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NCAPD3 contributes to lung cancer progression through modulated lactate-induced histone lactylation and MEK/ERK/LDHA axis

Zhibo Chang[1*]

Abstract

Lung cancer (LC) is one of the most common malignant tumors globally. Non-SMC condensin II complex subunit D3 (NCAPD3) has been involved in the progression of many kinds of tumors. However, the effects of NCAPD3 in LC remain unclear. NCAPD3 expression was investigated by the Ualcan database and using Western blot. The effect of NCAPD3 on prognosis was explored via the Kaplan-Meier plotter database. Cell viability, colony formation, apoptosis, and Transwell assays, and in vivo tumorigenesis were performed to reveal the biological roles of NCAPD3. Glycolysis was assessed via measurement of glucose consumption, extracellular acidification rate (ECAR), lactate production, and ATP levels. The deeper mechanisms of NCAPD3 were investigated by Western blot and rescue experiments. Upregulation of NCAPD3 levels in LC tissues was found in Ualcan and significantly associated with poor prognosis. The expression of NCAPD3 was up-regulated in LC cell lines compared to BEAS-2B cells. Knockdown and overexpression experiments suggested that proliferation, apoptosis, migration, invasion, and glycolysis were regulated by NCAPD3 via the MEK/ERK/LDHA pathway. Additionally, NCAPD3 knockdown inhibited tumor growth in vivo. Mechanistically, NCAPD3 overexpression-mediated activation of the MEK/ERK/LDHA pathway and proliferation, Glucose uptake, and glycolysis were attenuated by MEK inhibitor U0126. Also, histone lactylation helps in tumorigenesis by promoting NCAPD3 expression. Taken together, our results revealed that histone lactylation of NCAPD3 promoted proliferation, migration, invasion, and glycolysis through modulating the MEK/ERK/LDHA signaling pathway in LC, which highlights a novel understanding of NCAPD3 in LC.

Keywords Lung cancer, NCAPD3, Glycolysis, Histone lactylation

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Introduction

Lung cancer (LC) is the second most common cancer in the world, and studies reveal that it is the leading cause of cancer morbidity and mortality in men [1], and its incidence in the population continues to rise by 4.5% per year, posing a serious threat to human health [2]. Non-small-cell lung cancer (NSCLC) is the most common form of lung cancer, accounting for about 85% of all lung cancer cases [3]. Surgical resection, targeted therapy, immunotherapy, and novel adjuvant chemotherapy are currently the most effective treatments for NSCLC, but they are only effective for patients with stage I or II early-stage NSCLC, and there are some adverse effects [4]. However, these regimens are only effective for patients with stage I or II early-stage NSCLC and have some adverse effects [4]. Most of the patients are already in advanced stages at the time of diagnosis, and the 5-year survival rate is only 13.8% due to recurrence and metastasis [5]. Therefore, in addition to enhancing the early diagnosis of NSCLC, it is important to explore more effective therapeutic options and drugs targeting the molecular mechanisms associated with NSCLC progression to improve the prognosis of NSCLC patients.

Lactylation is a newly discovered post-translational modification of proteins in recent years [6]. It is a type of protein modification in which the lactyl group is covalently coupled with lysine residues of proteins to promote gene regulation, and is therefore also known as histone lysine lactylation (Kla) [7]. However, the biological function of this lactate modification remains unknown. In 2019, Zhang et al. [8]. found that histones in human and mouse cells can undergo lactate modification, and discovered that histone lactate modification can affect gene expression regulation in chromatin and act as an important epigenetic regulator in many cellular processes, which also pointed out a brand new direction for the study of the metabolism-epigenetic axis.

In recent years, some new non-metabolic functions of lactate have been discovered, including the regulation of macrophage polarization, T-cell activation, angiogenesis, and tumor growth [8–11]. Meanwhile, lactate is an important carbon-containing metabolite of the cellular glycolysis pathway, and its biological functions have received widespread attention due to the Warburg effect in tumor cells [11–13]. It is noteworthy that the Warburg effect is typically characterized by the production of large amounts of lactic acid by tumor cells, and it is not difficult to imagine that the level of protein lactation may also be significantly up-regulated in tumors. As a result, Luo et al. found that lactylation of HIF1α activated KIAA1199 transcription that promoted angiogenesis in prostate cancer [14]. Also, Gu et al. illustrated that lactate contributes to tumorigenesis through lactylation of MOESIN in regulatory T (Treg) cells [15]. Consequently, histone lactylation seems extremely likely to be abnormal in cancers, and investigating the role of possible targets with lactylation in carcinogenesis is intriguing.

As we know, the correct segregation of the genome during cell division is a prerequisite for the survival of all organisms, and the condensin complex is the main controller of this process [16]. Abnormal function or expression of subunits in the condensin complex has been found to be closely related to the development of cancer [17–18]. Non-SMC condensin II complex subunit D3 (NCAPD3) is localized at 11q25, contains 37 exon sequences, has a total length of 1498 amino acids, four HEAT repeats, and a coiled-coil structural domain. NCAPD3 is one of the non-structural maintenance of chromosomes (SMC) regulatory subunits of condensin II, which plays an important role in mitosis, mainly responsible for chromosome condensation and segregation [19, 20]. More importantly, Jing et al. reported that overexpressed NCAPD3 enhanced cellular aerobic glycolysis to promote cell growth in colorectal cancer [21]. Additionally, previous researchers have confirmed that NCAPD3 participated in the development of prostate cancer [20], diffuse large B-cell lymphoma [22], and pancreatic ductal adenocarcinoma [23]. However, there is a lack of studies of NCAPD3 in lung cancer and the underlying mechanism of NCAPD3 associated with lactylation remains mostly unknown. Hence, there is an urgent need to further investigate the biological functions and underlying mechanisms of NCAPD3 in lung cancer and develop promising therapeutic targets.

To explore the roles of NCAPD3, in this study, we first analyzed the gene expression levels and the prognostic reference value of NCAPD3 in lung cancer by using bioinformatics approaches. Then, we found the up-regulation of NCAPD3 in our LC cell lines and further assessed its biological function and potential molecular mechanism in vitro and in vivo. Generally, we aimed to identify the roles of NCAPD3 in lung cancer, which may highlight a possible target for diagnosis and treatment of lung cancer in the clinic.

Materials and methods

NCAPD3 expression analysis and Kaplan-Meier plotter analysis

The UALCAN database (https://ualcan.path.uab.edu/index.html) [24] was used to analyze the expression levels of NCAPD3 between lung squamous cell carcinoma (LUSC) primary tumor and normal tissues, as well as the expression of NCAPD3 in LUSC based on individual cancer stages. Furthermore, the potential prognostic value of NCAPD3 expression in LC patients was assessed by using a meta-analysis tool, Kaplan-Meier Plotter (http://kmplot.com/analysis/index.php?p=background) [25].

Cell culture and transfection

A human-immortalized lung epithelial cell line (BEAS-2B) and six human LC cell lines (HCC827, H460, PC-9, H1975, A549, and H1299) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cell lines in RPMI1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco), 100 IU/ml streptomycin, and 100 IU/ml penicillin were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. For the cell transfection, the specific NCAPD3 small interfering RNAs (si-NCAPD3 #1, #2, #3) and negative control (si-NC), and corresponding overexpression plasmids targeting NCAPD3 were purchased from GenePharma (Shanghai, China). Next, the oligonucleotides were transfected into the H1975 cell and the plasmids were transfected into HCC827 cells using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instruction. After incubation for 48 h, the protein expression levels of NCAPD3 in transfected cells were detected by western blot assays.

