

The STAU1-CD44v8-10 axis regulates the initiation and progression of lung squamous cell carcinoma through PI3K/AKT signaling

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Abstract

Background

Cluster of differentiation 44 (CD44) is a well-established cancer-associated stem cell marker closely linked to the initiation and progression of lung squamous cell carcinoma (LUSC). Furthermore, CD44 variable (CD44v) isoforms are positively correlated with tumorigenesis and progression across diverse cancer types. However, the precise functional role of the alternatively spliced variant CD44v8-10 in LUSC pathogenesis and early oncogenic signaling remains undefined.

Methods

CD44v8-10 expression and its clinical significance were evaluated in LUSC patient tissues using immunohistochemistry (IHC) and public clinical datasets. The functional role of CD44v8-10 was investigated in normal human bronchial epithelial cells (BEAS-2B) and LUSC cell lines (H226 and H520) via overexpression and siRNA-mediated knockdown, followed by proliferation, cell cycle, migration, invasion, and epithelial–mesenchymal transition (EMT) assays. The impact of cigarette smoke extract (CSE) on CD44v8-10 expression and cellular transformation was also examined. Additionally, the upstream regulatory mechanisms were explored, and the in vivo therapeutic efficacy of targeting CD44v8-10 was validated using an H226 xenograft mouse model.

Results

CD44v8-10 was preferentially upregulated over other isoforms in LUSC tissues and correlated with poor patient prognosis. Overexpression of CD44v8-10 in normal BEAS-2B cells induced abnormal proliferation and EMT, accelerated the G1/S cell cycle transition, enhanced DNA synthesis, and promoted migration by activating the PI3K/AKT signaling pathway. Conversely, CD44v8-10 depletion in LUSC cells significantly impaired proliferation, induced G1 arrest, and attenuated PI3K/AKT signaling. Furthermore, CSE exposure induced CD44v8-10 upregulation, thereby driving proliferative and invasive phenotypes. Mechanistically, Staufen double-stranded RNA-binding protein 1 (STAU1) was identified as an upstream regulator that sustained CD44v8-10 expression. ly, targeted silencing of CD44v8-10 effectively suppressed tumor growth and proliferative activity in the xenograft model.

Conclusion

The STAU1–CD44v8-10 axis promotes LUSC initiation and progression by sustaining PI3K/AKT signaling, thereby accelerating cell cycle progression and enhancing malignant traits. These findings highlight the critical role of alternative splicing dysregulation in carcinogen-driven tumorigenesis and

establish CD44v8-10 as a promising prognostic biomarker and therapeutic target for lung squamous cell carcinoma.

1. Introduction

Lung squamous cell carcinoma (LUSC), a smoking-related malignancy comprising 25–30% of non-small cell lung cancers (NSCLC)^{1, 2}, exhibits pronounced molecular heterogeneity and is thought to arise from bronchial basal cells.^{3, 4} Its development involves multistep epithelial transformation driven by aberrant clonal dynamics rather than isolated mutations^{3, 4}. Chronic tobacco exposure confers selective growth advantages to subsets of basal cells, promoting non-neutral clonal expansion and dominance^{5–7}. These clones undergo fate reprogramming toward squamous differentiation, generating KRT4/KRT13⁺ transitional populations that progress through squamous metaplasia, dysplasia, and carcinoma in situ⁸. Clonal spread along the airway epithelium facilitates multifocal colonization and precancerous field formation, consistent with field cancerization⁴. Genomic analyses highlight *TP53* mutations (80–90% prevalence) as central drivers, frequently accompanied by *CDKN2A/B* deletions and *SOX2* amplifications, collectively disrupting cell-cycle control and lineage specification while promoting epithelial-mesenchymal transition (EMT)⁷. Chromosomal instability and epigenetic reprogramming further exacerbate tumor heterogeneity, subclonal evolution, and metastatic potential⁹. Aberrant activation of PI3K/AKT/mTOR, FGFR, and NOTCH pathways sustains proliferation, survival, and microenvironment remodeling, maintaining a continuum from premalignant lesions to invasive carcinoma^{9–11}. These findings and integrated mechanisms¹² underscore the need for early interception strategies targeting clonal origins to halt LUSC progression and improve patient outcomes.

Although immune checkpoint inhibitors (ICIs) have improved outcomes in advanced LUSC, current therapies remain largely directed at invasive disease³. In contrast, molecular events underlying early initiation and progression are incompletely defined, limiting effective interception¹⁰. Advances in low-dose computed tomography (LDCT) screening enable the detection of early lung nodules, while subsequent multi-omics profiling has identified recurrent mutations and epigenetic alterations in genes such as *TP53*, *CDKN2A*, and *SOX2* associated with premalignant lesions⁷. However, these alterations have not yet yielded validated biomarkers for risk stratification or preventive intervention. The prolonged and heterogeneous natural history of precancerous lesions, challenges in patient recruitment, and lack of standardized endpoints further complicate prevention-oriented trials^{3, 14}. Consequently, comprehensive elucidation of the molecular mechanisms driving LUSC initiation and progression, the identification of critical oncogenic markers, and the establishment of reproducible and physiologically relevant experimental models have become central priorities and enduring challenges in the field.

Increasing evidence indicates that RNA splicing dysregulation, particularly alternative splicing (AS), represents a key molecular hallmark of tumorigenesis by generating diverse transcript isoforms that reshape the cancer transcriptome^{15–19}, splicing dysregulation drives oncogene activation and tumor

progression^{21–23}. CD44 is a transmembrane glycoprotein that mediates cell–cell and cell–matrix interactions and has been widely implicated in tumor progression^{24, 25}. AS generates multiple *CD44* isoforms, including the standard isoform (*CD44s*), which is predominantly expressed in normal tissues^{26–28}. In contrast, variant isoforms containing additional exons (*CD44v*) are frequently upregulated in malignancies and are associated with enhanced invasion, metastasis, and cancer stem cell–like properties^{29, 30}. Emerging evidence indicates that CD44 expression is elevated in lung cancer and correlates with tumor initiation, progression, metastasis, and therapeutic resistance^{31, 11}. In NSCLC, CD44 regulates cancer cell stemness and invasiveness, underscoring its role in sustaining malignant phenotypes³². Notably, CD44v8-10 has been identified as a predominant splice variant in NSCLC with potential diagnostic and therapeutic relevance³³. Despite substantial evidence implicating CD44v in diverse cancers^{25, 34}, its specific functions and underlying mechanisms in LUSC remain incompletely defined. Elucidating the role of CD44v in LUSC pathogenesis may provide insights into tumor heterogeneity and therapeutic resistance and inform the development of more precise targeted strategies.

Database analysis combined with patient validation revealed that the CD44v8-10 isoform is preferentially upregulated compared to other isoforms in LUSC. We confirmed that STAU1 activates the PI3K/AKT signaling pathway by regulating CD44v8-10 expression, leading to upregulation of the cell cycle regulators CCND1 and CDK2. This activation promotes G1/S transition, enhances DNA synthesis, and drives aberrant proliferation and malignant transformation. Concomitantly, reduced E-cadherin and increased N-cadherin and vimentin expression indicate induction of EMT, accompanied by enhanced invasive and metastatic capacities. Collectively, these findings demonstrate that CD44v8-10 contributes to LUSC initiation and progression through modulation of PI3K/AKT signaling.

2. Results

2.1 *CD44v8-10* is elevated in LUSC and correlates with enhanced proliferation and poor prognosis

Studies have shown that the expression level of *CD44v6* is higher in squamous cell carcinoma, and it is an independent prognostic indicator for NSCLC³⁵. An analysis of the HPA dataset revealed that *CD44* mRNA expression was significantly elevated in LUSC tissues compared with normal lung tissues (Fig. 1A), which is consistent with previous reports^{36, 19}. Survival analysis demonstrated that elevated *CD44* expression was associated with reduced overall survival, suggesting a potential prognostic value of *CD44* in LUSC (Fig. 1A). The following schematic representation of the *CD44* gene structure illustrates the generation of variant isoforms through alternative inclusion of variable exons (v2-v10) (Fig. 1B). An analysis of data extracted from the TCGA SpliceSeq database demonstrates that the *CD44v8-10* exhibited significantly higher PSI values than other CD44 exon skipping events (Fig. 1C), suggesting selective enrichment of the *CD44v8-10* isoform in LUSC. At the mRNA level, semi-quantitative PCR

analysis of paired clinical specimens confirmed increased *CD44v8-10* expression in tumors compared with matched normal tissues (Fig. 1D). Furthermore, quantitative RT-PCR analysis of six paired tumor samples revealed that, among the CD44 variant isoforms (v2-v10), CD44v8-10 was expressed at significantly higher levels than the other variants (Fig. 1E). These findings were further supported by densitometric quantification and immunohistochemical staining, which demonstrated prominent membranous CD44v8-10 expression in tumor samples but minimal staining in normal epithelium (Fig. 1F-G). Consistent with clinical observations, LUSC cell lines exhibited higher CD44v8-10 expression than the normal bronchial epithelial cell line BEAS-2B at both mRNA and protein levels (Fig. S1A-B). Functional assays indicated that LUSC cells displayed enhanced proliferative capacity, as reflected by increased CCK-8 absorbance, greater colony formation, and a higher proportion of EdU-positive cells compared with BEAS-2B cells (Fig. S1C-E). Collectively, these findings indicate that CD44v8-10 is preferentially enriched in LUSC and may be associated with increased proliferative potential and unfavorable clinical outcomes.

2.2 *CD44v8-10* enhances cell proliferation through activating the PI3K–AKT pathway and accelerating G1/S transition

To elucidate how *CD44v8-10* enhances cellular proliferative fitness and malignant traits in BEAS-2B cells, we performed transcriptomic profiling in *CD44v8-10*-overexpressed (*CD44v8-10* OE) and control cells. Hierarchical clustering revealed distinct transcriptional programs (Fig. 2A), and KEGG analyses highlighted significant enrichment of cancer-associated pathways, including PI3K-AKT, MAPK, Ras signaling, and focal adhesion (Fig. 2B). Given the prominent PI3K-AKT signature, we next assessed its functional relevance. Pharmacological inhibition of PI3K or AKT using LY294002 and MK2206, respectively, attenuated the CD44v8-10-induced proliferative advantage (Fig. 2C, D). Consistently, CD44v8-10 OE cells exhibited increased phosphorylation of PI3K and AKT without substantial changes in total protein levels (Fig. 2E, F), indicating activation of the PI3K-AKT pathway. Functionally, CD44v8-10 overexpression enhanced clonogenic capacity (Fig. 2G) and cell proliferation (CCK-8; Fig. 2H), supporting a PI3K-AKT-dependent pro-proliferative effect. Importantly, CD44v8-10 overexpression enhanced DNA synthesis activity, as evidenced by increased EdU incorporation (Fig. 2I). Flow cytometry analysis revealed a reduced G1 fraction accompanied by an increase in the S-phase population, while the G2 fraction remained relatively unchanged (Fig. 2J-L). These findings suggest accelerated cell cycle progression due to enhanced S-phase entry and increased DNA synthesis activity. Concurrently, the expression levels of CDK2 and CCND1, two pivotal regulators of the cell cycle, increased (Fig. 2K). Moreover, *CD44v8-10* overexpression promoted malignant-associated behaviors, including enhanced migration and invasion in Transwell assays and accelerated wound closure in scratch assays (Fig. S2A, B, D, E), accompanied by EMT-associated protein changes (decreased E-cadherin and increased N-cadherin and Vimentin; Fig. S2C). RT-qPCR analysis further confirmed increased expression of PI3K-AKT-related transcripts (*PIK3CA*, *PIK3CB*, *PIK3R1*, *SGK1*, *RPS6KB1*) and G1/S regulators (*CDK6*, *CCNE2*) (Fig. S3), reinforcing activation of a pro-proliferative transcriptional program. Collectively, these data

demonstrate that CD44v8-10 overexpression is sufficient to enhance BEAS-2B cell proliferation by promoting S-phase enrichment and DNA replication activity and to induce more aggressive, EMT-like motility phenotypes, at least in part through activation of the PI3K-AKT axis (Fig. 2, Fig. S2 and S3).

2.3 CD44v8-10 depletion restrains malignant phenotypes in H226 and H520 cells by impairing proliferation, enforcing cell-cycle blockade, and attenuating PI3K/AKT signaling

To elucidate the functional contribution of *CD44v8-10* to the malignant behavior of lung squamous cell carcinoma cells, we silenced *CD44v8-10* in H226 and H520 cells and quantified proliferation, cell-cycle progression, pathway activity, and motility. *CD44v8-10* depletion reduced clonogenicity and cell growth (Fig. 3A, B), accompanied by decreased EdU incorporation (Fig. 3C), indicating impaired DNA synthesis. Consistently, the protein expression of the key cell-cycle regulators CDK2 and CCND1 were downregulated following CD44v8-10 knockdown in both H226 and H520 cells (Fig. 3D, E). Flow cytometric analysis further demonstrated a significant accumulation of cells in the G1 phase accompanied by a decreased S-phase population after CD44v8-10 depletion, indicating impaired G1/S transition (Fig. 3F, G). In contrast, changes in the G2 population were relatively modest and not consistently significant between the two cell lines. Collectively, these findings suggest that CD44v8-10 depletion suppresses cell-cycle progression primarily through induction of G1 arrest and inhibition of DNA synthesis. Given that NCI-H226 cells exhibited the highest endogenous CD44v8-10 expression and the strongest proliferative activity among the examined lung squamous cell carcinoma cell lines (Fig. S1), subsequent transcriptomic and pathway analyses were performed primarily in H226 cells following CD44v8-10 silencing. KEGG enrichment analysis revealed that the downregulated pathways were predominantly associated with cancer-related proliferative and motility signaling, including PI3K-AKT signaling, MAPK signaling, focal adhesion, regulation of the actin cytoskeleton, ECM–receptor interaction, and TGF- β signaling (Fig. 3H). GO biological process analysis further demonstrated significant suppression of extracellular matrix organization, protein kinase B signaling, cell–matrix adhesion, and positive regulation of cell migration (Fig. 3I). Consistent with these transcriptomic findings, Western blot analysis showed markedly reduced phosphorylation levels of PI3K and AKT, whereas total PI3K and AKT protein levels remained relatively stable after CD44v8-10 depletion, indicating attenuation of PI3K-AKT pathway activity (Fig. 3J). In parallel, qRT-PCR analysis demonstrated significant downregulation of proliferation-associated genes, including *AKT1*, *MCM2*, *E2F1*, and *PCNA*, accompanied by upregulation of the cell-cycle inhibitory factor *CDKN1A* (Fig. 3K). Collectively, these findings suggest that CD44v8-10 promotes the malignant proliferative and migratory phenotype of lung squamous cell carcinoma cells, at least in part through activation of the PI3K-AKT signaling axis. Moreover, CD44v8-10 knockdown suppressed migration and invasion in transwell assays in NCI-H226 cells (Fig. S4A, B). EMT-associated changes were also reversed following CD44v8-10 depletion, as evidenced by increased E-cadherin and decreased N-cadherin and Vimentin expression (Fig. S4C), consistent with the downregulation of EMT-related pathways and biological processes identified in the enrichment analyses performed in H226 cells (Fig. 3I). Consistently, wound-healing assays demonstrated delayed wound closure at both 24 h and 48 h after CD44v8-10 silencing in H226 cells (Fig. S4D, E). Collectively, these findings indicate that CD44v8-10 promotes the proliferative and invasive phenotypes of lung squamous

cell carcinoma cells, at least in part through sustaining PI3K/AKT activation, facilitating G1/S cell-cycle progression, and maintaining an EMT-like state (Fig. 3, Fig. S4).

