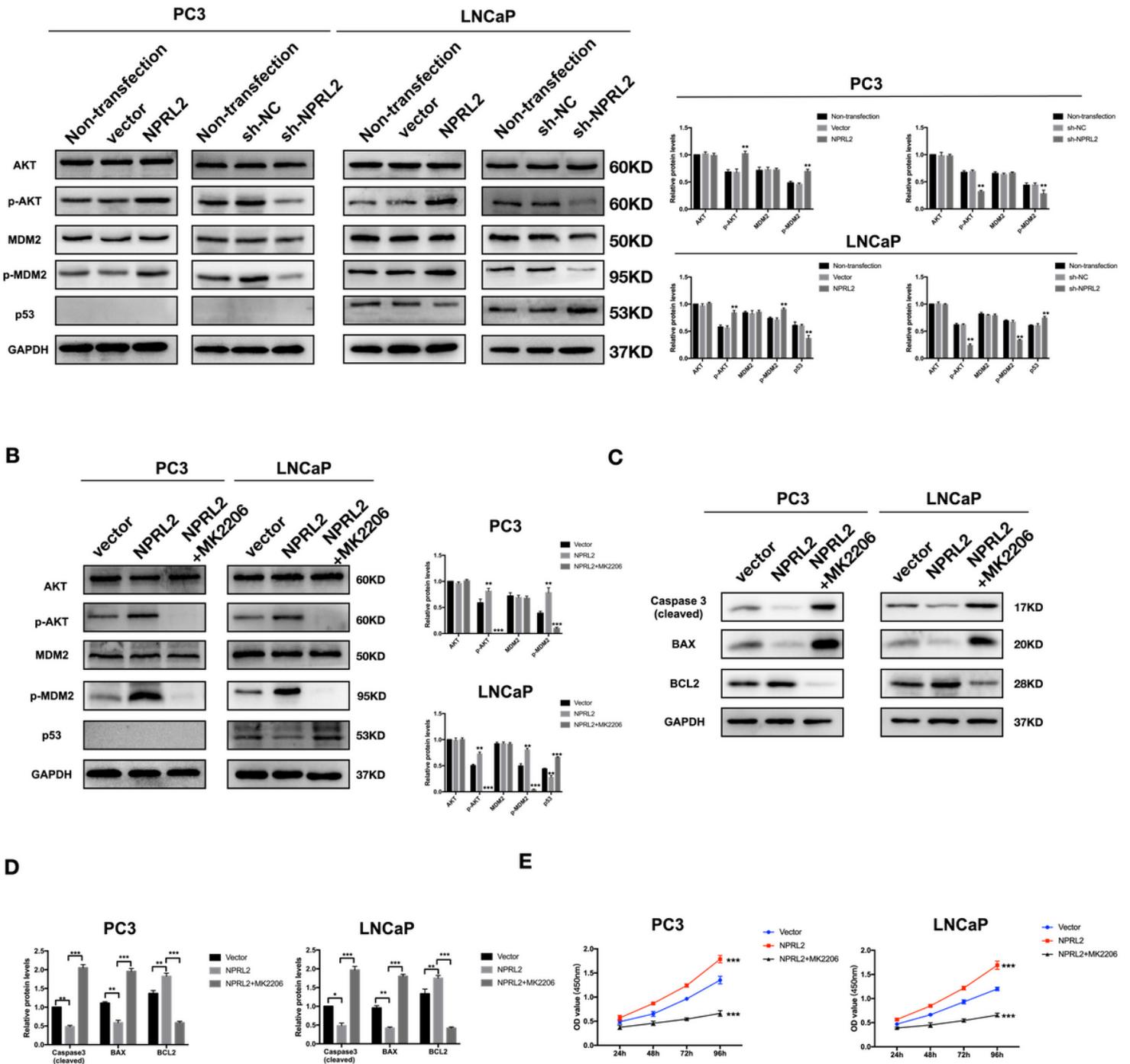


Figure 6

NPRL2 promotes proliferation via activating AKT/MDM2/p53 axis. a Western blotting analysis was used to determine the effect of NPRL2 overexpression and knockdown on