Cell viability and colony formation assay

For cell viability assays, the transfected H1975 and HCC827 cells (3×10^3/well) were cultivated in 96-well plates with or without oxamate (20 mM, Shanghai Yuanye Biotechnology Co., LTD, China) and lactate (5 mM, Yuanye, Shanghai, China) treatment and the absorption value at 450 nm was recorded at appointed time 24 h, 48 h, 72 h, and 96 h by using by a microplate reader (CMaxPlus, Molecular Devices, USA). For colony formation assays, transfected cells (800 cells/well) with or without U0126 (50 μM, Beyotime Biotechnology, China) were seeded into 12-well plates. After 14 days, the colonies were fixed with 4% paraformaldehyde (Sangon, China) for 1 h and then stained with 0.1% crystal violet solution (Shanghai Qiangshun Chemical Reagent Co., Ltd, China). Finally, the number of colonies was quantified by ImageJ software.

Cell apoptosis assay

The transfected H1975 and HCC827 cells were collected, washed, and suspended in binding buffer and then stained with 5 μL of Annexin V-FITC and 10 μL propidiumiodide (Beyotime, China) in a dark environment for 30 min. After that, the apoptosis of cells was measured by flow cytometry (BD Biosciences, USA) and analyzed by FlowJo software (FlowJo LLC).

Transwell assay

To investigate the effect of NCAPD3 on the migration and invasion ability of LC cells, the transwell assays were performed by using Transwell chambers (Corning, NY, USA). For the invasion assay, the upper chambers were covered with Matrigel (BD Biosciences CA, USA), but not in the migration assay. The transfected H1975 and HCC827 cells were cultivated in the upper chamber with or without oxamate and lactate treatment in 200 μL serum-free RPMI-1640 medium. A total of 600 μL medium with 20% FBS was added into the lower chamber. After incubation for 24 h, the cells on the lower surface of the chamber were fixed with 4% methanol for 10 min and then stained with 0.1% crystal violet for 15 min. Finally, the images were captured by a light microscope (ICX41, Sunny Optical Technology (group) co., Ltd., Xianggang, China).

Glucose uptake assay

The transfected H1975 and HCC827 cells were placed into 24-well plates at a density of 5×10^5 cells per well overnight. After that, the transfected HCC827 cells with or without U0126 treatment were also starved for 4 h in the glucose-free medium. Next, a total of 100 μM fluorescent glucose analog (2-NBDG, Sigma) was added into the culture medium for 2 h. Then, the cellular fluorescence was analyzed and quantitated by Agilent NovoCyte flow cytometry (Agilent, USA).

Detection of lactate production and ATP levels

The lactate production and ATP levels in the transfected H1975 and HCC827 cells with or without U0126 treatment were assessed using a Lactic Acid (LA) Content Assay Kit (Beijing Solarbio Science & Technology Co., Ltd, China) and ATP Assay Kit (Beyotime, China), respectively, according to the manufactures' instruction.

Glycolysis stress test

Extracellular Acidification Rate Test (ECAR) was used to estimate metabolic alternations in transfected H1975 and HCC827 cells by using Seahorse Bioscience XF-24 Extracellular Flux Analyzer (Agilent). Briefly, the NCAPD3-knockdown H1975 cells and NCAPD3-over-expression HCC827 cells were cultured in XF24-well microplates at a density of 20,000 cells/well for 24 h. Then, the cells were incubated in pyruvate- and glucose-free medium supplemented with 2 mM glutamine for 1 h followed by sequential addition of 10 mM glucose, 1 μM oligomycin (oxidative phosphorylation inhibitor), and 50 mM 2-deoxyglucose (2-DG, glycolytic inhibitor) from the Seahorse XF glycolysis stress test Kit (Seahorse Bioscience) according to the manufactures'instruction. At last, the basic glycolysis levels of transfected H1975 and HCC827 cells were analyzed using Wave 2.3 software and ECAR was shown in mpH/min.

Xenograft assay

A total of 14 male BALB/c (nu/nu) mice (age: 6–7 weeks, weight: 21–23 g) were purchased from Shanghai Slac Laboratory Animals Ltd (SCXK(Hu)2017-0005,

Shanghai, China) and kept in a controlled environment. For tumor growth assay in vivo, 1×10^6 (100 μL) H1975 cells with si-NCAPD3 or si-NC were separately injected subcutaneously into the right flank of the BALB/c mice. The tumor volumes were measured every 3 days and calculated as following the equation: Tumor volume (mm^3) = (width)×(height)^2/2. All the mice were sacrificed 21 days later. Finally, the tumor tissues were collected and weighted and then used for Immunohistochemistry (IHC) and Western blotting analyses. All animal protocols were approved by the Animal Experimentation Ethics Committee of Zhejiang Eyong Pharmaceutical Research and Development Center [(SYXK (Zhe) 2021-0003, Hangzhou, China)].

IHC assay

Collected tumor tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and spliced into 5 μm thickness. After that, the sections were incubated with primary antibodies anti-NCAPD3 (1:300, Bioss, Beijing, China), anti-LDHA (1:500, Bioss, Beijing, China), and anti-Ki67 (1:500, Affinity, Jiangsu, China) at 4 °C overnight and then incubated with corresponding HRP-conjugated secondary antibody (1:5000, ab97080, Abcam) at room temperature for 1 h. Next, the IHC reactions were developed with a diaminobenzidine (DAB) kit (Beyotime, China). And then, the sections were counterstained by Hematoxylin (Beyotime). Finally, the images were photographed under a microscope (200×, Nikon, Japan).

Western blot assay

Total protein from LC cell lines and tumor tissues was extracted using RIPA lysis buffer (Beyotime) and quantified with a BCA kit (Beyotime). Equal amounts of protein samples were separated through sodium dodecyl sulfonate-polyacrylamide gel (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare Life). Next, the membranes were blocked with 5% fat-free milk and then incubated with primary antibodies against NCAPD3 (1:1000, DF9413, Affinity, Jiangsu, China), Bax (1:1000, AF0120, Affinity), Bcl-2 (1:1000, AF6139, Affinity), Caspase-3 (1:1000, AF6311, Affinity), PCNA (1:2000, 10205-2-AP, proteintech), E-cadherin (1:1000, 3195T, Cell Signaling Technology), Vimentin (1:1000, ab20346, Abcam), MMP-2 (1:1000, 66366-1-Ig, proteintech, Proteintech Group, Inc, China), HK2 (1:1000, BF0283, Affinity), PKM2 (1:1000, AF5234, Affinity), GLUT1 (1:1000, AF5462, Affinity), LDHA (1:1000, bs-34202R, Bioss), p-MEK (1:1000, AF8035, Affinity), MEK (1:1000, AF6385, Affinity), p-ERK (1:1000, AF1015, Affinity), ERK (1:1000, 4695s, Cell Signaling Technology), Pan Kla (1:1000, PTM-1401, PTM-biolab, Hangzhou, China), H3K18la (1:1000, A18807, ABclonal Technology Co., Ltd., Wuhan, China), Histone H3 (1:1000, AF0863, Affinity), GAPDH (1:5000, D190090, Sangon, China), and β-actin (1:5000, 81115-1-RR, proteintech) overnight at 4 °C. After being washed with TBST for 3 times, the membranes were then incubated with horseradish-peroxidase (HRP)-linked goat anti-rabbit antibody (1:6000, #7074, Cell Signaling Technology) or HRP-linked goat anti-mouse antibody (1:6000, #7076, Cell Signaling Technology) for 1 h at room temperature. The protein bands were captured using the enhanced chemiluminescence assay kit (Beyotime) and quantified by the Image J software (V1.8.0.112, USA).