2.4 CSE induces CD44v8-10 upregulation that promotes proliferative and invasive phenotypes in BEAS-2B cells.

CSE has been reported to drive the initiation and progression of lung squamous cell carcinoma by promoting epithelial cell proliferation, inducing malignant transformation, and activating pro-tumorigenic signaling pathways such as PI3K/AKT and IL-6-mediated inflammatory responses^{37–39}. To determine whether CD44v8-10 mediates CSE-driven malignant phenotypes in BEAS-2B normal lung epithelial cell line, we compared Control, CSE and CSE+siCD44v8-10 groups. CSE exposure increased *CD44v8-10* protein expression, whereas CD44v8-10 siRNA efficiently suppressed this induction (Fig. 4A). Functionally, CSE enhanced clonogenic growth and cell viability, both of which were attenuated by *CD44v8-10* knockdown (Fig. 4A, B). Consistently, EdU incorporation was increased by CSE and reduced upon CD44v8-10 depletion, indicating that *CD44v8-10* is required for CSE-induced proliferative activity (Fig. 4C). Cell-cycle profiling further demonstrated that cigarette smoke extract (CSE) treatment reduced the G1-phase population and increased the S-phase fraction, indicating enhanced cell-cycle progression in NCI-H226 cells. In contrast, CD44v8-10 knockdown partially reversed these effects, leading to an increased G1-phase fraction and reduced S-phase population, consistent with impaired G1/S transition (Fig. 4D, E). Notably, the G2-phase population showed a modest increase following CD44v8-10 depletion. In line with this, CCND1 and CDK2 levels were elevated by CSE and decreased after CD44v8-10 silencing (Fig. 4F). Given that growth-promoting programs often couple to metastatic traits, we next assessed motility phenotypes in the same experimental framework. CSE markedly increased migration and invasion in transwell assays, whereas *CD44v8-10* knockdown significantly reduced both processes (Fig. 4G, H). At the molecular level, CSE induced EMT-associated transcripts (*SNAI1*, *TWIST1*, *ZEB2*, *FOXC2*, *AXL*, *MMP2*), and this EMT-like transcriptional shift was partially reversed by *CD44v8-10* depletion (Fig. 4I). Furthermore, transcriptomic analysis revealed significant gene expression differences between CSE and control cells (Fig. 4J). KEGG enrichment analysis identified cancer-relevant signaling pathways, including those related to the PI3K/AKT pathway (Fig. 4K). Consistently, WB demonstrated that *CD44v8-10* knockdown reduced PI3K/AKT activation, reflected by decreased p-PI3K and p-AKT with comparatively stable total PI3K and AKT levels (Fig. 4L). Collectively, these data indicate that CSE promotes proliferation, cell-cycle progression, migration/invasion, and EMT-associated programs in BEAS-2B cells through upregulation of CD44v8-10, at least in part via PI3K/AKT pathway activation (Fig. 4A-L).

2.5 STAU1 acts as an upstream regulator of CD44v8-10 in H226 and H520 cells

To identify the upstream regulators of *CD44v8-10* in lung squamous cell carcinoma, we first surveyed alternative splicing-associated factors in the CPTAC proteomic datasets and found that *STAU1* was elevated in LUSC compared to normal tissues (Fig. 5A). STAU1 is an RNA-binding protein involved in

mRNA stability and processing. It has also been reported to regulate alternative splicing events, which suggests that it may play a role in tumorigenesis^{40, 41}. Guided by these clinical associations, we next investigated the functional role of the STAU1-CD44 axis in H226 and H520 cells. STAU1 knockdown selectively reduced CD44 variant isoform expression, with the most pronounced inhibitory effect observed for CD44v8-10 in both cell lines (Fig. 5B), supporting STAU1 as an upstream regulator of CD44v8-10 expression. Functionally, STAU1 depletion significantly suppressed cell proliferation, as demonstrated by reduced CCK-8 signals over time and decreased EdU incorporation in both H226 and H520 cells (Fig. 5C, D). Flow cytometric analysis further revealed that STAU1 silencing increased the G1-phase population while reducing the S-phase fraction, consistent with impaired G1/S cell-cycle progression in both cell lines (Fig. 5E, F). In contrast, changes in the G2-phase population were relatively modest. Concordantly, Western blot analysis demonstrated reduced protein expression of the G1/S regulators CDK2 and CCND1 following STAU1 depletion (Fig. 5G). qRT-PCR analysis performed in H226 cells further showed significant downregulation of proliferation-associated genes, including CCNE1, CDK4, and E2F1, accompanied by upregulation of the cell-cycle inhibitory factor CDKN1A (Fig. 5H). Mechanistically, STAU1 knockdown attenuated PI3K/AKT pathway activation in H226 cells, as evidenced by reduced phosphorylation of PI3K and AKT, whereas total PI3K and AKT protein levels remained relatively stable (Fig. 5I). Collectively, these findings support a model in which STAU1 functions upstream of CD44v8-10 and promotes lung squamous cell carcinoma cell proliferation by sustaining PI3K/AKT signaling and facilitating G1/S cell-cycle progression (Fig. 5B-I).

2.6 CD44v8-10 silencing suppresses H226 xenograft growth in vivo

To evaluate the in vivo relevance of CD44v8-10 in lung squamous cell carcinoma, we established H226 xenografts in NCG mice and initiated intertumoral treatment with siCD44v8-10 or siNC once tumors reached ~ 100–150 mm³ following previously established siRNA delivery protocols in xenograft models^{42–44} (Fig. 6A). *CD44v8-10* silencing markedly restrained tumor progression, as evidenced by reduced tumor burden in representative xenografts (Fig. 6B). Notably, mouse body weight remained comparable between groups throughout the treatment course (Fig. 6C), suggesting that the regimen was well tolerated under these conditions. Consistently, endpoint tumor weight was significantly lower in the siCD44v8-10 group (Fig. 6D), and longitudinal measurements showed a sustained reduction in tumor volume compared with siNC controls (Fig. 6E). Mechanistically, immunohistochemistry confirmed effective target suppression in vivo. CD44v8-10 staining intensity was reduced in tumors from siCD44v8-10-treated mice and quantified by a lower positive area (%) (Fig. 6F, G). Concordantly, tumors with CD44v8-10 depletion exhibited decreased proliferative activity, as indicated by reduced Ki67 staining and a significantly lower Ki67 positive area (%) (Fig. 6H, I). Collectively, these data demonstrate that CD44v8-10 is required for efficient H226 xenograft growth, and that its silencing suppresses tumor expansion in vivo in association with reduced tumor-cell proliferation (Fig. 6A-I).

3. Discussion

Across patient specimens and cell models, CD44v8-10 is preferentially enriched in LUSC and associates with increased proliferation and unfavorable clinical outcome. In this study, we provide evidence that CD44v8-10 may promote the initiation and progression of LUSC through the activation of PI3K/AKT signaling axis. We further identify STAU1 as an upstream determinant of CD44v8-10, thereby linking splicing-associated regulation to oncogenic signaling output. Intriguingly, we found that the mRNA level of CD44s remains unchanged upon STAU1 knockdown, despite a significant decrease in variant exons (v8-10) (Fig. 5B). These results reflect isoform-specific regulation potentially mediated via selective exon inclusion or transcript stability rather than a global splicing switch. This is consistent with previous studies demonstrating that DNA methylation-dependent regulation by DNMT1 primarily affects the inclusion of specific CD44 variant exons rather than inducing a global splicing switch or altering constitutive exon usage^{45, 46}. However, the detail mechanism of this isoform-specific regulation needs to be further investigated. Together, these findings support a model in which an STAU1-CD44v8-10 axis sustains PI3K/AKT activity, enabling cell-cycle acceleration and malignant traits that are relevant to LUSC initiation and progression.

A notable aspect of our observations is that the disease association appears to concentrate on the CD44v8-10-containing isoform rather than CD44 expression²⁴ as a single, uniform entity. Given the extensive alternative splicing of CD44, the preferential inclusion of variant exons v8–v10 in LUSC suggests that isoform selection may represent a more informative layer than total CD44 abundance (Fig. 1C). This is conceptually important because isoform-level regulation can uncouple CD44's broad roles in adhesion/signaling from the specific oncogenic activities conferred by variant exons. Therefore, focusing on CD44v8-10 provides a more mechanistically tractable explanation for clinical correlations with poor outcome and for functional phenotypes observed in LUSC cells, and it may help rationalize why CD44 has shown context-dependent prognostic associations across NSCLC subtypes. LUSC remains strongly linked to tobacco exposure and exhibits substantial molecular heterogeneity, yet effective early interception strategies are still limited by incomplete understanding of early initiating mechanisms^{1, 47}. In this context, AS dysregulation has emerged as a hallmark of cancer biology and a potential source of actionable vulnerabilities^{48, 49}. Our findings contribute to this framework by identifying a specific splice isoform, CD44v8-10, as a driver of pro-proliferative and pro-migratory programs and by linking the splicing-associated regulator STAU1 to oncogenic PI3K/AKT signaling. The convergence of dysregulated splicing events on canonical growth and EMT pathways suggests that aberrant isoform switching may actively reinforce oncogenic signaling programs in LUSC, especially in carcinogen-driven tumorigenesis^{34, 50}.

The in vivo xenograft experiments further substantiate the functional importance of CD44v8-10, as silencing significantly suppressed tumor growth and reduced proliferative indices, including Ki67 expression. Combined with the in vitro findings, these data indicate that CD44v8-10 is not merely associated with LUSC but functionally required for efficient tumor expansion. This requirement is consistent with its role in sustaining PI3K/AKT signaling and promoting cell-cycle progression.

Collectively, these results provide a mechanistic rationale for targeting CD44v8-10 or its upstream splicing regulators as potential therapeutic or preventive strategies.

Several limitations merit consideration. First, although our data demonstrate a functional association between CD44v8-10 expression and PI3K/AKT activation, the precise molecular mechanism by which this isoform engages the PI3K/AKT pathway remains undefined. In particular, it will be important to determine whether specific co-receptors, ligand interactions, or membrane microdomain organization facilitate pathway activation. Secondly, while STAU1 is implicated as an upstream regulator of CD44v isoform expression, the underlying splicing regulatory mechanism has not been fully elucidated. Key questions include whether STAU1 directly binds CD44 pre-mRNA, cooperates with additional splicing factors, or exerts context-dependent effects under carcinogen exposure. Thirdly, validation in additional LUSC models and larger, well-annotated clinical cohorts will be necessary to assess the generalizability of our findings and to define the prognostic or predictive value of CD44v8-10 relative to total CD44 expression. Addressing these limitations will strengthen causal inference, clarify regulatory architecture within the STAU1-CD44v8-10-PI3K/AKT axis, and determine whether isoform-specific targeting strategies can be rationally developed for early interception or therapeutic intervention in LUSC. Together, these findings support a model in which an STAU1-CD44v8-10 axis sustains PI3K/AKT activity, enabling cell-cycle acceleration and malignant traits that are relevant to LUSC initiation and progression. These findings are summarized schematically in the graphical abstract.

4. Materials and Methods

4.1 Cell lines and culture conditions

BEAS-2B, H226, SK-MES-1, H520, and 293T cells were obtained from the Chinese National Stem Cell Resource Center. BEAS-2B, H226 and H520 cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific, #11875119) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, #F8318) and 1% penicillin–streptomycin (Thermo Fisher Scientific, #15140122). SK-MES-1 cells were maintained in Minimum Essential Medium (MEM, 1X; Thermo Fisher Scientific, #11095-080) supplemented with 10% FBS and 1% penicillin–streptomycin. 293T cells were cultured in DMEM (Thermo Fisher Scientific, #11995-073) supplemented with 10% FBS and 1% penicillin–streptomycin. All cells were maintained at 37°C in a humidified incubator with 5% CO₂ (Thermo Fisher Scientific, #51032874).

4.2 Antibodies

The following primary antibodies were used: Anti CD44v9(CAC-LKG-M003, Cosmo Bio), anti-PI3K p110 β (Proteintech, #20584-1-AP), anti-CDK2 (Proteintech, #22060-1-AP), anti-Cyclin D1 (Proteintech, #60186-1-Ig), anti-AKT (Proteintech, #10176-2-AP), anti-CD44 (Proteintech, #60224-1-Ig), anti-phospho-AKT (Ser473) (Proteintech, #66444-1-Ig), anti-phospho-PI3K p85 (Tyr458)/p55 (Tyr199) (Cell Signaling Technology, #4228T), and anti-STAU1 (Proteintech, #14225-1-AP). Secondary antibodies: Anti-Rat IgG(7077, Cell Signaling Technology), HRP-conjugated Goat Anti-Mouse IgG(H + L) (Proteintech, #SA00001-1), HRP-conjugated Goat Anti-Rabbit IgG(H + L) (Proteintech, #SA00001-2).