AKT, p-AKT, MDM2, p-MDM2, and p53 expression levels in PC3 and LNCaP cells. b The effect of MK2206 (AKT inhibitor, 5  $\mu$ M) on AKT/MDM2/p53 pathway proteins with or without NPRL2 overexpression. c, d Western blot analysis

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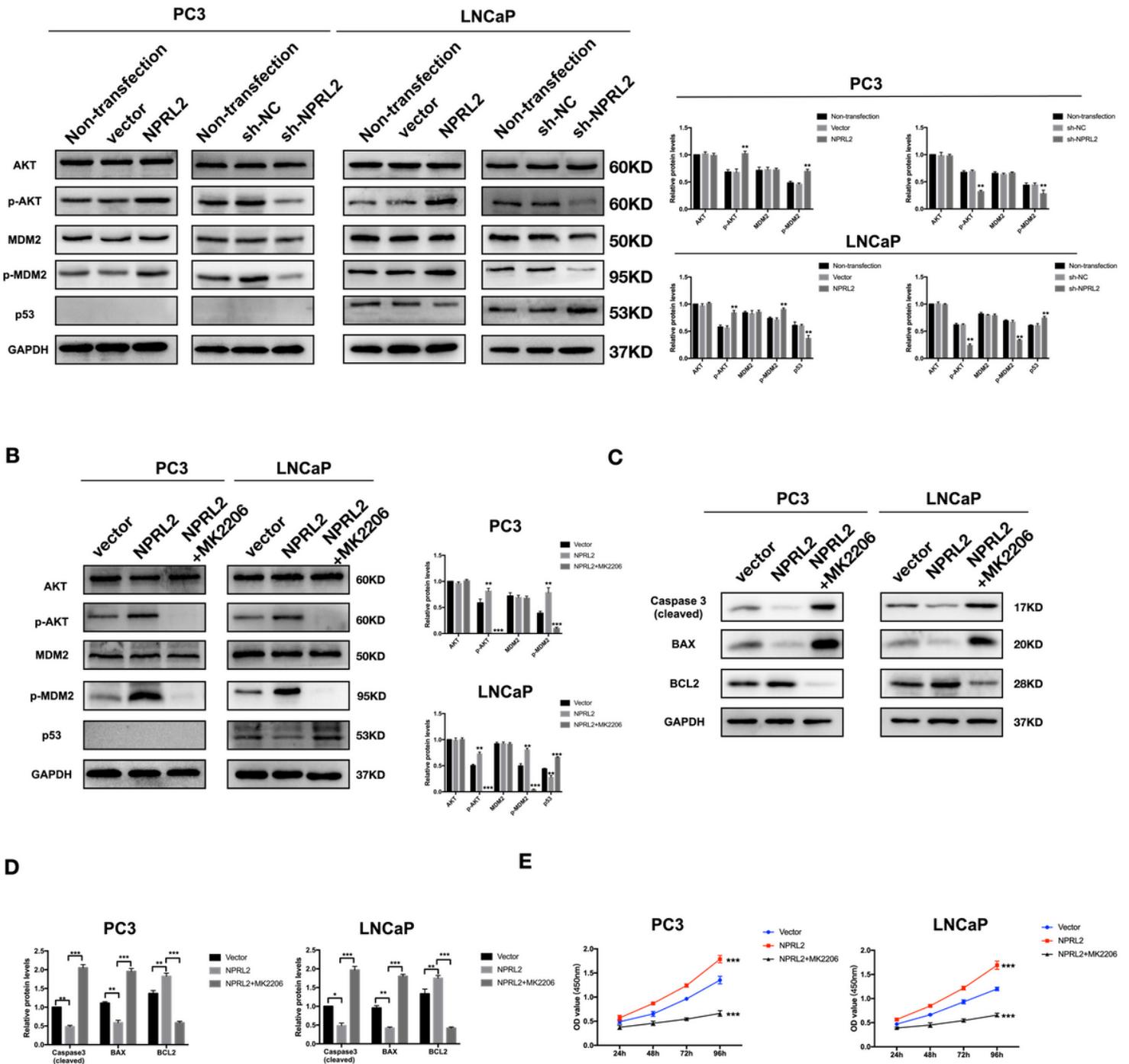


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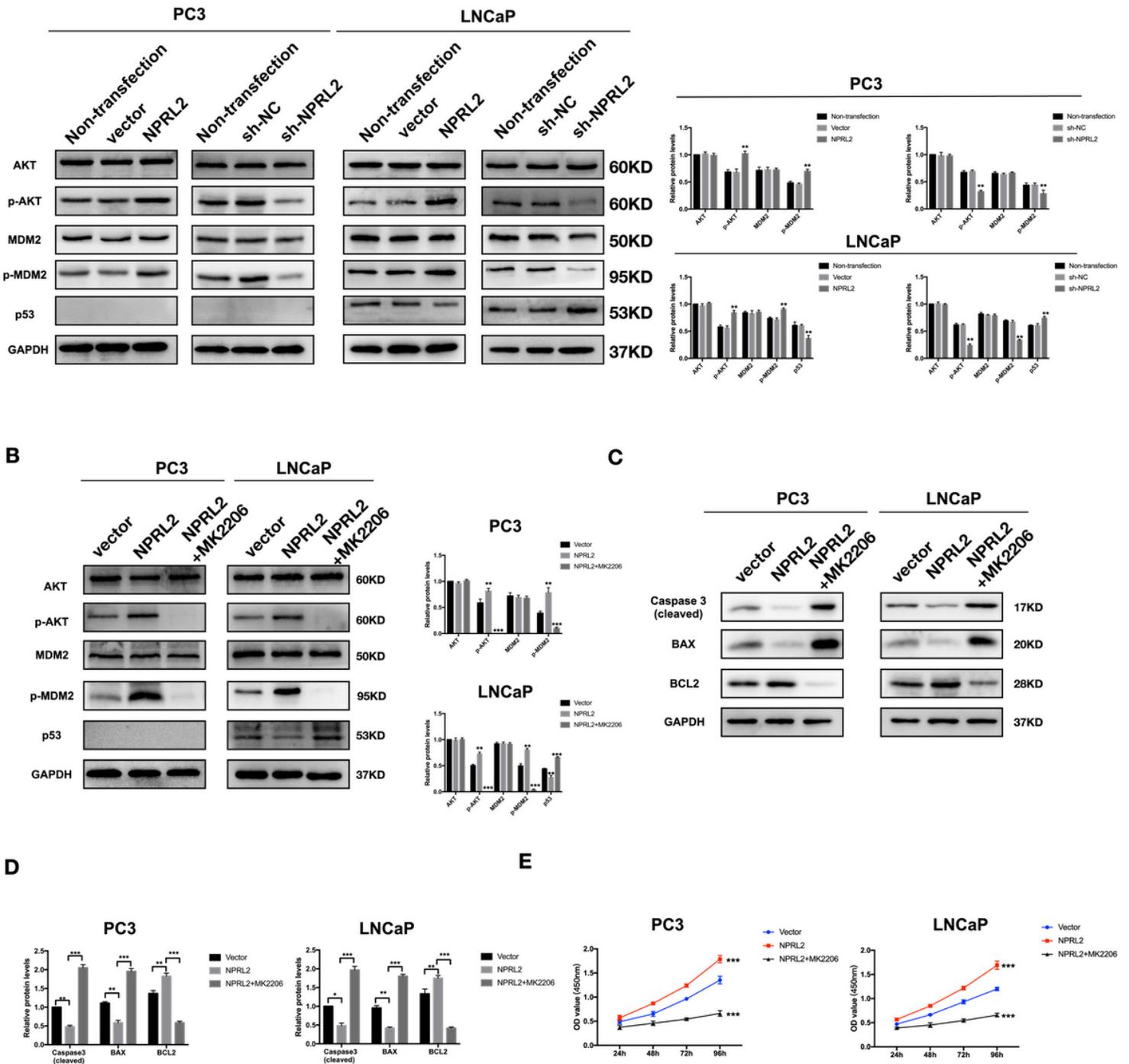
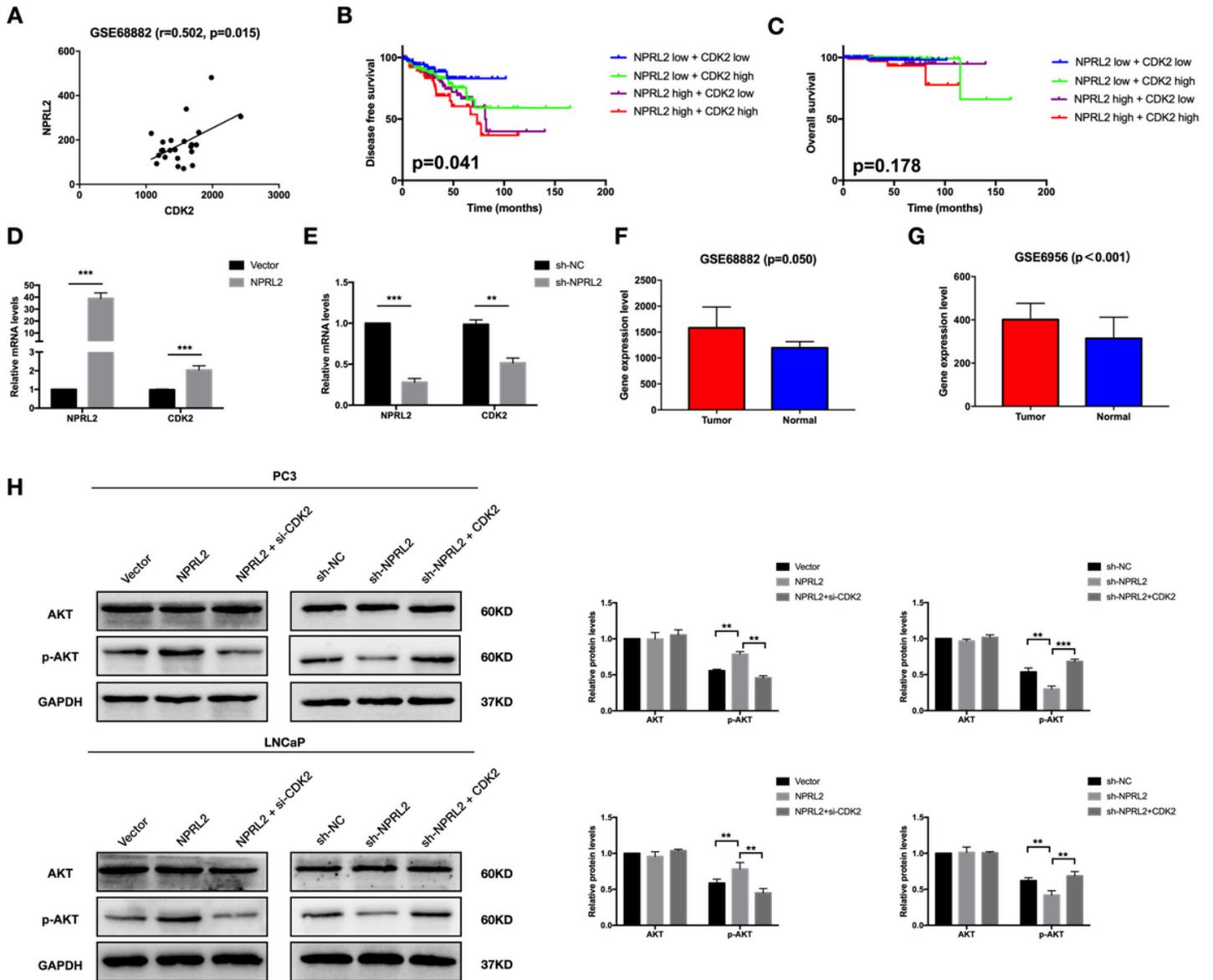