Statistical analysis

Data were shown as mean ± standard deviation (SD). Comparisons within groups were analyzed using one-way analysis of variance (ANOVA) or Student's *t-test*. $P < 0.05$ was considered to statistically significant difference.

Results

NCAPD3 is upregulated in LC and affects the proliferation capacity of H1975 and HCC827 cells

To evaluate the expression and prognostic value of NCAPD3, we used the UALCAN database and Kaplan-Meier Plotter. The results showed that the expression levels of NCAPD3 were significantly increased in primary tumors compared to that in normal tissues in LUSC patients (Fig. 1A). Additionally, the differences in the expression of NCAPD3 mRNA between stages of LUSC were observed in the UALCAN database (Fig. 1B). Meanwhile, as shown in Fig. 1C, the result of the Kaplan-Meier Plotter revealed that high NCAPD3 expression was significantly related to poor overall survival. Further, we found that NCAPD3 also exhibited a higher expression than the normal cell in the LC cell lines, especially in H1975 cells (Fig. 1D). Given that H1975 showed the highest level of NCAPD3 and HCC827 presented a relatively lower expression level among LC cell lines, we chose H1975 and HCC827 cells for subsequent functional experiments. To investigate the biological roles of NCAPD3, we knock-downed the expression of NCAPD3 in H1975 cells and over-expressed the levels of NCAPD3 in HCC827 cells, respectively (Fig. 1E). As revealed by CCK-8 assays, the cell proliferation of the H1975 cells was apparently decreased after a knockdown of NCAPD3 (Fig. 1F). Knockdown of NCAPD3 could also attenuate the colony-formation potential of H1975 cells (Fig. 1G). Moreover, up-regulation of NCAPD3 could remarkably promote the proliferation capacity of H1975 and HCC827 cells (Fig. 1F and G).

NCAPD3 is involved in apoptosis, migration and invasion in LC cells

As shown in Fig. 2A, flow cytometry assays found that inhibition of NCAPD3 remarkably promoted apoptosis

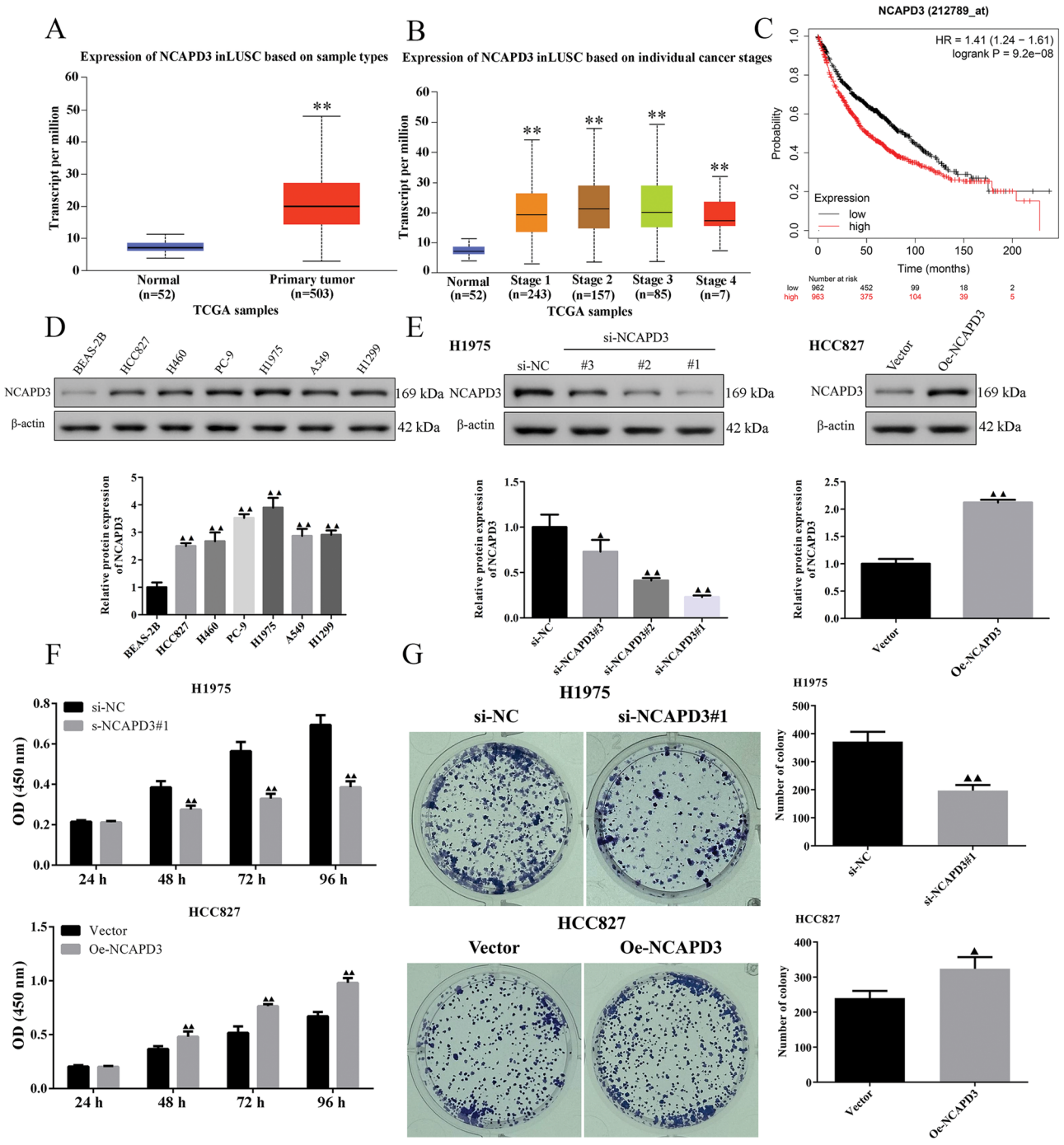

Fig. 1 NCAPD3 contributes to cell proliferation in LC cells. (**A**-**B**) NCAPD3 expression was raised in LUSC patients with different cancer stages using the UALCAN database. (**C**) Survival analysis of LUSC patients according to the mRNA expression of the NCAPD3 using the log-rank test in Kaplan-Meier Plotter. (**D**) Protein expression levels of NCAPD3 in LC cell lines were detected by Western blotting. (**E**) Western blot assay was used to measure the knockdown and over-expression efficiency in LC cells. (**F**) CCK-8 assay and (**G**) colony formation assay were applied to estimate the influences of knockdown and over-expression of NCAPD3 on the proliferation of H1975 and HCC827, respectively. $^{**}P < 0.01$, vs. normal group; $^{▲}P < 0.05$, $^{▲▲}P < 0.01$, vs. si-NC group or vector group

of H1975 cells but that of cell apoptosis in HCC827 cells with NCAPD3 overexpression was significantly decreased in this study. Moreover, the expression of proliferation/apoptosis-associated targets, including Bax and Caspase-3, was markedly upregulated after NCAPD3 knockdown, while Bcl-2 and PCNA levels were significantly decreased in NCAPD3-knockdown H1975 cells (Fig. 2B). Furthermore, Transwell assays showed that