4.3 Lentiviral vector construction and stable cell line generation

The lentiviral plasmid pLenti-CMV-eGFP was obtained from Addgene (plasmid #228271) and used as the backbone for stable cell line construction. The coding sequence of CD44v8-10 was cloned into the pLenti-CMV-eGFP vector to generate the recombinant plasmid pLenti-CMV-CD44v8-10-eGFP. All constructs were verified by DNA sequencing. For lentiviral production, the recombinant plasmid was co-transfected with packaging plasmids into 293T cells using PEI MAX transfection reagent (Polysciences, #24765). The culture medium was replaced 24 h after transfection. Viral supernatants were collected at 72 h post-transfection and filtered to remove cell debris. Target cells were infected with the harvested viral supernatants. Cells exhibiting stable green fluorescent protein (eGFP) expression were selected as CD44v8-10 overexpressing stable cell lines.

4.4 Cigarette smoke extract (CSE) treatment and vitro cell model establishment

CSE was generously provided by Professor Wenguang Yin (Guangzhou National Laboratory, China). The stock solution was freshly diluted in RPMI 1640 medium to a final concentration of 0.1% (v/v) immediately prior to use. BEAS-2B cells were exposed to 0.1% CSE, and the culture medium was replaced with freshly prepared 0.1% CSE every 48 hours to maintain consistent exposure. Continuous stimulation was performed for approximately 10 days. All CSE working solutions were prepared immediately before each treatment to ensure stability and reproducibility. Successful establishment of the in vitro model was confirmed prior to subsequent experiments.

4.5 RNA extraction and quantitative real-time PCR

Total RNA was isolated from cultured cells using the RNeasy Mini Kit (QIAGEN, #74106) according to the manufacturer's instructions. RNA quantity and purity were assessed with a NanoDrop One spectrophotometer (Thermo Fisher Scientific). One microgram of total RNA was reverse-transcribed into cDNA using the PrimeScript RT Reagent Kit (Takara, #RR037). The resulting cDNA was diluted to 50 ng/ μ L and used as the template for quantitative real-time PCR (qRT-PCR). qRT-PCR was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad) using the TB Green Premix Ex Taq II kit (Takara, #RR820) following the manufacturer's recommended thermal cycling program. Primer sequences are listed in Table.

4.6 siRNA transfection

Cells were transfected with siRNAs (final concentration, 50 nM) targeting CD44v8-10, STAU1 or a scrambled negative control using Lipofectamine 3000 (Invitrogen) following the manufacturer's protocol. siRNAs were synthesized by Tsingke Biotechnology (Beijing, China). Cells were collected 48–72 h after transfection for subsequent assays. Knockdown efficiency was verified by qRT-PCR and Western blotting.

4.7 Collection clinical lung cancer samples

Tumor tissues and matched adjacent normal tissues was generously provided by Professor Wenzhao Zhong (Guangdong Provincial People's Hospital). None of the patients received chemotherapy or radiotherapy prior to surgery. The study protocol was approved by the Ethics Committee of Guangdong Provincial People's Hospital (Approval No. KY2026-266-01). All procedures involving human samples were conducted in accordance with the Declaration of Helsinki. Fresh tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until further use.

4.8 Immunohistochemistry

Mouse tissues were fixed in 4% paraformaldehyde (PFA) at 4°C for 24 h, embedded in paraffin, and sectioned at $4\text{ }\mu\text{m}$. Human clinical specimens stored at -80°C were cryosectioned at $6\text{--}8\text{ }\mu\text{m}$ thickness. Paraffin sections were deparaffinized in xylene and rehydrated through graded ethanol. Both paraffin-embedded mouse sections and human frozen sections were subjected to heat-mediated antigen retrieval in citrate buffer (pH 6.0) at $95\text{--}100^{\circ}\text{C}$ for 15–20 min. After cooling to room temperature, endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. Sections were then blocked with 5% normal serum for 30 min and incubated overnight at 4°C with primary antibodies CD44v9 (CAC-LKG-M003, Cosmo Bio, 1:200-1:500 dilution) and Ki67 (Proteintech, #27309-1-AP, 1:2000-1:5000). After washing, sections were incubated with Goat anti-Rat IgG (H + L) (Thermo Fisher Scientific, #31470, 1:100-1:500) and Goat anti-Rabbit IgG (H + L) (Thermo Fisher Scientific, #31460, 1:100-1:500) secondary antibodies for 30–60 min at room temperature. IHC-stained sections were scanned using a whole-slide imaging system (VS200, Evident, Tokyo, Japan). Digital images were analyzed using ImageJ software (NIH, Bethesda, MD, USA). DAB staining was separated from hematoxylin using the color deconvolution plugin. The percentage of positive staining area was calculated from five randomly selected fields per section. The average value was used for statistical analysis.

4.9 Cell proliferation assay

For CCK8 assay, cells were seeded at 1000 cells per well in 96-well plates with fresh medium. Cell viability was assayed using Cell Counting Kit-8 (Beyotime, #C0037) at the time in 0, 48, 72h. Absorbance was read at 450 nm using a multiskan microplate reader (Thermo Scientific).

To evaluate the cell proliferation, cells were incubated with 5-ethynyl-2'-deoxyuridine (EdU) for the indicated time, and DNA synthesis was detected using the EdU Cell Proliferation Kit (Beyotime, #C0075S) following the manufacturer's protocol. The proportion of EdU-positive cells was determined by fluorescence microscopy (Nikon) or flow cytometry (Agilent Technologies).

4.11 Colony formation assay

Cells were seeded in six-well plates at a density of 500 cells per well and cultured for 14 days to allow colony formation. The culture medium was replaced every 3 days. For CSE treatment, cells were continuously exposed to CSE from the time of seeding until the end of the experiment. For gene

silencing experiments, cells were transfected with siCD44v8-10 or siSTAU1 during the final 3 days of culture using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. At the end of the incubation period, colonies were fixed with 4% paraformaldehyde for 15 min and stained with 0.1% crystal violet for 20 min. Colonies containing more than 50 cells were counted under a light microscope. Colony formation efficiency was calculated as the number of colonies divided by the number of seeded cells.

4.12 Cell cycle analysis

Cell cycle distribution was analyzed using a Cell Cycle and Apoptosis Analysis Kit (Beyotime, #C1052) according to the manufacturer's instructions. Cells were harvested, washed with cold PBS, and fixed with 70% ethanol at 4°C overnight. After fixation, cells were washed with PBS and incubated with propidium iodide (PI) staining solution containing RNase A for 30 min at room temperature in the dark. DNA content was analyzed by flow cytometry using a NovoCyte Advanteon flow cytometer (Agilent Technologies). The percentages of cells in G1, S, and G2 phases were determined using FlowJo software.

4.13 Cell migration and invasion assay

Cell migration and invasion abilities were evaluated using Transwell chambers (Corning, USA) with 8-μm pore polycarbonate membrane inserts in 24-well plates.

For migration assays, 5×10^4 cells per well were suspended in 200 μL serum-free medium and seeded into the upper chamber. The lower chamber was filled with 600 μL complete medium containing 10% fetal bovine serum (FBS) as a chemoattractant.

For invasion assays, the upper chamber membranes were pre-coated with Matrigel (Corning, #354277) diluted 1:7 in serum-free medium, with a final coating volume of 50 μL per insert (approximately 200–300 μg/mL final concentration). The coated inserts were incubated at 37°C for 1 h to allow gel polymerization before cell seeding.

A total of 1×10^5 cells per well in 200 μL serum-free medium were added to the upper chamber. The lower chamber contained 600 μL complete medium with 10% FBS. For both migration and invasion assays. After 24h, non-invaded/migrated cells were removed, cells on the lower surface of the membrane were fixed with 4% paraformaldehyde for 15 min and stained with 0.1% crystal violet for 20 min at room temperature. Stained cells were photographed under a light microscope (×4 magnification), and cells in five randomly selected fields per membrane were counted. The percentage of invaded/migrated cells area was calculated from five randomly selected fields per section. The average value was used for statistical analysis.

4.14 Western blotting

Cells were washed with ice-cold PBS and lysed on ice using strong RIPA lysis buffer (Yeasten, #20101ES60) supplemented with protease and/or phosphatase inhibitors as indicated. Lysates were

incubated on ice for 20–30 min with intermittent mixing and clarified by centrifugation at 12,000–16,000 × g for 10–15 min at 4°C. The supernatants were collected, and protein concentrations were determined using a BCA Protein Assay Kit (Beyotime, #P0012). Equal amounts of protein (typically 20–40 µg per lane) were mixed with SDS sample buffer, denatured at 95°C for 5 min, and separated on SurePAGE precast gels (GenScript, #M00610). Proteins were transferred onto 0.45-µm PVDF membranes (Merck Millipore, #IPVH00010) and blocked with Western blocking buffer (Beyotime, #P0023B) for 1 h at room temperature. Membranes were incubated with primary antibodies diluted in Western primary antibody dilution buffer (Beyotime, #P0023A) overnight at 4°C. After washing with 1×TBST prepared from 10×TBST wash buffer (Sangon Biotech, #C520009-0500), membranes were incubated with HRP-conjugated secondary antibodies diluted in Western secondary antibody dilution buffer (Beyotime, #P0023D) for 1 h at room temperature. Signals were developed using BeyoECL Moon (Beyotime, #P0018FS) and captured with a digital imaging system. Detailed information for all primary and secondary antibodies (including supplier, catalog number, and dilution) is provided in Table.

4.15 Bulk RNA sequencing data analysis

All cells used for sequencing (OE CD44v8-10, siCD44v8-10, CSE-treated BEAS-2B, BEAS-2B, and H226) were validated for overexpression or knockdown efficiency by qRT-PCR and Western blotting. mRNA was enriched using Oligo(dT) magnetic beads, fragmented, and reverse-transcribed into double-stranded cDNA. Libraries were prepared via end repair, A-tailing, adapter ligation, PCR amplification, and circularization into single-stranded circular DNA for sequencing on a DNBSEQ-T7 platform. Library quality was assessed by Qubit 3.0 and Agilent 2100, and sequencing was performed in paired-end mode (PE150). Raw reads were aligned to the human GRCh38 genome using HISAT2 (v2.1.0). Gene-level expression was quantified with StringTie (v1.3.4d). Differential expression analysis between groups was performed using the limma-trend method, with $|\log_2 \text{fold change}| > 1$ and $\text{FDR} < 0.05$ as thresholds. DEGs were visualized by volcano plots and hierarchical clustering heatmaps, with FPKM values normalized using Z-score transformation for cross-sample comparison.

Functional enrichment analysis was conducted to explore the biological relevance of DEGs. GO enrichment analysis included molecular function (MF), cellular component (CC), and biological process (BP) categories. KEGG pathway enrichment analysis was performed using the clusterProfiler package. DEGs were separated into up- and down-regulated groups for enrichment calculations, and $\text{FDR} < 0.05$ was considered statistically significant. Enriched terms and pathways were visualized using bar plots, dot plots, and network diagrams to illustrate functional associations.

4.16 Animal study

All animal experiments were approved by the Animal Care and Use Committee of the Guangzhou National Laboratory (GZLAB-AUCP-2025-11-A01). H226 cells (5×10^6) were suspended in saline and injected subcutaneously into the dorsal flank of Male NOD SCID IL-2R $\gamma^{-/-}$ (NSG) mice (6-week-old; Shanghai Model Organisms) mice. Tumor dimensions (length and width) and body weight were measured every 3 days. Tumor volume was calculated using the formula: $V = (\text{length} \times \text{width}^2)/2$. When

tumors reached 100–150 mm³, mice were randomized into the siCD44v8–10 group or negative control (siNC) group. The maximal tumour burden approved by the institutional ethics committee was 1500 mm³, and this limit was not exceeded in the present study. Humane endpoints were defined according to institutional guidelines. Mice received siCD44v8–10 (5 nmol in 200μL) or an equal volume of siNC twice weekly for 3 consecutive weeks via intertumoral injection as described previously. At the endpoint, mice were euthanized, and tumors were excised, photographed, and weighed. Tumor tissues were sectioned and fixed in 4% paraformaldehyde (PFA) for downstream immunohistochemistry. Source data for tumor growth curves are provided in the Source Data file.

4.17 Statistical analysis

Statistical analyses were performed in GraphPad Prism (v8.0.2). Differences between two groups were evaluated using an unpaired two-tailed Student's t-test, whereas comparisons among multiple groups were assessed by one-way or two-way analysis of variance (ANOVA), as appropriate. Data are presented as mean ± s.e.m. unless otherwise specified. A P value < 0.05 was considered statistically significant.

During the preparation of this work, the author(s) used GPT-5.3 Instant (OpenAI) for language polishing and editing. After using this tool, the author(s) reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declarations

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval and consent to participate

The use of human tissue samples in this study was approved by the Ethics Committee of Guangdong Provincial People's Hospital (Approval No. KY2026-266-01). The requirement for informed consent was waived due to the retrospective use of archived specimens stored in the hospital biobank, with all data anonymized prior to analysis. All procedures involving human samples were conducted in accordance with the Declaration of Helsinki.

Authors' contributions

HL and LC conceived the project. HL, LC and FS supervised the study. LS conducted most of the experiments and performed data analysis. SJ contributed to RNA-seq data analysis. NW, QL, and WZ helped with the clinical data analysis and animal study. WZ and YC provided clinical samples and contributed to data interpretation and discussion. HL, LC, FS, YC and LS wrote and reviewed the

manuscript. All authors discussed the data, critically reviewed the manuscript, and approved the final version.