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**Figure 7**

CDK2 mediates the effect of NPRL2 on the p-AKT. a The correlation between NPRL2 and CDK2 expression PCa in the GSE68882 database. b, c Association between disease-free survival and overall survival and NPRL2 mRNA and CDK2 expression in the PCa from the TCGA database. d NPRL2 overexpression upregulated CDK2 mRNA expression in the PC3 cell line. e NPRL2 knockdown suppressed

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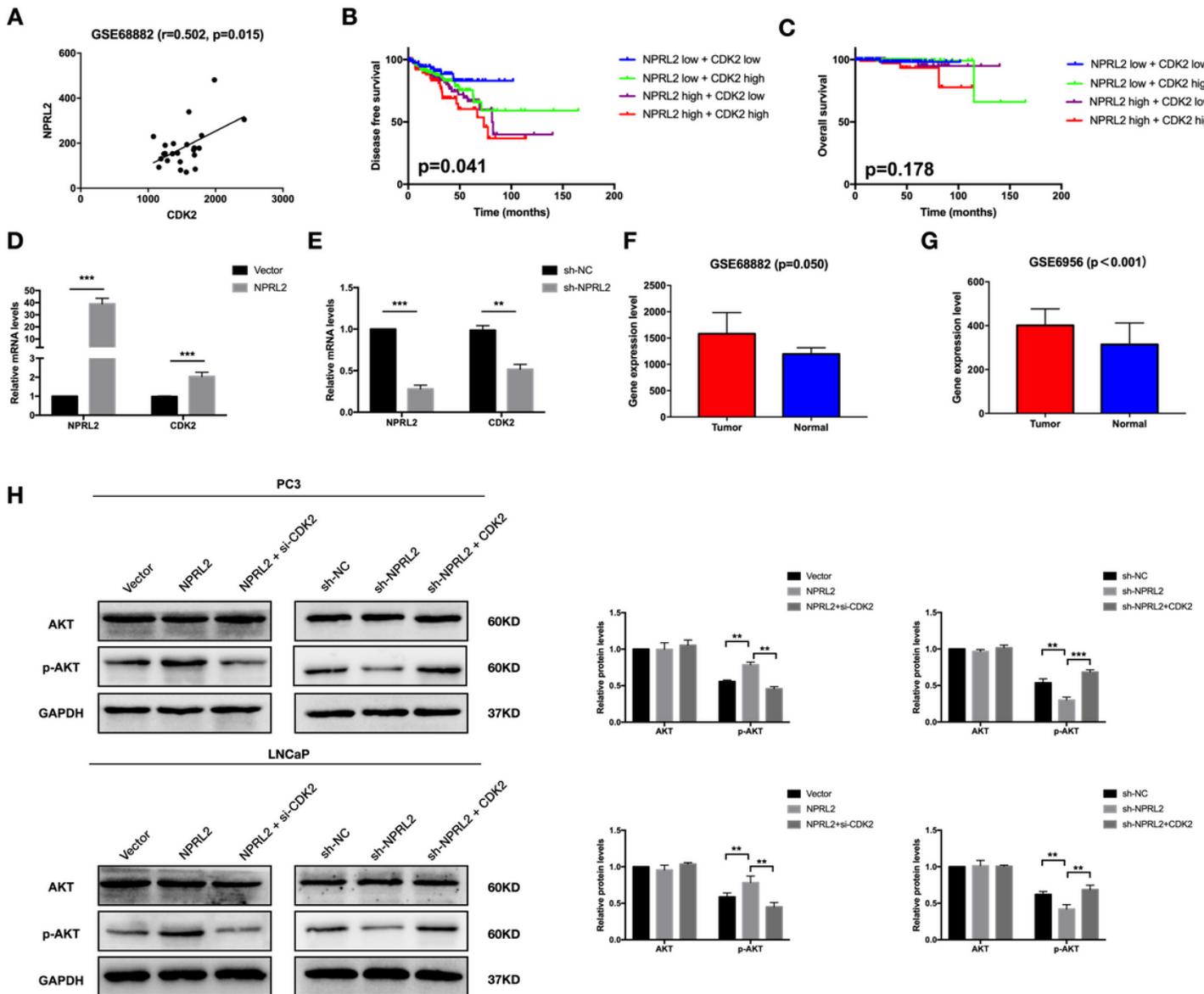


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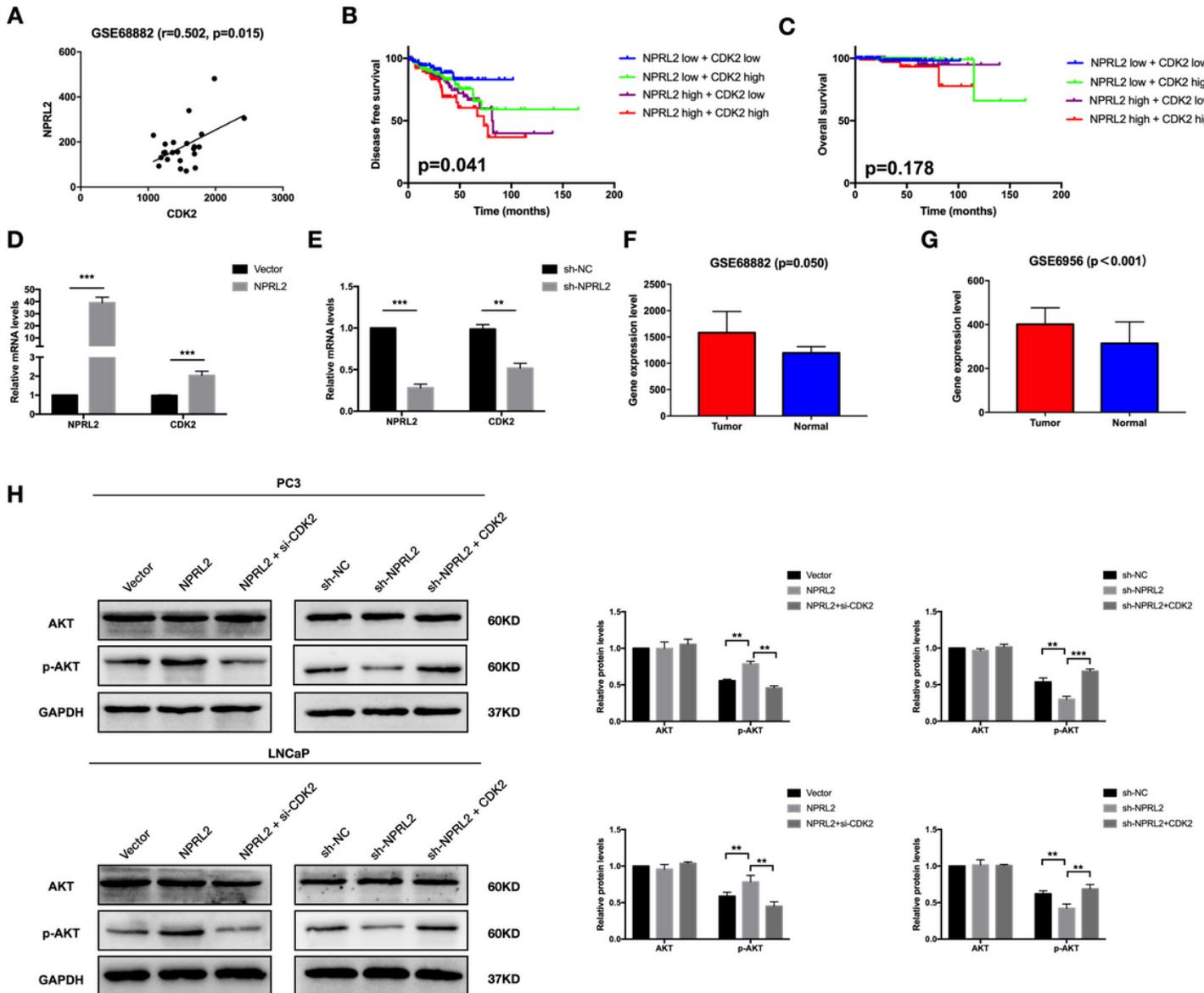


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