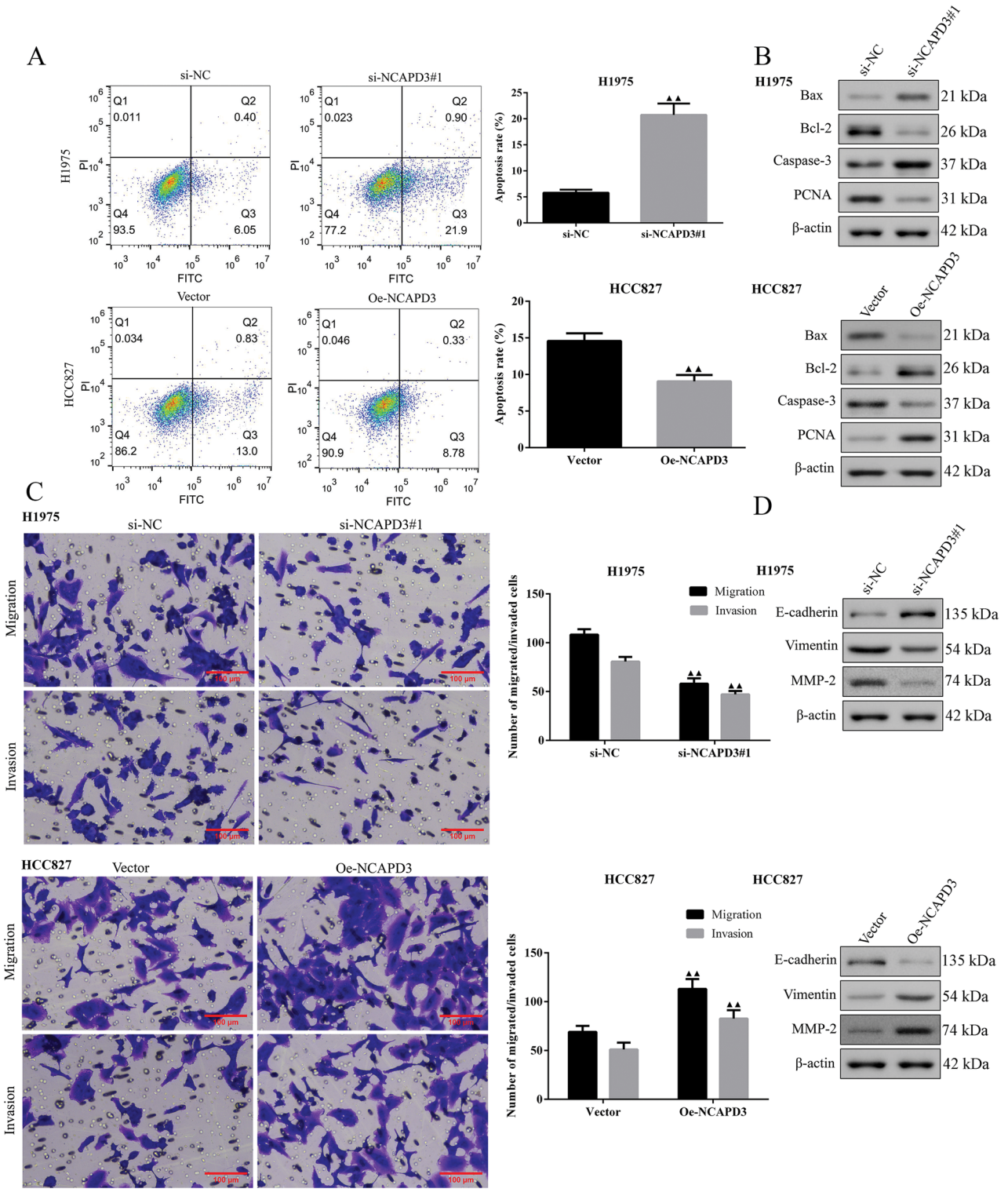

Fig. 2 NCAPD3 inhibits apoptosis and promotes migration and invasion in LC cells. (**A**) Cell apoptosis in H1975 transfected with NCAPD3 siRNA and HCC827 transfected with NCAPD3 overexpression plasmids was assessed by Flow cytometry. (**B**) The levels of cell apoptosis-associated proteins were determined by Western blotting in transfected H1975 and HCC827 cells. (**C**) Cell migration and invasion in LC cell lines H1975 and HCC827 were assessed by Transwell assay. Scale bar, 100 μm. (**D**) The protein expression levels of EMT-related targets were detected through western blotting after transfection in H1975 and HCC827 cells. ▲$P < 0.05$, ▲▲$P < 0.01$, vs. si-NC group or vector group

NCAPD3 knockdown significantly inhibited H1975 cell migration and invasion (Fig. 2C). For the primary role of epithelial-to-mesenchymal transition (EMT) in the progression of tumors, we also investigated whether NCAPD3 could trigger the EMT process. As a result, the Western blotting showed that knockdown of NCAPD3 resulted in an increased expression of the E-cadherin and a decreased expression of the N-cadherin and MMP2 (Fig. 2D). Besides, overexpression of NCAPD3 in HCC827 cells caused an increase in migration and invasion abilities, and a decrease in cell apoptosis, as accompanied by an EMT phenotype (Fig. 2A and D). Together, these results suggest that NCAPD3 plays an important role in cell proliferation, apoptosis, migration, and invasion in LC cells.

NCAPD3 promotes aerobic Glycolysis of LC cells

Subsequently, single-gene Gene Set Enrichment Analysis (GSEA) was conducted to investigate the potential molecular mechanisms of NCAPD3 by using the Sangerbox 3.0 (http://sangerbox.com/) platform. The results shown that NCAPD3-associated targets might participate in cell cycle, glycolysis, TCA cycle, and MAPK pathway (Fig. 3A). Aerobic glycolysis, also known as the Warburg effect, is recognized as a crucial element of metabolic reprogramming and is implicated in the onset and progression of tumors [25]. To confirm the findings, we estimated whether NCAPD3 could regulate glycolytic flux in LC cells. As shown in Fig. 3B, the knockdown of NCAPD3 weakened the uptake of the 2-NBDG into H1975 cells. The contrary finding was observed in HCC827 overexpressing NCAPD3. Congruously, NCAPD3-deficient in H1975 cells further promoted the decrease of intracellular lactate and ATP levels and the extracellular acidification rate (ECAR) compared to the negative control cells (Fig. 3C and E). Conversely, upregulated NCAPD3 in HCC827 cells heightened lactate and ATP production as well as the ECAR (Fig. 3C and E). Additionally, the protein level of HK2, PKM2, GLUT1, and LDHA was also reduced after NCAPD3 knockdown in H1975 cells and increased after NCAPD3 overexpression in HCC827 cells (Fig. 3F). Altogether, these results highlighted that NCAPD3 could also contribute to aerobic glycolysis in LC cells.

NCAPD3 aggravates malignant behaviors in LC cells through the MEK/ERK pathway

Glycolysis in cancers is associated with the regulation of LDHA via activation of the mitogen-activated protein kinase kinase (MEK)/extracellular signal-related protein kinase (ERK) pathway [26, 27]. Subsequently, the effect of NCAPD3 on the MEK/ERK pathway was investigated in LC cells using western blot assays. Results revealed that the phosphorylation of MEK and ERK was also significantly reduced after NCAPD3 knockdown in H1975 cells and increased after NCAPD3 overexpression in HCC827 cells, respectively (Fig. 4A). To further confirm the effects of NCAPD3 on MEK/ERK/LDHA axis, the MEK1/2 inhibitor U0126 (50 μM) was used to inhibit the MEK/ERK pathway in NCAPD3-overexpression HCC827 cells. As shown in Fig. 4B and D, U0126 treatment decreased clone formation, glucose uptake, and lactate production, which were inconsistent with the results of overexpressing NCAPD3 in HCC827 cells. More importantly, we found that the stimulative effect of NCAPD3 overexpression on cell proliferation, glucose uptake, and lactate production was noticeably weakened through the combined intervention of U0126 (Fig. 4B and D). Interestingly, the promoted expression of HK2, PKM2, GLUT1, LDHA and the phosphorylation of MEK and ERK by overexpressing NCAPD3 was rescued because of the addition of U0126, suggesting that the inhibition of MEK/ERK/LDHA axis might neutralize the promoting effect of NCAPD3 on malignant characteristics in LC (Fig. 4E).