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Data Availability Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

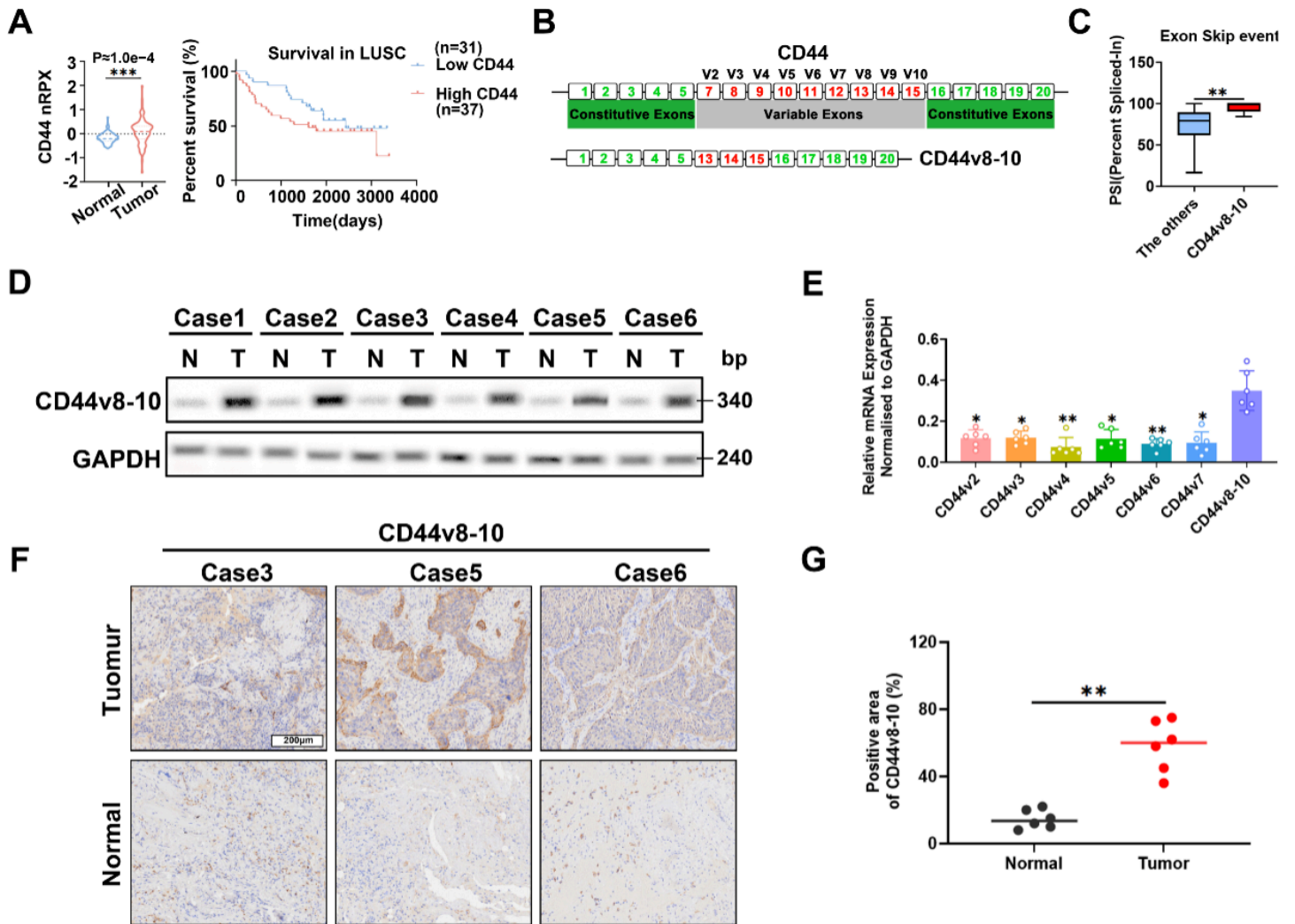


Figure 1

CD44v8-10 expression is elevated in LUSC and is associated with poor prognosis.

(A) CD44 expression and its prognostic relevance in LUSC. Left: CD44 expression levels in normal and tumor tissues obtained from the HPA database. Expression values are presented as normalized relative protein expression (nRPX). Statistical significance between Normal and Tumor groups was assessed using the two-tailed Mann–Whitney U test, as data were not normally distributed. Data are shown as median (interquartile range). *** $P < 0.0001$. RihKaplan-Meier survival analysis of lung squamous cell carcinoma (LUSC) patients stratified by CD44 expression. Patients were divided into high and low expression groups based on the median value of CD44 expression. Overall survival was compared using the log-rank test. The number of patients in each group is indicated (Low CD44, $n = 31$; High CD44, $n = 37$).

(B) *CD44* gene structure and variant isoforms. Schematic representation of the full-length *CD44* gene structure and the *CD44v8-10* variant. Exon composition and splicing events are highlighted.

(C) Box plots show the percent spliced-in (PSI) values of *CD44* exon skip events in LUSC samples. “The others” represent *CD44* exon skipping events excluding the v8-10 cassette, while “*CD44v8-10*” corresponds to splice events containing the v8-10 cassette exons. *CD44v8-10* shows significantly higher PSI values compared with other *CD44* exon skipping events. Data are presented as median with interquartile range. ** $P < 0.01$. PSI values were calculated based on RNA-seq data as implemented in the TCGA SpliceSeq pipeline.

(D) *CD44v8-10* isoform expression in tumor and normal tissues. Semi-quantitative RT-PCR of *CD44v8-10* isoform in 6 paired normal (N) and tumor (T) tissues from 6 patients. *GAPDH* was used as the loading control.

(E) Quantitative reverse transcription PCR (qRT-PCR) analysis of relative mRNA expression of *CD44* alternative splicing isoform in tumor tissues (n=6). Data are presented as mean $\pm$ SD.

(F) Immunohistochemical staining of *CD44v8-10* in tumor and normal tissues (n=2). Representative images of *CD44v8-10* expression in tumor and matched normal tissues from cases 3, 7, and 6. Scale bar = 600 μ m. Brown DAB staining was defined as positive staining for *CD44v8-10*, while hematoxylin-stained blue areas represented nuclei/background tissue.

(G) Quantification of the positive staining area was performed using ImageJ software, and the positive area (%) was calculated as the ratio of DAB-positive area to total tissue area. Each dot represents one tissue sample (n = 6). Data are shown as mean $\pm$ SEM. NS, not significant ($P > 0.05$); **, $P < 0.01$.

Statistical significance was analyzed using the two-tailed Wilcoxon matched-pairs signed-rank test. (E).

Statistical analyses were performed using the two-tailed unpaired t test for (A, C and G). NS, not significant for $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

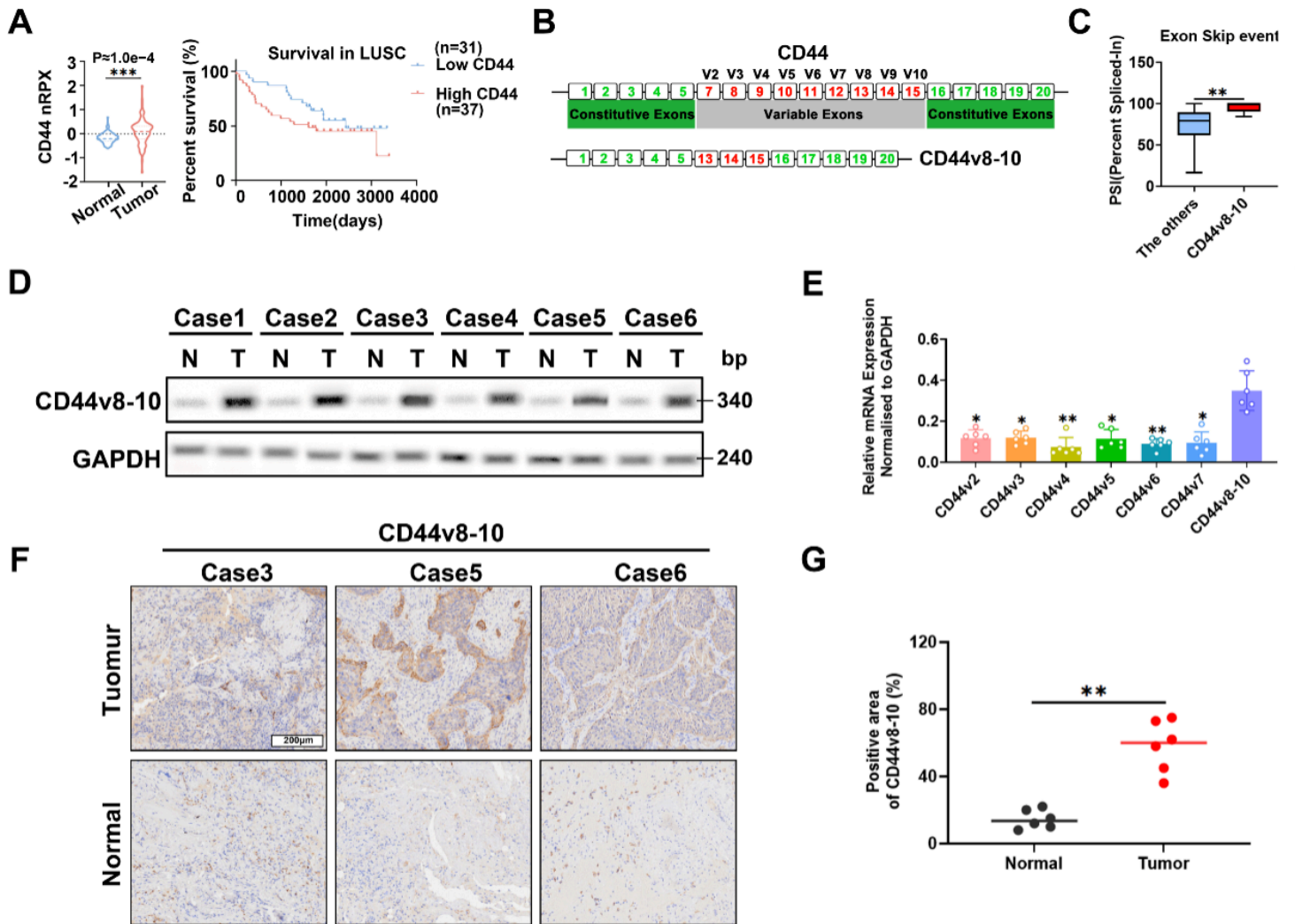


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CD44v8-10 expression is elevated in LUSC and is associated with poor prognosis.

(A) CD44 expression and its prognostic relevance in LUSC. Left: CD44 expression levels in normal and tumor tissues obtained from the HPA database. Expression values are presented as normalized relative protein expression (nRPX). Statistical significance between Normal and Tumor groups was assessed using the two-tailed Mann–Whitney U test, as data were not normally distributed. Data are shown as median (interquartile range). ***P < 0.0001. RihKaplan-Meier survival analysis of lung squamous cell carcinoma (LUSC) patients stratified by CD44 expression. Patients were divided into high and low expression groups based on the median value of CD44 expression. Overall survival was compared using the log-rank test. The number of patients in each group is indicated (Low CD44, n = 31; High CD44, n = 37).

(B) *CD44* gene structure and variant isoforms. Schematic representation of the full-length *CD44* gene structure and the *CD44v8-10* variant. Exon composition and splicing events are highlighted.

(C) Box plots show the percent spliced-in (PSI) values of *CD44* exon skip events in LUSC samples. “The others” represent *CD44* exon skipping events excluding the v8-10 cassette, while “*CD44v8-10*” corresponds to splice events containing the v8-10 cassette exons. *CD44v8-10* shows significantly higher PSI values compared with other *CD44* exon skipping events. Data are presented as median with interquartile range. ** $P < 0.01$. PSI values were calculated based on RNA-seq data as implemented in the TCGA SpliceSeq pipeline.

(D) *CD44v8-10* isoform expression in tumor and normal tissues. Semi-quantitative RT-PCR of *CD44v8-10* isoform in 6 paired normal (N) and tumor (T) tissues from 6 patients. *GAPDH* was used as the loading control.

(E) Quantitative reverse transcription PCR (qRT-PCR) analysis of relative mRNA expression of *CD44* alternative splicing isoform in tumor tissues (n=6). Data are presented as mean $\pm$ SD.

(F) Immunohistochemical staining of *CD44v8-10* in tumor and normal tissues (n=2). Representative images of *CD44v8-10* expression in tumor and matched normal tissues from cases 3, 7, and 6. Scale bar = 600 μ m. Brown DAB staining was defined as positive staining for *CD44v8-10*, while hematoxylin-stained blue areas represented nuclei/background tissue.

(G) Quantification of the positive staining area was performed using ImageJ software, and the positive area (%) was calculated as the ratio of DAB-positive area to total tissue area. Each dot represents one tissue sample (n = 6). Data are shown as mean $\pm$ SEM. NS, not significant ($P > 0.05$); **, $P < 0.01$.

Statistical significance was analyzed using the two-tailed Wilcoxon matched-pairs signed-rank test. (E).