Histone lactylation of NCAPD3 promotes tumorigenesis of LC

Given the well-documented connection between glycolysis and histone lactylation [8, 28], we wondered that if NCAPD3 signaling might result in histone lactylation. As shown in Fig. 5A, lower levels of global lactylation and H3K18la were observed in NCAPD3-knockdown H1975 cells, whereas overexpression of NCAPD3 existed a higher level of global lactylation and H3K18la in HCC827 cells. In addition, the protein expression levels of NCAPD3 were significantly decreased after treatment with glycolytic inhibitors (2-deoxy-D-glucose (2-DG) and oxamate) in H1975 and HCC827 cells (Fig. 5B). Furthermore, oxamate treatment significantly inhibited the cellular proliferation and migratory ability of HCC827 cells. And, overexpression of NCAPD3 obviously recovered the cell proliferation and migration ability of oxamate-treated HCC827 cells (Fig. 5C and E). In contrast to oxamate, the cell proliferation and migration ability showed the opposite trend when treating lactate in H1975 cells. Accordingly, opposite results were observed in lactate-treated H1975 cells with NCAPD3 silencing (Fig. 5F and H). Overall, these results may provide a new perspective that histone lactylation encourages LC progression through activating NCAPD3.

NCAPD3 knockdown inhibited tumor growth in vivo

To investigate whether NCAPD3 affects tumor formation in vivo, we conducted a tumor growth experiment in nude mice bearing H1975 cells subcutaneous tumors. As illustrated in Fig. 6A and C, the volume and weight of the mice in the si-NCAPD3 group were significantly

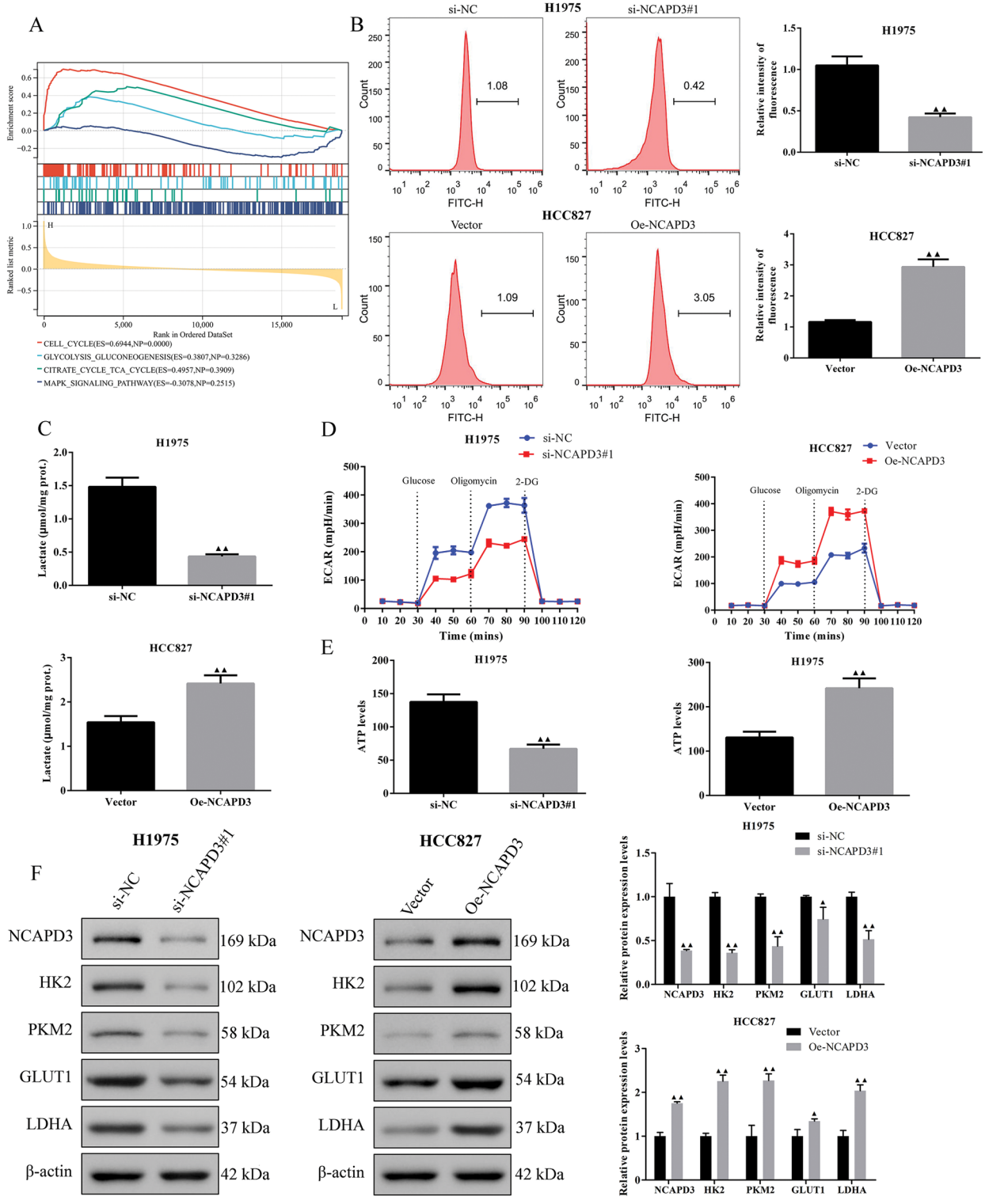

Fig. 3 NCAPD3 induces aerobic glycolysis in LC cells. (**A**) Single-gene GSEA analysis of NCAPD3. (**B**) Glucose uptake in NCAPD3-knockdown H1975 cells and NCAPD3-over-expression HCC827 cells was measured with flow cytometry by using the fluorescent glucose analog 2-NBDG. (**C**) Lactate production was measured in LC cells with NCAPD3-knockdown or NCAPD3-over-expression using the Lactic Acid (LA) Content Assay Kit. (**D**) The extracellular acidification rate (ECAR) of NCAPD3-knockdown H1975 cells and NCAPD3-over-expression HCC827 cells was also monitored. (**E**) The ATP levels in NCAPD3-knockdown H1975 cells and NCAPD3-over-expression HCC827 cells were quantified using the ATP Assay Kit. (**F**) The protein levels of NCAPD3 and aerobic glycolysis-related targets HK2, PKM2, GLUT1, and LDHA were determined by Western blotting. ▲$P < 0.05$, ▲▲$P < 0.01$, vs. si-NC group or vector group

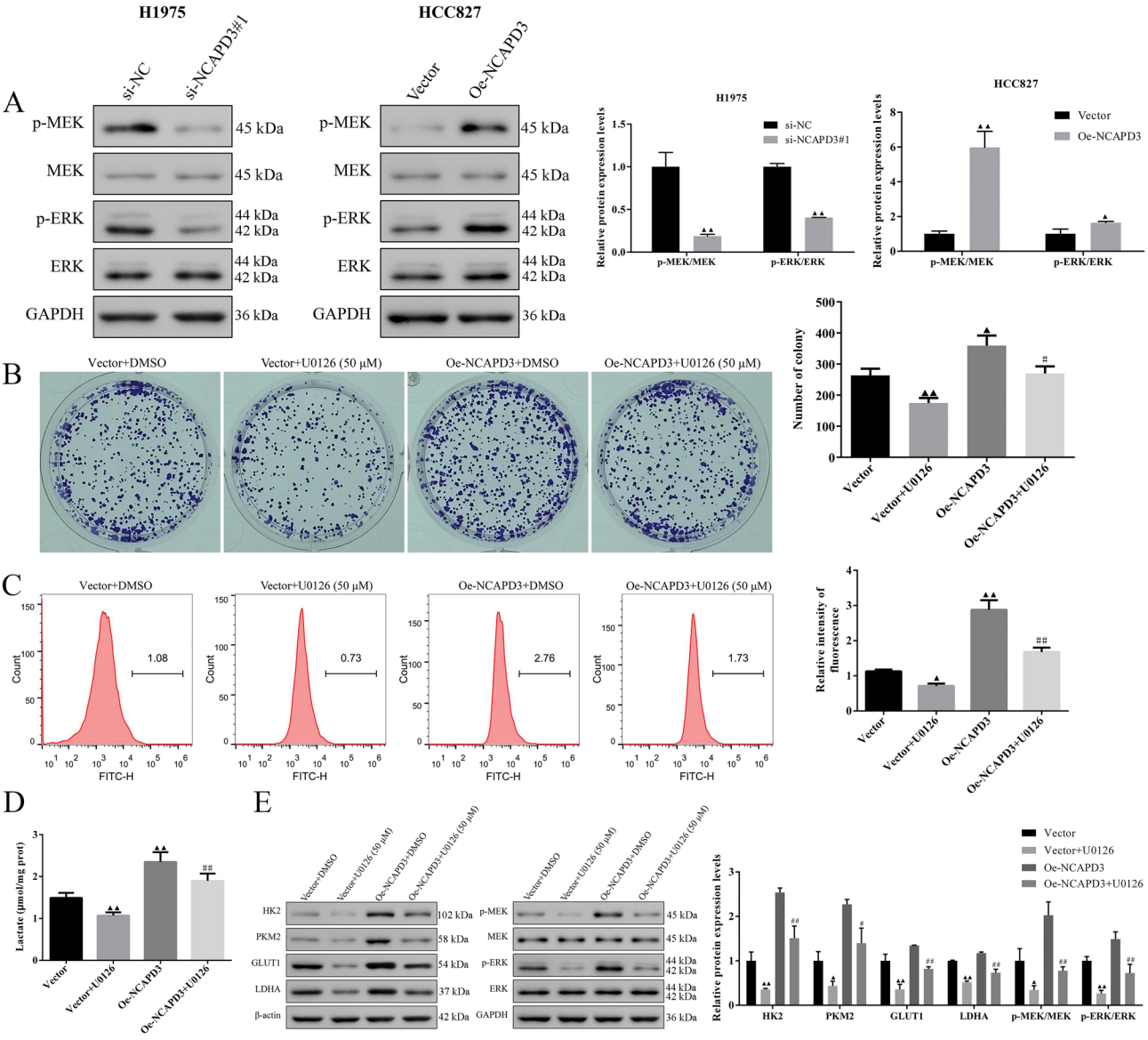

Fig. 4 NCAPD3 regulated aerobic glycolysis in LC cells through the MEK/ERK/LDHA signaling pathway. (**A**) The protein levels of MEK/ERK pathway-associated targets were detected by western blotting in NCAPD3-knockdown H1975 cells and NCAPD3-over-expression HCC827 cells. (**B**) Cell proliferation in HCC827 cells treated with U0126 (50 μM) and NCAPD3 overexpression plasmids in alone or combination was assessed by a colony formation assay. (**C**) Glucose uptake in HCC827 cells treated with U0126 (50 μM) and NCAPD3 overexpression plasmids in alone or combination was assessed by Flow cytometry. (**D**) The lactate levels in HCC827 cells treated with U0126 (50 μM) and NCAPD3 overexpression plasmids in alone or combination were assessed by the Lactic Acid (LA) Content Assay Kit. (**E**) The protein expression levels of HK2, PKM2, GLUT1, LDHA, p-MEK, MEK, p-ERK, and ERK in HCC827 cells treated with U0126 (50 μM) and NCAPD3 overexpression plasmids in alone or combination were detected through western blotting. ▲$P < 0.05$, ▲▲$P < 0.01$, vs. vector group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs. Oe-NCAPD3 group

decreased when compared to those in the si-NC group. Furthermore, IHC analysis also showed the NCAPD3, Ki67, and LDHA expressions of tumor tissues were substantially downregulated in the si-NCAPD3 group when compared with that in the si-NC group (Fig. 6D). Similarly, Western blot results showed that the expression levels of global lactylation, H3K18la, Ki67, NCAPD3, and LDHA, as well as the phosphorylation of MEK and ERK, were impaired by NCAPD3 silencing in tumor tissues (Fig. 6E). Taken together, these results suggested that NCAPD3 knockdown might inhibit tumor growth in vivo by blocking the communication between histone lactylation and NCAPD3 in LC.

Discussion

Under the influence of environment, hereditary conditions, and unhealthy behaviors such as smoking, LC has a high incidence rate, which is among the most common