Statistical analyses were performed using the two-tailed unpaired t test for (A, C and G). NS, not significant for $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

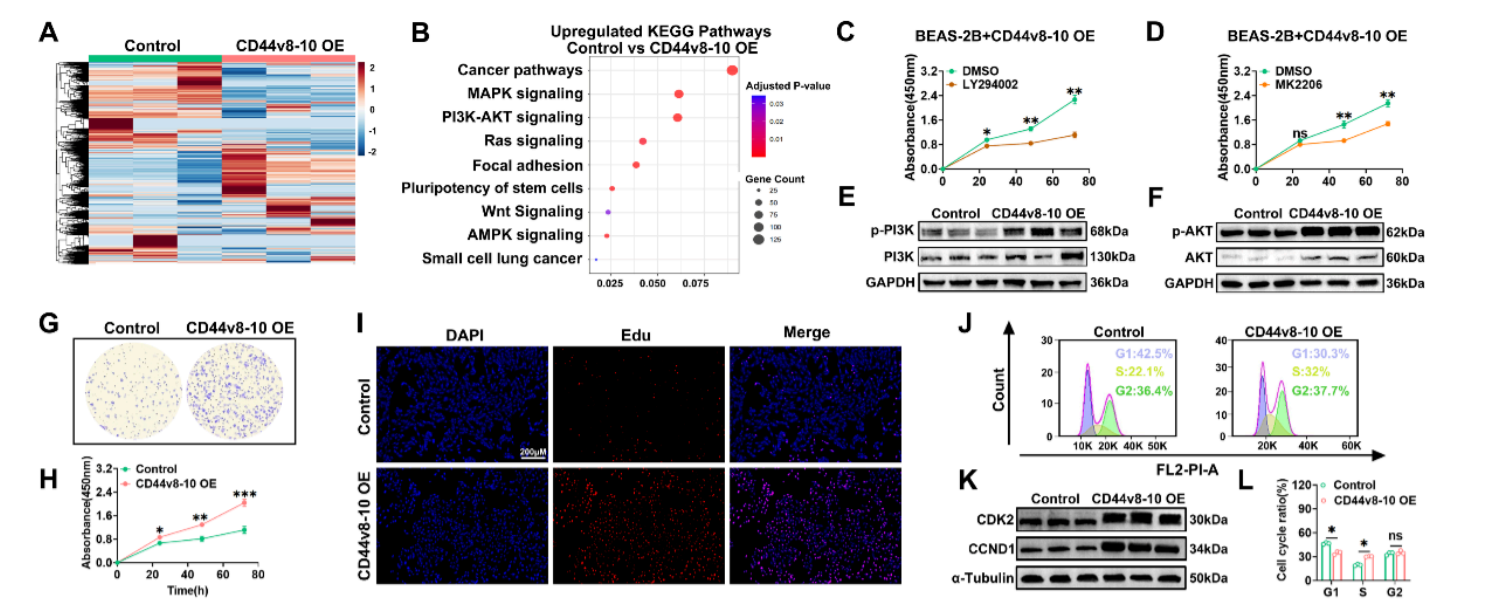


Figure 2

Overexpressing *CD44v8-10* (*CD44v8-100E*) promotes cell proliferation through the activation of PI3K/AKT and cell cycle progression.

(A) Heatmap of differentially expressed genes identified between Control and *CD44v8-10* OE BEAS-2B cells. Hierarchical clustering shows distinct gene expression patterns, with red indicating upregulation and blue indicating downregulation.

(B) Upregulated and KEGG pathway enrichment analysis of differentially expressed genes. Bubble plot shows significantly enriched pathways in *CD44v8-10* OE cells. Bubble size represents gene count and color indicates adjusted P-value. Enriched pathways include Cancer pathways, MAPK signaling (MAPK signaling pathway), PI3K-AKT signaling (PI3K-Akt signaling pathway), Ras signaling (Ras signaling pathway), Focal adhesion, Pluripotency of stem cells (Signaling pathways regulating pluripotency of stem cells), Wnt signaling (Wnt Signaling pathway), AMPK signaling (AMPK signaling pathway), and small cell lung cancer.

(C) CCK8 assay of *CD44v8-10* OE cells treated with or without the PI3K inhibitor LY294002. Absorbance at 450 nm was measured at the indicated time points (n=3). Data are presented as mean $\pm$ SD.

(D) CCK8 assay of *CD44v8-10* OE cells treated with or without the AKT inhibitor MK2206. Absorbance at 450 nm was measured at the indicated time points (n=3). Data are presented as mean $\pm$ SD.

(E) Western blot (WB) analysis of phospho-PI3K (p-PI3K) and total PI3K protein levels in Control and *CD44v8-10* OE cells. GAPDH was used as a loading control.

(F) Western blot analysis of phospho-AKT (p-AKT) and total AKT protein levels in Control and *CD44v8-10* OE cells. GAPDH was used as a loading control.

(G) Representative images of colony formation assays in Control and *CD44v8-10* OE cells.

(H) CCK8 assay showing cell proliferation in Control and *CD44v8-100E* cells at the indicated time points (n=3). Data are presented as mean $\pm$ SD.

(I) Representative immunofluorescence images of EdU incorporation assay in Control and *CD44v8-10* OE cells. DAPI (blue) stains nuclei and EdU (red) indicates proliferating cells. Merged images are shown.

(J) Cell cycle analysis by flow cytometry in Control and *CD44v8-100E* cells. Representative histograms show the distribution of cells in G1, S, and G2 phases.

(K) WB analysis of cell cycle-related proteins CDK2 and CCND1 in Control and *CD44v8-10* OE cells. α -Tubulin was used as a loading control.

(L) Quantification of cell cycle distribution in Control and *CD44v8-10* OE cells. Bar graphs show the percentages of cells in G1, S, and G2 phases (n=3). Data are presented as mean $\pm$ SD.

Statistical analyses were performed using the two-tailed unpaired t test for (C and D, H and L). NS, not significant for $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

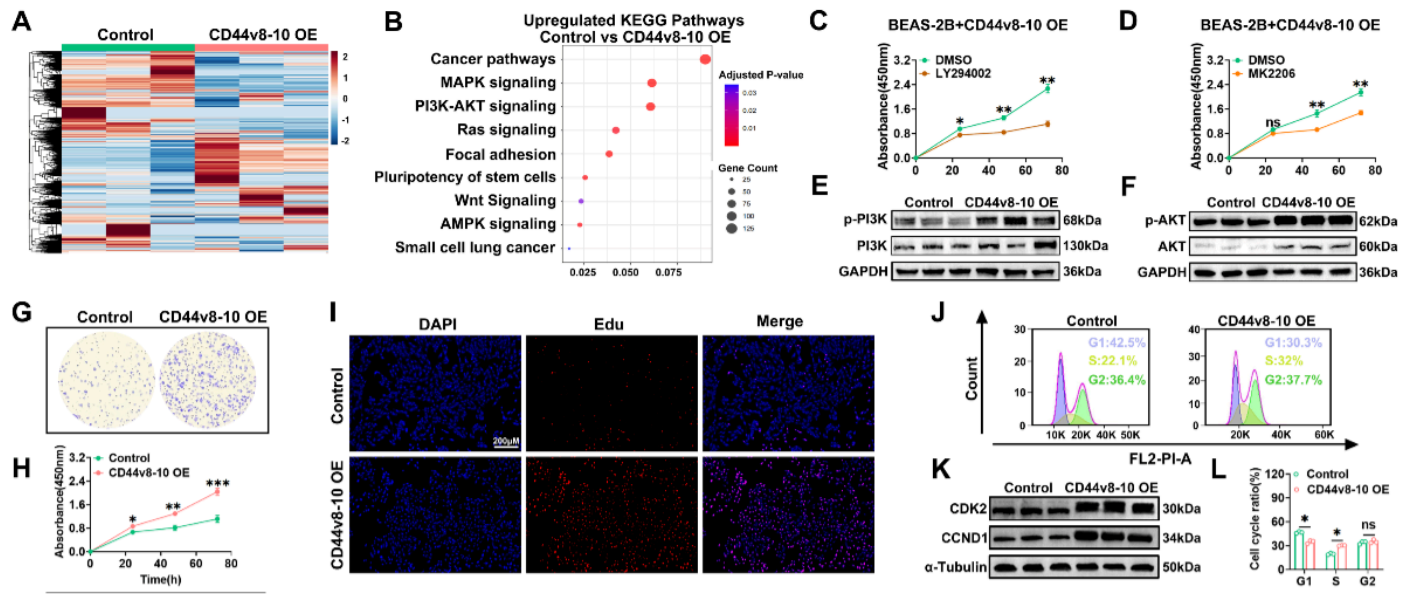


Figure 2

Overexpressing *CD44v8-10* (*CD44v8-10OE*) promotes cell proliferation through the activation of PI3K/AKT and cell cycle progression.

(A) Heatmap of differentially expressed genes identified between Control and CD44v8-10 OE BEAS-2B cells. Hierarchical clustering shows distinct gene expression patterns, with red indicating upregulation and blue indicating downregulation.

(B) Upregulated and KEGG pathway enrichment analysis of differentially expressed genes. Bubble plot shows significantly enriched pathways in CD44v8-10 OE cells. Bubble size represents gene count and color indicates adjusted P-value. Enriched pathways include Cancer pathways, MAPK signaling (MAPK signaling pathway), PI3K-AKT signaling (PI3K-Akt signaling pathway), Ras signaling (Ras signaling pathway), Focal adhesion, Pluripotency of stem cells (Signaling pathways regulating pluripotency of stem cells), Wnt signaling (Wnt Signaling pathway), AMPK signaling (AMPK signaling pathway), and small cell lung cancer.

(C) CCK8 assay of CD44v8-10 OE cells treated with or without the PI3K inhibitor LY294002. Absorbance at 450 nm was measured at the indicated time points (n=3). Data are presented as mean $\pm$ SD.

(D) CCK8 assay of CD44v8-10 OE cells treated with or without the AKT inhibitor MK2206. Absorbance at 450 nm was measured at the indicated time points (n=3). Data are presented as mean $\pm$ SD.

(E) Western blot (WB) analysis of phospho-PI3K (p-PI3K) and total PI3K protein levels in Control and CD44v8-10 OE cells. GAPDH was used as a loading control.

(F) Western blot analysis of phospho-AKT (p-AKT) and total AKT protein levels in Control and CD44v8-10 OE cells. GAPDH was used as a loading control.

(G) Representative images of colony formation assays in Control and *CD44v8-10* OE cells.

(H) CCK8 assay showing cell proliferation in Control and *CD44v8-10*OE cells at the indicated time points (n=3). Data are presented as mean $\pm$ SD.

(I) Representative immunofluorescence images of EdU incorporation assay in Control and *CD44v8-10* OE cells. DAPI (blue) stains nuclei and EdU (red) indicates proliferating cells. Merged images are shown.

(J) Cell cycle analysis by flow cytometry in Control and *CD44v8-10*OE cells. Representative histograms show the distribution of cells in G1, S, and G2 phases.

(K) WB analysis of cell cycle-related proteins CDK2 and CCND1 in Control and CD44v8-10 OE cells. α -Tubulin was used as a loading control.

(L) Quantification of cell cycle distribution in Control and *CD44v8-10* OE cells. Bar graphs show the percentages of cells in G1, S, and G2 phases (n=3). Data are presented as mean $\pm$ SD.

Statistical analyses were performed using the two-tailed unpaired t test for (C and D, H and L). NS, not significant for $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

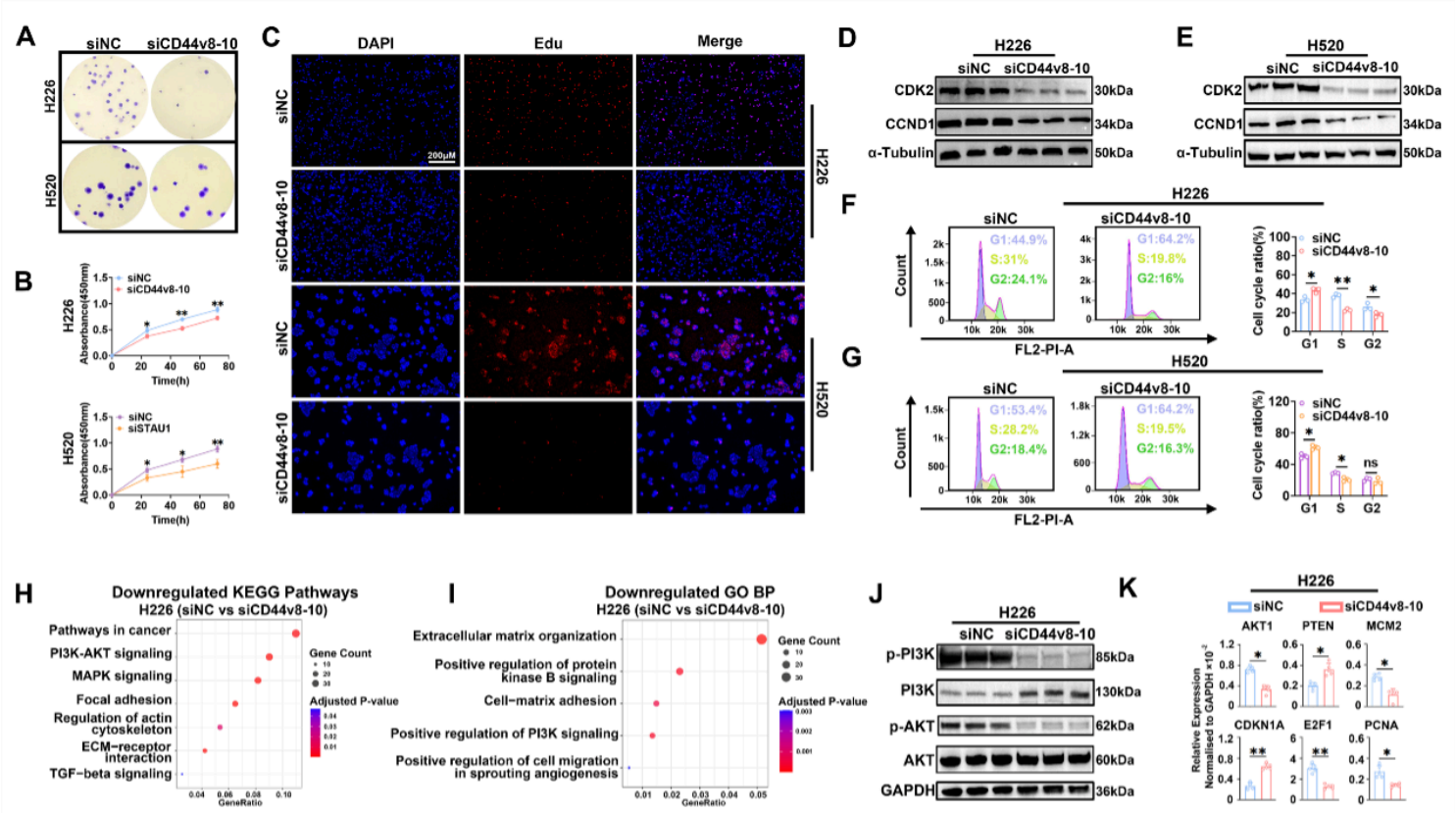


Figure 3

Knockdown of *CD44v8-10* suppresses PI3K/AKT signaling, leading to cell cycle arrest and inhibition of cell proliferation.

(A) Representative images of colony formation assays in siNC and si*CD44v8-10* cells.

(B) CCK8 assay showing cell proliferation in siNC and si*CD44v8-10* cells at the indicated time points. Absorbance was measured at 450 nm (n=3). Data are presented as mean $\pm$ SD.

(C) Representative immunofluorescence images of EdU incorporation assay in siNC and si*CD44v8-10* cells. DAPI (blue) stains nuclei and EdU (red) indicates proliferating cells. Merged images are shown. Scale bars, 1000 μ m.

(D and E) Protein expression of the cell cycle-related proteins CDK2 and CCND1 in siNC and si*CD44v8-10* cells. α -Tubulin was used as a loading control.

(F and G) Cell cycle analysis by flow cytometry in siNC and si*CD44v8-10* cells. Representative histograms show the distribution of cells in G1, S, and G2 phases. Quantification of cell cycle distribution in siNC and si*CD44v8-10* cells (n=3). Bar graphs show the percentages of cells in G0/G1, S, and G2/M phases. Data are presented as mean $\pm$ SD.

(H) Downregulated KEGG pathway enrichment analysis of differentially expressed genes following *CD44v8-10* knockdown. Bubble size represents gene count and color indicates adjusted P-value.

(I) Downregulated GO biological process enrichment analysis of genes altered by *CD44v8-10* knockdown. Bubble size represents gene count and color indicates adjusted P-value.

(J) Western blot analysis of PI3K/AKT signaling pathway proteins in siNC and si*CD44v8-10* cells. Protein levels of p-PI3K, PI3K, p-AKT, and AKT were detected, with GAPDH as a loading control.

(K) qRT-PCR analysis relative mRNA expression of PI3K/AKT pathway (*AKT1*, *PTEN*, *CDKN1A*, *E2F1*, *PCNA*, and *MCM2*) in siNC and si*CD44v8-10* cells (n=3). Expression levels were normalized to GAPDH (n=3). Data are presented as mean $\pm$ SD.

Statistical analyses were performed using the two-tailed unpaired t test for (B, E, G and K). NS, not significant for $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

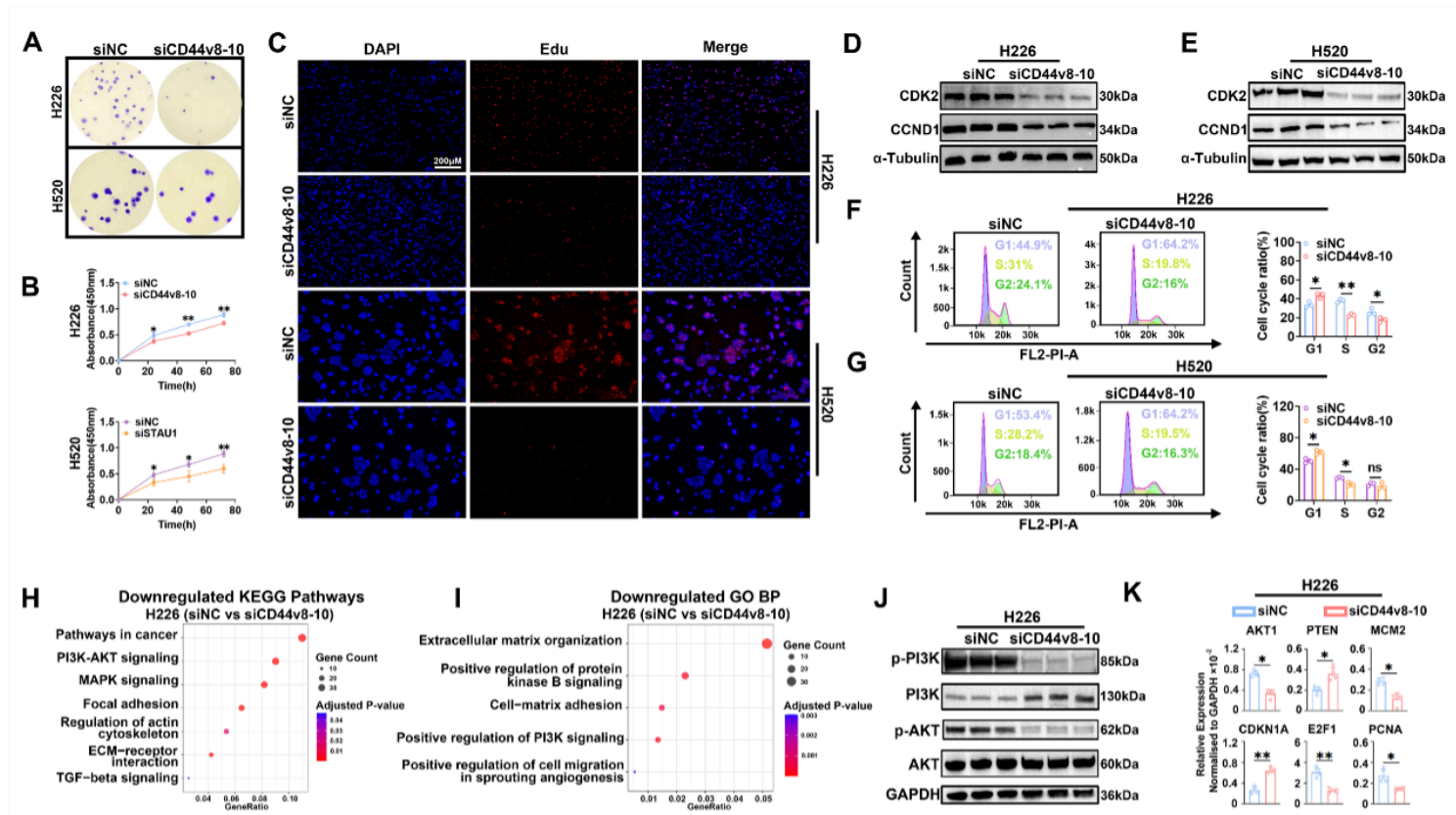


Figure 3

Knockdown of *CD44v8-10* suppresses PI3K/AKT signaling, leading to cell cycle arrest and inhibition of cell proliferation.

(A) Representative images of colony formation assays in siNC and siCD44v8-10 cells.

(B) CCK8 assay showing cell proliferation in siNC and siCD44v8-10 cells at the indicated time points. Absorbance was measured at 450 nm (n=3). Data are presented as mean ± SD.

(C) Representative immunofluorescence images of EdU incorporation assay in siNC and siCD44v8-10 cells. DAPI (blue) stains nuclei and EdU (red) indicates proliferating cells. Merged images are shown. Scale bars, 1000 μm.

(D and E) Protein expression of the cell cycle-related proteins CDK2 and CCND1 in siNC and siCD44v8-10 cells. α-Tubulin was used as a loading control.

(F and G) Cell cycle analysis by flow cytometry in siNC and siCD44v8-10 cells. Representative histograms show the distribution of cells in G1, S, and G2 phases. Quantification of cell cycle distribution in siNC and siCD44v8-10 cells (n=3). Bar graphs show the percentages of cells in G0/G1, S, and G2/M phases. Data are presented as mean ± SD.

(H) Downregulated KEGG pathway enrichment analysis of differentially expressed genes following CD44v8-10 knockdown. Bubble size represents gene count and color indicates adjusted P-value.

(I) Downregulated GO biological process enrichment analysis of genes altered by *CD44v8-10* knockdown. Bubble size represents gene count and color indicates adjusted P-value.

(J) Western blotanalysis of PI3K/AKT signaling pathway proteins in siNC and si*CD44v8-10* cells. Protein levels of p-PI3K, PI3K, p-AKT, and AKT were detected, with GAPDH as a loading control.

(K) qRT-PCR analysis relative mRNA expression of PI3K/AKT pathway (*AKT1*, *PTEN*, *CDKN1A*, *E2F1*, *PCNA*, and *MCM2*) in siNC and si*CD44v8-10* cells (n=3). Expression levels were normalized to GAPDH (n=3). Data are presented as mean $\pm$ SD.

Statistical analyses were performed using the two-tailed unpaired t test for (B, E, G and K). NS, not significant for $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

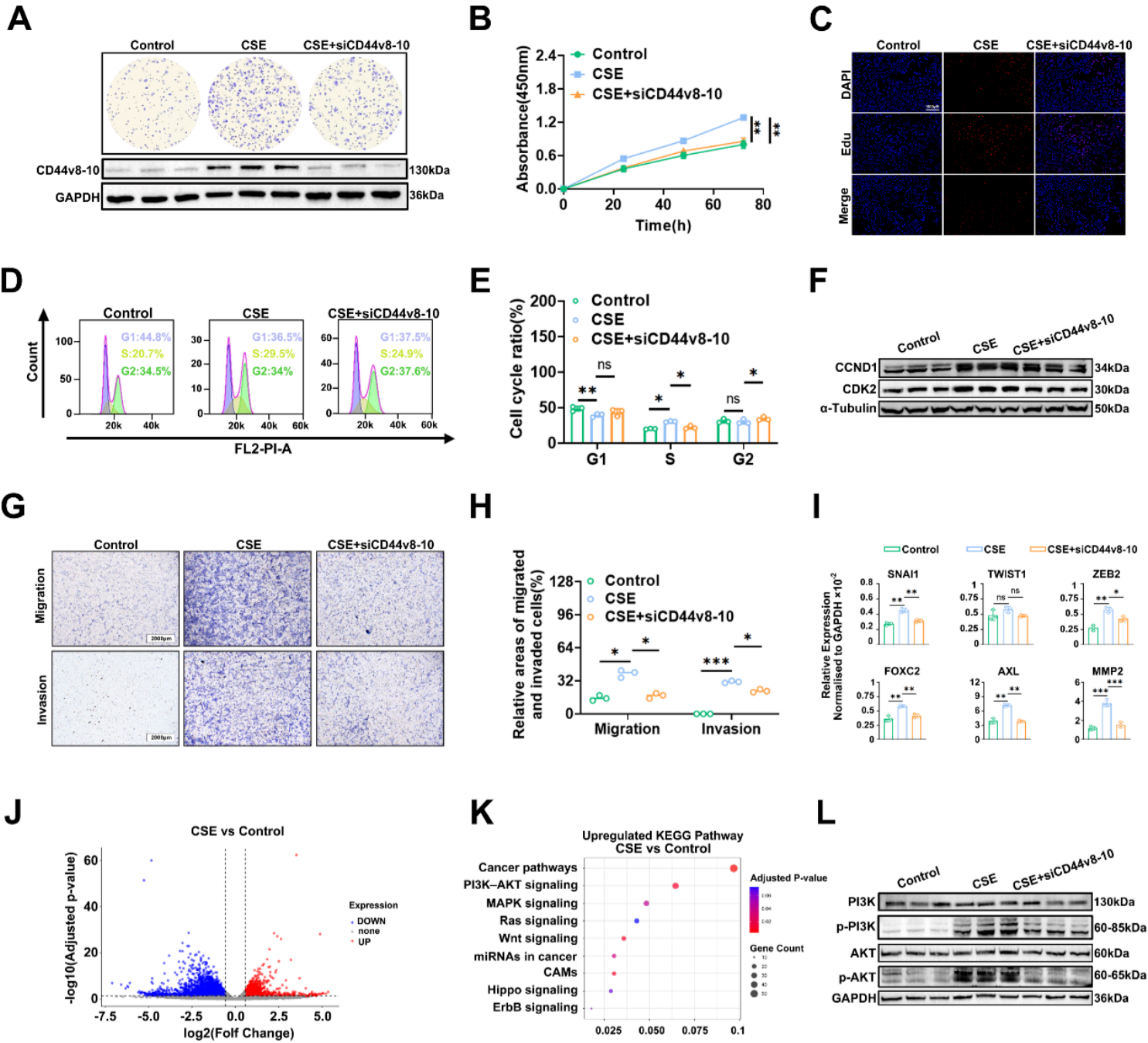


Figure 4

CSE induces carcinogenesis by upregulating CD44v8–10.

(A) Representative images of colony formation assays in Control, CSE, and CSE+siCD44v8-10 cells (upper). WB analysis of CD44v8-10 protein expression in the indicated groups (lower). GAPDH was used as a loading control.

(B) CCK8 assay showing cell proliferation in Control, CSE, and CSE+siCD44v8-10 cells. Absorbance was measured at 450 nm at the indicated time points. Data are presented as mean $\pm$ SD (n = 3).

(C) Representative immunofluorescence images of EdU incorporation assay in the indicated groups. DAPI (blue) stains nuclei and EdU (red) indicates proliferating cells. Merged images are shown.

(D) Cell cycle distribution analyzed by flow cytometry in Control, CSE, and CSE+siCD44v8-10 cells. Representative histograms show the percentages of cells in G1, S, and G2 phases.

(E) Quantification of cell cycle distribution in the indicated groups. Bar graphs show the percentages of cells in G1, S, and G2 phases (n=3). Data are presented as mean $\pm$ SD.

(F) WB analysis of the cell cycle-related proteins CCND1 and CDK2 in the indicated groups. α -Tubulin was used as a loading control.

(G) Representative images of Transwell migration and invasion assays in Control, CSE, and CSE+siCD44v8-10 cells. Scale bars, 200 μ m.

(H) Quantification of migrated and invaded cells in the indicated groups (n = 3). Relative areas of migrated and invaded cells (%) are shown. Data are presented as mean $\pm$ SD.

(I) qRT-PCR analysis relative mRNA expression of EMT-related genes (*SNAI1*, *TWIST1*, *ZEB2*, *FOXC2*, *AXL*, and *MMP2*) in the indicated groups (n = 3). Expression levels were normalized to GAPDH. Data are presented as mean $\pm$ SD.

(J) Volcano plot of differentially expressed genes between CSE and Control cells. Red dots indicate upregulated genes and blue dots indicate downregulated genes.

(K) Upregulated KEGG pathway enrichment analysis of differentially expressed genes. Bubble size represents gene count and color indicates adjusted P value.

(L) WB analysis of PI3K/AKT signaling pathway proteins (PI3K, p-PI3K, AKT, and p-AKT) in the indicated groups. GAPDH was used as a loading control.