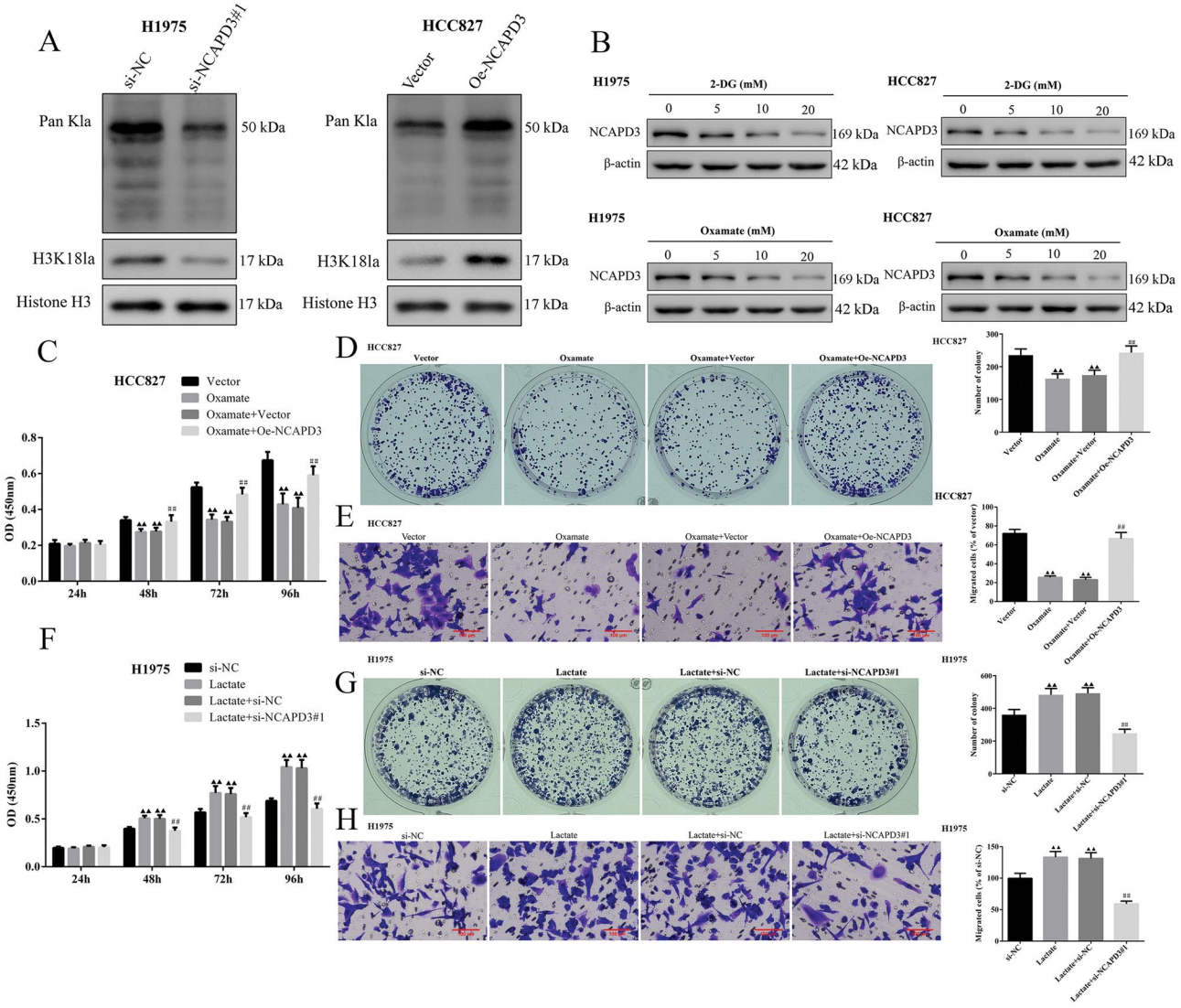

Fig. 5 Histone lactylation-NCAPD3 promotes aerobic glycolysis, cell proliferation, and migration in LC cells. (**A**) Western blotting reveals the global lactylation and H3K18la levels of NCAPD3-knockdown H1975 cells and NCAPD3-over-expression HCC827 cells. (**B**) The protein expression levels of NCAPD3 in H1975 and HCC827 cells with treatment of a series of concentrations of 2-DG or oxamate. (**C**-**D**) Proliferation and migration (**E**) of HCC827 cells with or without NCAPD3 over-expression treated with oxamate (20 mM) were evaluated by CCK-8, colony formation, and transwell assays, respectively. (**F**-**G**) Proliferation and migration (**H**) of H1975 cells with or without NCAPD3 knockdown treated with lactate (5 mM) were evaluated by CCK-8, colony formation, and transwell assays, respectively. ▲$P < 0.05$, ▲▲$P < 0.01$, vs. vector or si-NC group. $^{\#\#}P < 0.01$, vs. oxamate + vector or lactate + si-NC group

malignant tumors [29]. Furthermore, the early stage of LC is not noticeable, and it is frequently detected in the late stage, which complicates therapy in the latter stage of the disease. With the advancement of medical technology, in addition to conventional surgery, chemotherapy, and radiotherapy, with the development of molecular biology and human genomics, people's understanding of the molecular mechanism of invasion and metastasis in LC, as well as some of the bio-signaling pathways has been further deepened, which provides an opportunity for early diagnosis and development of new therapeutic methods. And, the emerging molecular targeted therapies have been widely used in the clinic. However, intriguing novel targets must be investigated extensively, as well as their underlying mechanisms.

Condensin complexes have critical roles in controlling chromosomal condensation and segregation within cell cycles [21]. More recently, studies have confirmed that NCAPD3 plays an essential role in the progression of cancers [20, 21]. For example, Yin et al. suggested that overexpression of androgen receptor increased the expression of NCAPD3 and also promoted cell proliferation, migration, and invasion in prostate cancer [30]. However, little is known about the bio-functions and molecular mechanisms of NCAPD3 in LC. In this study, we discovered that NCAPD3 was upregulated in LC cell

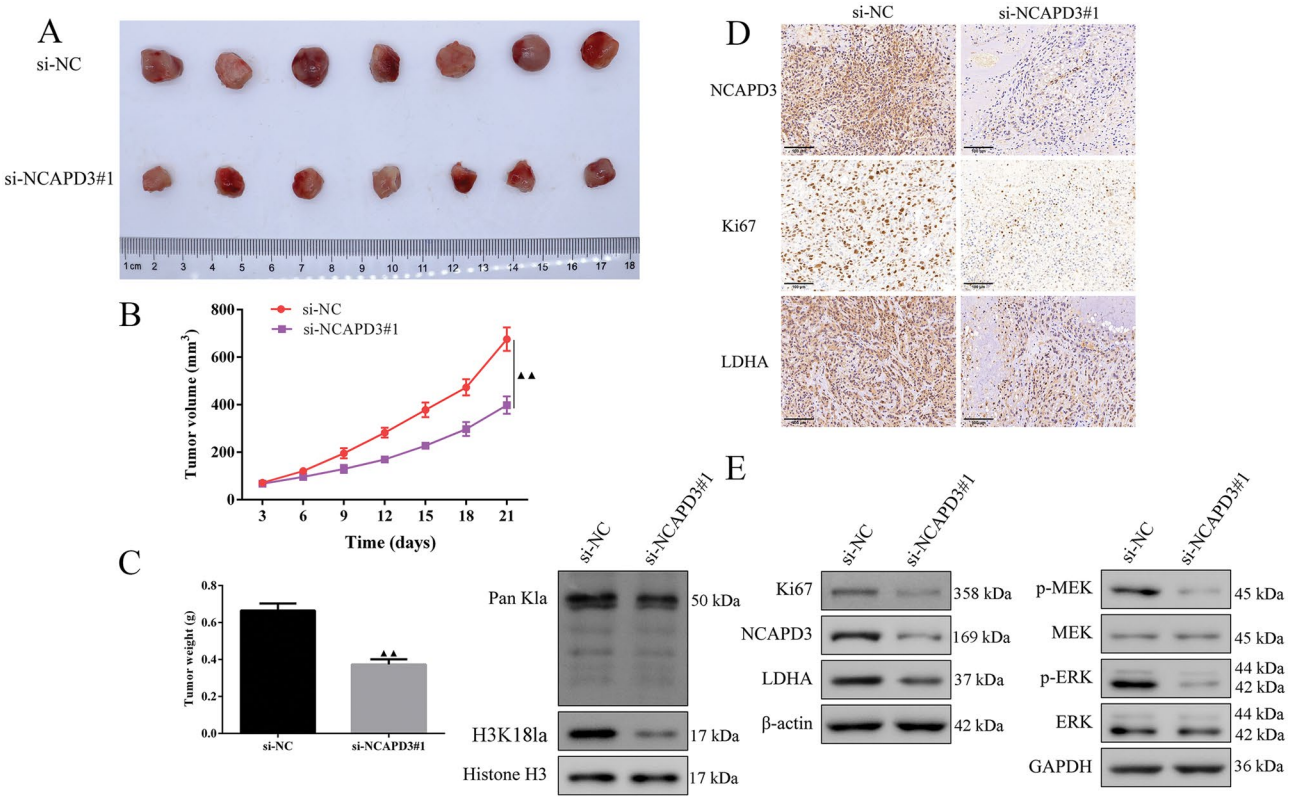

Fig. 6 NCAPD3 knockdown inhibited tumor growth of H1975 by MEK/ERK/LDHA axis in vivo. (**A**) Representative images of xenograft tumors in H1975-bearing tumor mice. (**B**) Tumor volume and (**C**) weight in mice. (**D**) Immunohistochemical (IHC) staining results showing the NCAPD3, Ki67, and LDHA protein expressions in tumor tissues of mice. Scale bar, 100 μm. (**E**) Western blot assays showing the global lactylation and H3K18la levels, the expression of Ki67, NCAPD3, Ki67, and LDHA, as well as MEK and ERK phosphorylation levels in tumor tissues of mice. ▲▲$P < 0.01$, vs. si-NC group

lines. High NCAPD3 levels were associated with unfavorable overall survival. Moreover, NCAPD3 knockdown or overexpression led to significantly inhibited or promoted cell proliferation and metastasis, respectively.