Statistical analyses for panels B, E, H, and I were performed using one-way ANOVA followed by Tukey's multiple-comparisons test. Data are presented as mean $\pm$ SD unless otherwise indicated. NS, not significant ($P > 0.05$); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

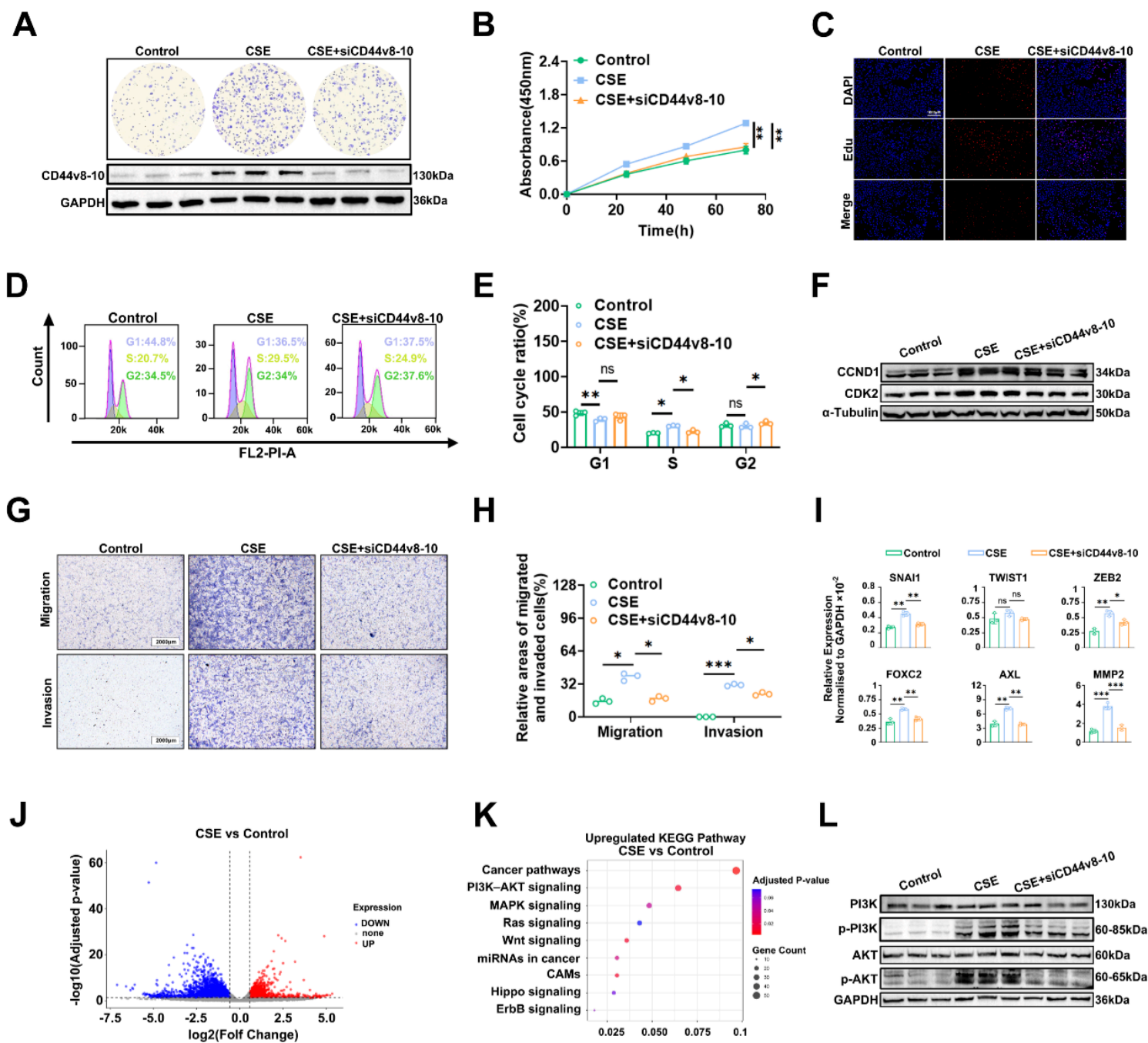


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CSE induces carcinogenesis by upregulating CD44v8-10.

(A) Representative images of colony formation assays in Control, CSE, and CSE+siCD44v8-10 cells (upper). WB analysis of CD44v8-10 protein expression in the indicated groups (lower). GAPDH was used as a loading control.

(B) CCK8 assay showing cell proliferation in Control, CSE, and CSE+siCD44v8-10 cells. Absorbance was measured at 450 nm at the indicated time points. Data are presented as mean $\pm$ SD (n = 3).

(C) Representative immunofluorescence images of EdU incorporation assay in the indicated groups. DAPI (blue) stains nuclei and EdU (red) indicates proliferating cells. Merged images are shown.

(D) Cell cycle distribution analyzed by flow cytometry in Control, CSE, and CSE+si*CD44v8-10* cells. Representative histograms show the percentages of cells in G1, S, and G2 phases.

(E) Quantification of cell cycle distribution in the indicated groups. Bar graphs show the percentages of cells in G1, S, and G2 phases (n=3). Data are presented as mean $\pm$ SD.

(F) WB analysis of the cell cycle-related proteins CCND1 and CDK2 in the indicated groups. α -Tubulin was used as a loading control.

(G) Representative images of Transwell migration and invasion assays in Control, CSE, and CSE+si*CD44v8-10* cells. Scale bars, 200 μ m.

(H) Quantification of migrated and invaded cells in the indicated groups (n = 3). Relative areas of migrated and invaded cells (%) are shown. Data are presented as mean $\pm$ SD.

(I) qRT-PCR analysis relative mRNA expression of EMT-related genes (*SNAI1*, *TWIST1*, *ZEB2*, *FOXC2*, *AXL*, and *MMP2*) in the indicated groups (n = 3). Expression levels were normalized to GAPDH. Data are presented as mean $\pm$ SD.

(J) Volcano plot of differentially expressed genes between CSE and Control cells. Red dots indicate upregulated genes and blue dots indicate downregulated genes.

(K) Upregulated KEGG pathway enrichment analysis of differentially expressed genes. Bubble size represents gene count and color indicates adjusted P value.

(L) WB analysis of PI3K/AKT signaling pathway proteins (PI3K, p-PI3K, AKT, and p-AKT) in the indicated groups. GAPDH was used as a loading control.

Statistical analyses for panels B, E, H, and I were performed using one-way ANOVA followed by Tukey's multiple-comparisons test. Data are presented as mean $\pm$ SD unless otherwise indicated. NS, not significant (P > 0.05); *P < 0.05, **P < 0.01, ***P < 0.001.

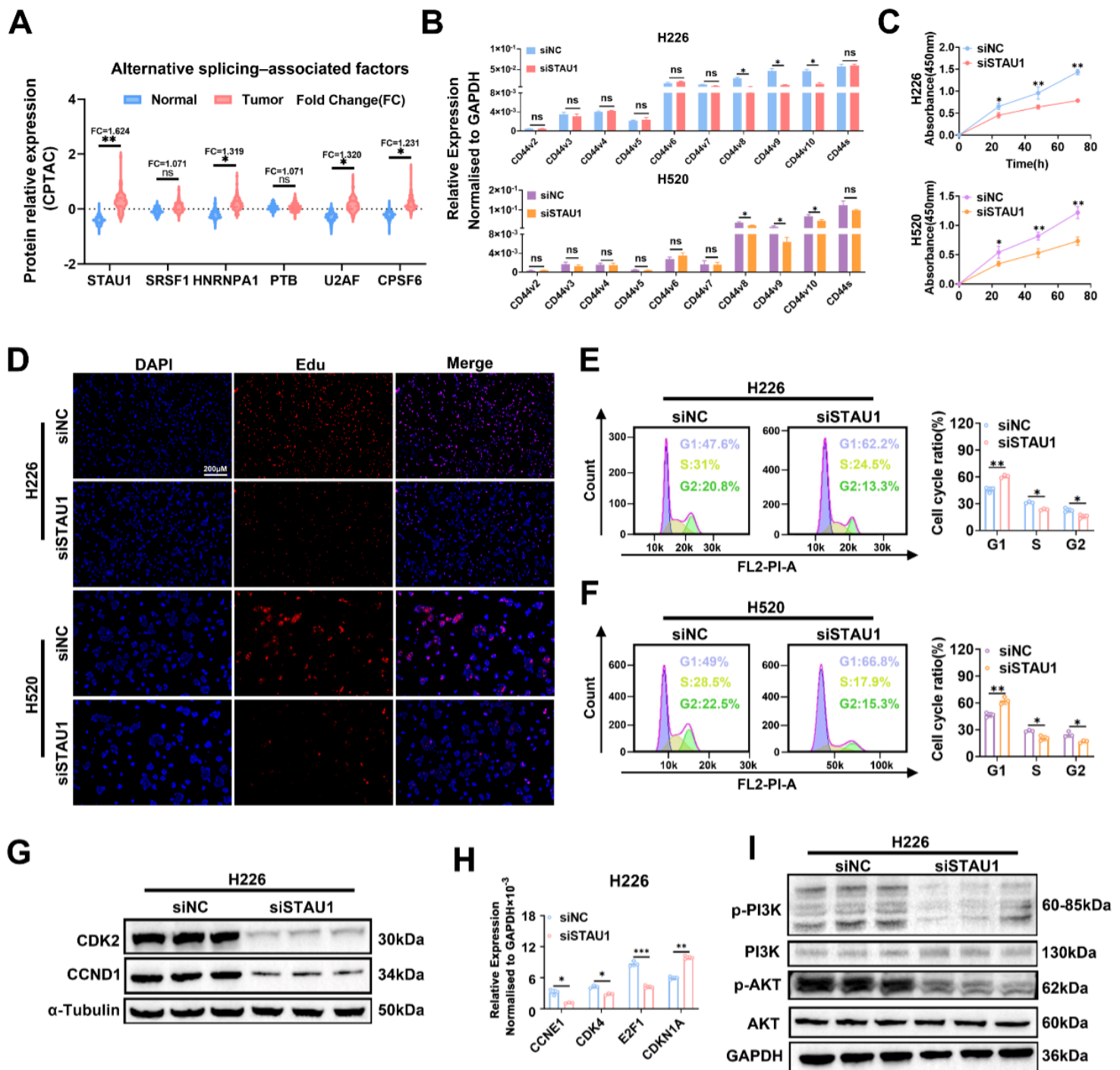


Figure 5

STAU1 acts as an upstream regulator of CD44v8-10 expression.

(A) Protein expression levels of alternative splicing-associated factors in Normal and LUSC samples from the CPTAC cohort. Protein abundance distributions are shown as violin plots, with blue and red representing Normal and Tumor samples, respectively. The fold change (FC) of protein expression between Tumor and Normal samples is indicated above each factor. Statistical significance was determined by comparison between the two groups. ns, not significant; * $P < 0.05$; ** $P < 0.01$.

(B) Relative expression levels of *CD44* splice variants (*CD44v2-v10* and *CD44s*) in siNC and siSTAU1 cells (n=3). Expression levels were normalized to GAPDH. Data are presented as mean $\pm$ SD.

(C) CCK8 assay showing cell proliferation in siNC and siSTAU1 cells at the indicated time points. Absorbance was measured at 450 nm (n=3). Data are presented as mean $\pm$ SD.

(D) Representative immunofluorescence images of EdU incorporation in siNC and siSTAU1 cells. DAPI (blue) stains nuclei and EdU (red) marks proliferating cells. Merged images are shown. Scale bars, 100 μ m.

(E and F) Cell cycle distribution analyzed by flow cytometry in siNC and siSTAU1 cells (left). Representative histograms show the percentages of cells in G1, S, and G2 phases. Quantification of cell cycle distribution in siNC and siSTAU1 cells (n=3). Bar graphs show the percentages of cells in G0/G1, S, and G2/M phases. Data are presented as mean $\pm$ SD.

(G) WB analysis of CDK2 and CCND1 protein levels in siNC and siSTAU1 cells. α -Tubulin was used as a loading control.

(H) qRT-PCR analysis relative mRNA expression of cell cycle-related genes (*CCNE1*, *CDK4*, *E2F1*, and *CDKN1A*) in siNC and siSTAU1 cells (n=3). Expression levels were normalized to GAPDH. Data are presented as mean $\pm$ SD.

(I) WB analysis of PI3K/AKT pathway-related proteins (p-PI3K, PI3K, p-AKT, and AKT) in siNC and siSTAU1 cells. GAPDH was used as a loading control.

Statistical analyses were performed using the two-tailed unpaired t test for (A, C, G and H). NS, not significant for $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Data are presented as mean $\pm$ SD unless otherwise indicated.