Reprogramming of glucose metabolism is a fundamental characteristic of tumor cells. A substantial body of evidence exists to support the crucial functions of glycolytic metabolism in carcinogenesis and progression in diverse cancer types [31, 32]. Despite the fact that glycolysis is extremely active (Warburg effect) and plays a key role in cell tumorigenesis and the onset of cancer, the exact mechanism by which glycolysis becomes exceptionally activated is not well understood. As a result, NCAPD3 knockdown or overexpression obviously inhibited or increased aerobic glycolysis, glucose uptake, and lactate production in H1975 and HCC827 cells, respectively. In addition, NCAPD3 also increased the expression of HK2, PKM2, and GLUT1, indicating that NCAPD3 could promote glycolysis in LC cells. More importantly, the in vivo role of NCAPD3 in LC tumor growth was also explored. As a result, NCAPD3 knockdown also inhibited tumor growth *in vivo*. More recently, another study by Yang et al. also reported that knockdown of NCAPD3 suppressed the proliferation, invasion, and migration of A549 and SPCA-1 cells in NSCLC by inhibiting the PI3K/Akt pathway [33]. Consistently, these findings suggest that NCAPD3 may serve as an oncogene in LC.

Additionally, compared to the current literature [33], this study mainly focused on the effects of NCAPD3 on aerobic glycolysis and histone lactylation. To further investigate the underlying mechanism of NCAPD3 promotion of LC cell proliferation, glucose uptake, and lactate production, we found that NCAPD3-knockdown and NCAPD3-over-expression respectively reduced or elevated or phosphorylation of MEK/ERK pathway. These results suggest that NCAPD3 could promote LC proliferation and aerobic glycolysis by activating the MEK/ERK pathway. It is commonly established that LDH regulates glycolysis in malignant cells by way of the MEK/ERK signaling pathway [34, 35]. Consequently, respective lower or higher expression of LDHA was also observed in NCAPD3-knockdown H1975 cells and NCAPD3-over-expression HCC827 cells in the current study. Noticeable, we demonstrated that MEK inhibitor U0126 significantly attenuated the effect of NCAPD3-over-expression on cell proliferation, glucose uptake, lactate production, the expression levels of glycolysis enzymes and MEK/ERK signaling pathway in HCC827 cells. Overall, these results

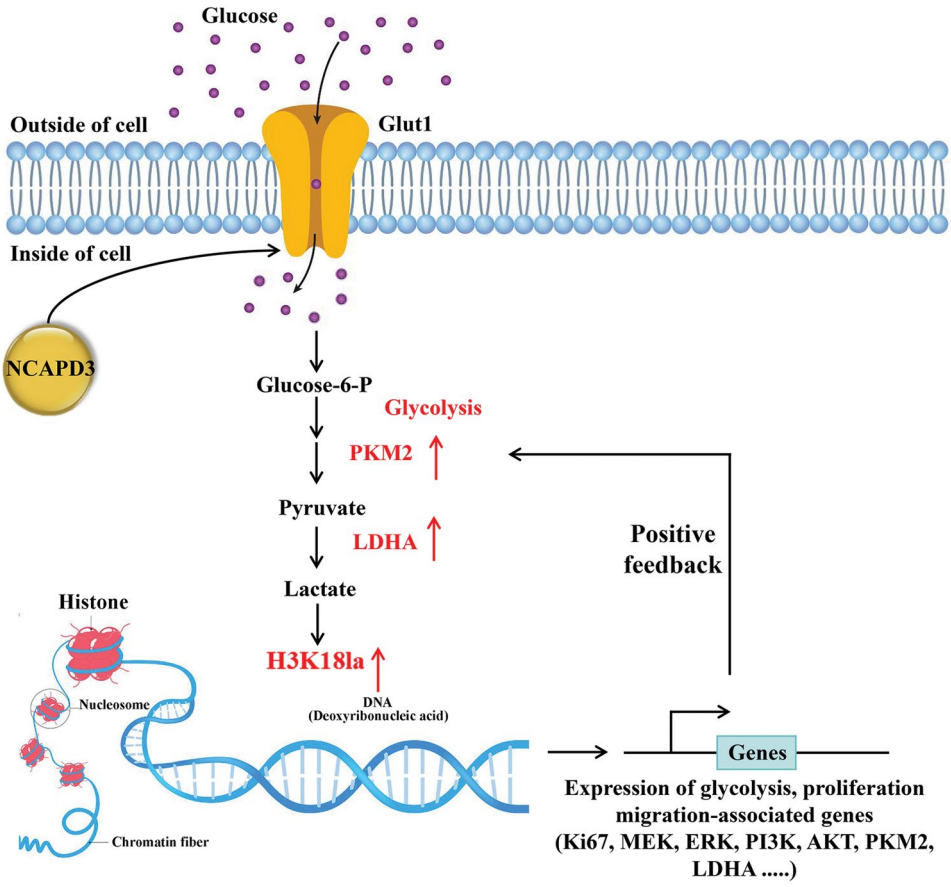

Fig. 7 Schematic diagram illustrating effects of NCAPD3 in promoting lung cancer progression. NCAPD3 promotes cell proliferation and metastasis of LC cells by increasing LDHA and lactate production. Then, lactate-induced histone lactylation activates MEK/ERK pathway. Hence, it is probable that there is a synergistic reaction between histone lactylation and NCAPD3 in Lung cancer cell metabolism

suggest that the NCAPD3/MEK/ERK/LDHA axis could act as a potential target for LC treatment.

The recent understanding of histone lactylation connects lactate to transcription in physiological and illness [7, 28, 36]. Given that LC exhibited active glycolysis that produced a considerable amount of lactate as substrates for histone lactylation in this study, we then examined the possible functions of histone lactylation in tumorigenesis. We found that NCAPD3 knockdown or overexpression in LC cells presented with decreased or elevated levels of histone lactylation. Moreover, cell proliferation and migration were effectively suppressed by target repair of faulty histone lactylation. Decreased histone lactylation inhibits the expression of NCAPD3. Here, these findings provide an alternative explanation for the significant involvement of NCAPD3 in tumor growth. However, the specific regulatory mechanism of NCAPD3 lactylation with histone modifications needs to be further investigated, and we will prioritize this matter in our upcoming work.

Conclusion

In summary, our findings showed that the expression of NCAPD3 was increased in LC cell lines and related to poor overall survival. NCAPD3 facilitated LC cell growth, migration, invasion, and aerobic glycolysis by activating the MEK/ERK/LHDA pathway and triggering histone lactylation (Fig. 7). These findings provide novel insights between NCAPD3 and histone lactylation, suggesting that NCAPD3 is a new therapeutic target for LC.

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Author contributions
Zhibo Chang: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing-original draft, Writing -review & editing.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

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