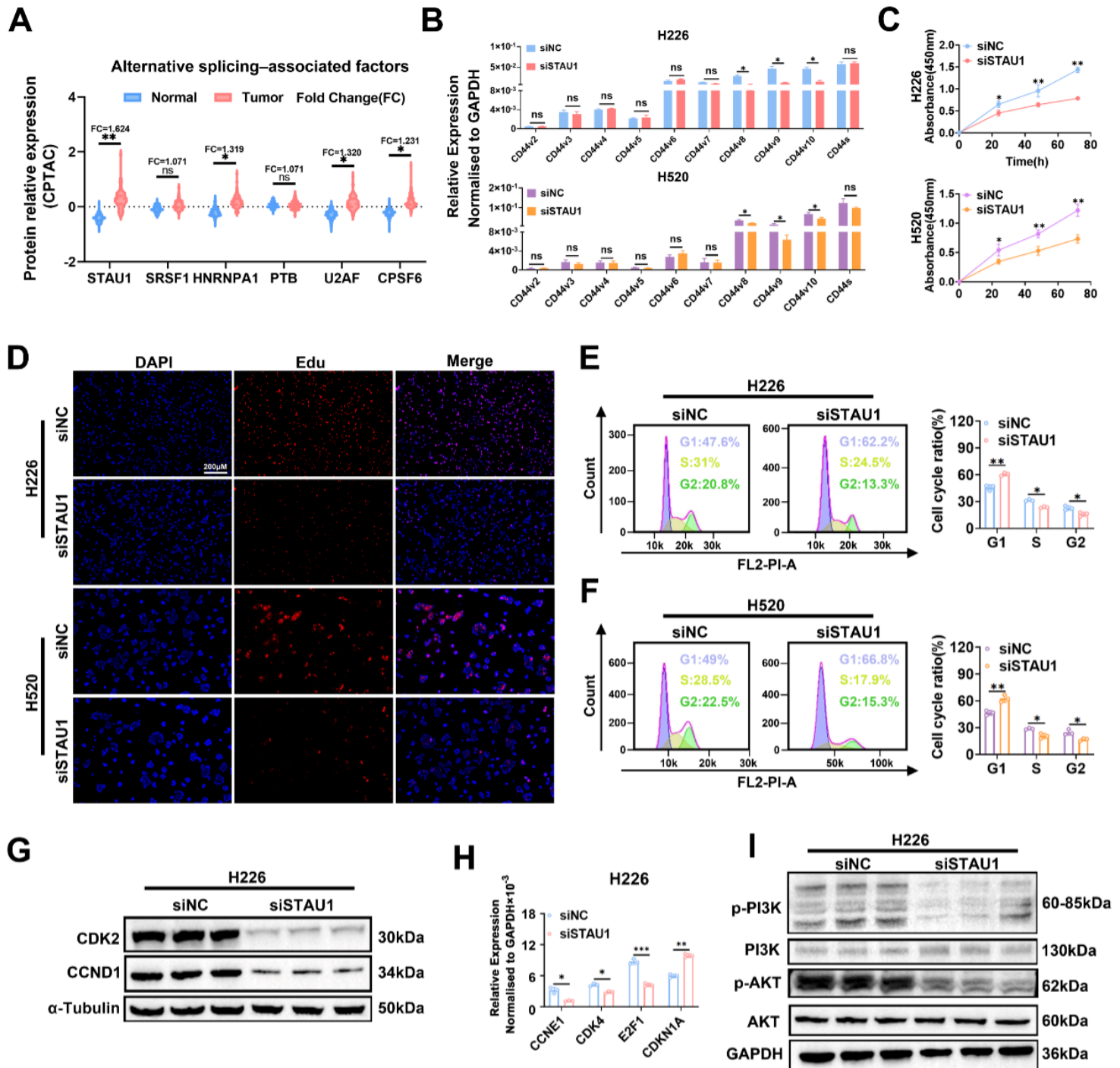


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(B) Relative expression levels of *CD44* splice variants (*CD44v2-v10* and *CD44s*) in siNC and siSTAU1 cells (n=3). Expression levels were normalized to GAPDH. Data are presented as mean $\pm$ SD.

(C) CCK8 assay showing cell proliferation in siNC and siSTAU1 cells at the indicated time points. Absorbance was measured at 450 nm (n=3). Data are presented as mean $\pm$ SD.

(D) Representative immunofluorescence images of EdU incorporation in siNC and siSTAU1 cells. DAPI (blue) stains nuclei and EdU (red) marks proliferating cells. Merged images are shown. Scale bars, 100 μ m.

(E and F) Cell cycle distribution analyzed by flow cytometry in siNC and siSTAU1 cells (left). Representative histograms show the percentages of cells in G1, S, and G2 phases. Quantification of cell cycle distribution in siNC and siSTAU1 cells (n=3). Bar graphs show the percentages of cells in G0/G1, S, and G2/M phases. Data are presented as mean $\pm$ SD.

(G) WB analysis of CDK2 and CCND1 protein levels in siNC and siSTAU1 cells. α -Tubulin was used as a loading control.

(H) qRT-PCR analysis relative mRNA expression of cell cycle-related genes (*CCNE1*, *CDK4*, *E2F1*, and *CDKN1A*) in siNC and siSTAU1 cells (n=3). Expression levels were normalized to GAPDH. Data are presented as mean $\pm$ SD.

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Statistical analyses were performed using the two-tailed unpaired t test for (A, C, G and H). NS, not significant for $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Data are presented as mean $\pm$ SD unless otherwise indicated.

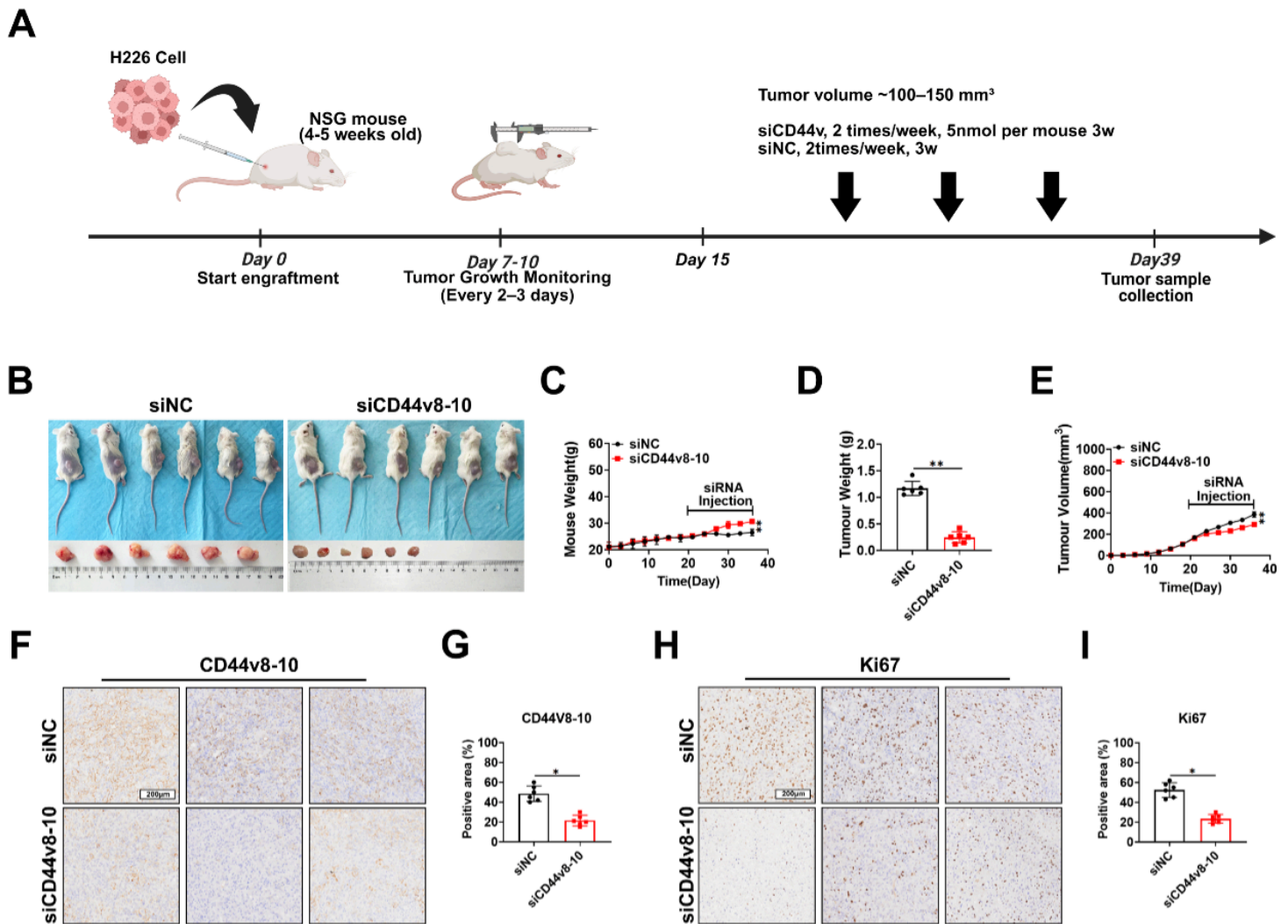


Figure 6

CD44v8-10 silencing suppresses tumor growth in vivo in a xenograft mouse model.

(A) Schematic illustration of the in vivo xenograft experiment. H226 cells were subcutaneously injected into 4-5-week-old NSG mice (Day 0). Tumor growth was monitored every 2-3 days. When tumor volume reached approximately 100-150 mm³ (around Day 15), mice received siCD44v8-10 (5 nmol per mouse, twice per week for 3 weeks) or siNC control. Tumor samples were collected on Day 39.

(B) Representative images of excised tumors from the siNC and siCD44v8-10 groups.

(C) Mouse body weight measured at the indicated time points during treatment (n=6). Data are presented as mean ± SD.

(D) Tumor weight of xenografts in the siNC and siCD44v8-10 groups at the endpoint (n=6). Data are presented as mean ± SD.

(E) Tumor growth curves showing tumor volume measured at the indicated time points (n=6). The arrow indicates the initiation of siRNA injection. Data are presented as mean $\pm$ SD.

(F) Representative immunohistochemical (IHC) staining of *CD44v8-10* in xenograft tumor sections from siNC and si*CD44v8-10* groups. Scale bars, 50 μ m.

(G) Quantification of *CD44v8-10* protein expression by positive area (%) in the indicated groups (n=6). Data are presented as mean $\pm$ SD.

(H) Representative IHC staining of Ki67 in xenograft tumor sections from siNC and si*CD44v8-10* groups. Scale bars, 50 μ m.

(I) Quantification of Ki67 protein expression by positive area (%) in the indicated groups. Data are presented as mean $\pm$ SD.

Statistical analyses were performed using the two-tailed unpaired t test for (D, G, and I) and two-way ANOVA followed by multiple comparisons test for (C and E). NS, not significant for $P > 0.05$, * $P < 0.05$, ** $P < 0.01$. Data are presented as mean $\pm$ SD unless otherwise indicated.

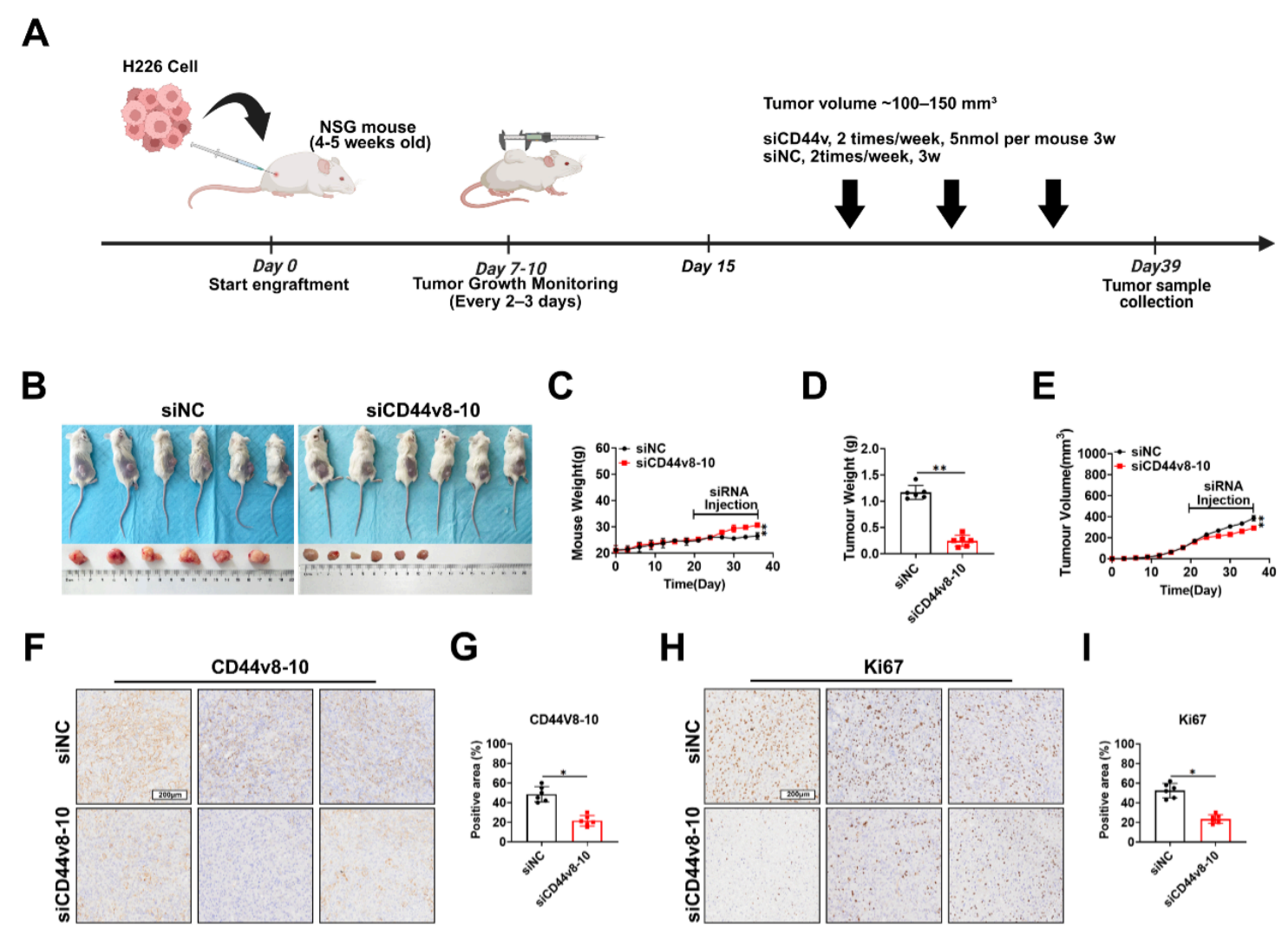


Figure 6

***CD44v8-10* silencing suppresses tumor growth in vivo in a xenograft mouse model.**

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(B) Representative images of excised tumors from the siNC and si*CD44v8-10* groups.

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(F) Representative immunohistochemical (IHC) staining of *CD44v8-10* in xenograft tumor sections from siNC and si*CD44v8-10* groups. Scale bars, 50 µm.

(G) Quantification of *CD44v8-10* protein expression by positive area (%) in the indicated groups (n=6). Data are presented as mean ± SD.

(H) Representative IHC staining of Ki67 in xenograft tumor sections from siNC and si*CD44v8-10* groups. Scale bars, 50 µm.

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Supplementary Files

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- floatimage1.jpeg