

Integrated Proteomics Reveal ALDH1A1 as an Oncogenic Driver and Regulatory Hub in Hepatocellular Carcinoma

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Abstract

Background/Aim: Although aldehyde dehydrogenases 1 family member A1 (ALDH1A1) has been extensively studied in cancer, its role in hepatocellular carcinoma (HCC) remains poorly understood. This study was designed to characterize the expression pattern and functional roles of ALDH1A1 in HCC, and to further investigate its underlying molecular mechanisms using integrated proteomic analysis.

Materials and Methods: This study performed a pan-cancer analysis of ALDH1A1 expression, followed by validation of its expression levels in HCC tissue samples and cell lines. The expression of ALDH1A1 was manipulated using small interfering RNAs and lentiviral vector in HCC cell lines. And then the proliferation, migration, invasion, and tumorigenicity of HCC cells were observed *in vitro* and *in vivo*. Quantitative proteomic and phosphoproteomic profiling were conducted by liquid chromatography-tandem mass spectrometry, with comprehensive analyses and key findings validated by experiments.

Results: ALDH1A1 was highly expressed in HCC tissues but exhibited notable expression heterogeneity among HCC cell lines. Functionally, ALDH1A1 promoted the proliferation, migration, and tumorigenicity of HCC cells, while suppressing apoptosis and modestly attenuates invasion. Quantitative proteomic and phosphoproteomic analyses revealed that ALDH1A1 profoundly reshaped both protein expression and phosphorylation landscapes in HCC. Furthermore, we validated that ALDH1A1 positively regulates the expression of DMPK, PCMTD2, VAMP4, ARHGAP19, NOL4L, and ST7, while suppressing SLC31A1, DSTYK, CYP4F12, GPNMB expression. Rescue experiments indicated that ALDH1A1 may promote HCC *via* activating Rho family small GTPase. ALDH1A1 also significantly upregulated mTOR phosphorylation.

Conclusion: ALDH1A1 acts as an oncogenic driver and regulatory hub in HCC.

Keywords: ALDH1A1, hepatocellular carcinoma, HCC, proteomics, phosphoproteomics.



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Introduction

Globally, primary liver cancer ranks sixth in incidence and third in mortality among all malignant tumors, with its disability-adjusted life years reaching 12.5 million (1-3). Among the three pathological subtypes of primary liver cancer, hepatocellular carcinoma (HCC) accounts for approximately 80% of cases (4).

In our previous study, we summarized and discussed that examined the role of aldehyde dehydrogenases 1 family member A1 (ALDH1A1) in cancers. Research showed that ALDH1A1 induces cancers progression *via* the maintenance of cancer stem cell properties, modification of metabolism, promotion of DNA repair. And the detoxification of ALDH1A1 often causes chemotherapy failure. ALDH1A1 also acted as a tumor suppressor in certain cancers (5). Molecular probes targeting ALDH1A1 have also been developed for the diagnosis of breast cancer (6). However, the role of ALDH1A1 in HCC is not fully understood. Although recent study highlighted the importance of ALDH1A1 in inherent drug resistance and cell differentiation of HCC, the roles of ALDH1A1 in HCC progression remain poorly understood (7-9).

This work aimed to systematically assess the regulatory roles of ALDH1A1 in HCC progression and comprehensively explore its underlying molecular mechanisms. In this study, we evaluated the expression patterns of ALDH1A1 and genetically modulated ALDH1A1 expression in HCC cell lines. Subsequently, the proliferation, migration, invasion, apoptosis and tumorigenicity of HCC cells were observed. Furthermore, integrated proteomic profiling was performed to investigate ALDH1A1-mediated alterations in protein expression and phosphorylation, with key findings validated by experiments (Figure 1).

Materials and Methods

Cell culture and samples. Human HCC cell lines (Huh-7, SK-HEP-1, and HCCLM3) were obtained from Pricella (Wuhan, PR China) with genetic authentication, while

human normal liver cell line THLE-2 was obtained from Immocell (Xiamen, PR China). All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM; Solarbio, Beijing, PR China) supplemented with 10% fetal bovine serum (FBS; Solarbio) and 1% penicillin-streptomycin (Beyotime, Shanghai, PR China), and maintained at 37°C in a humidified incubator (Thermo Fisher Scientific, Waltham, MA, USA) containing 5% CO₂.

Complementary DNA (cDNA) samples of HCC and adjacent normal tissues were collected from the Department of Gastroenterology, the First Hospital of Lanzhou University.

Gene expression manipulation and drugs. Small interfering RNA specifically targeting ALDH1A1 (siRNA-ALDH1A1) and corresponding negative control (siRNA-NC) were designed and synthesized by GenePharma (Shanghai, PR China). Three siRNA-ALDH1A1 sequences were provided for screening. Transfection was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions.

To establish ALDH1A1-overexpressing HCC cell lines, a customized lentiviral vector was purchased from Genechem. Transfection efficiency was assessed *via* fluorescence intensity. Stably transfected cells were selected using neomycin (MCE, Monmouth Junction, NJ, USA), and successful ALDH1A1 overexpression was verified by quantitative real-time PCR (qRT-PCR) and western blot.

MLS000532223 (C₁₅H₉NO₃), and EHT1864 (C₂₅H₂₉Cl₂F₃N₂O₄S) were obtained from MCE and utilized in accordance with the manufacturer's guidelines.

RNA extraction and qRT-PCR. Total RNA was extracted from cells using TransZol reagent (TransGen, Beijing, PR China) following the manufacturer's instructions. Cytoplasmic and nucleic RNA fractions were isolated and purified using EasyPure RNA kit (TransGen). RNA concentration and purity were assessed using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized on D100 thermal cycler (Novogene, Beijing, PR China) from purified RNA

with TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen). PCR was performed on QIAquant 96 2Plex system (QIAGEN, Hilden, Germany) with TransStart Top Green qPCR SuperMix (TransGen). Target gene mRNA expression levels were quantified using $2^{-\Delta C_t}$ method [$\Delta C_t = C_t (\text{Target}) - C_t (\text{Reference})$] and normalized to GAPDH. The experiments were performed in triplicate. The primer sequences used in this study are listed below: ALDH1A1, forward: 5'-CTTACCTGTCTACTCACCATT-3'; reverse: 5'-TCCTTATCTCCTTCTTCTACCTGG-3'; GAPDH, forward: 5'-ACAACCTTTGGTATCGTGGAAGG-3'; reverse: 5'-GCCATCACGCCACAGTTTC-3'. The reserved transcription condition was 25°C (10 s), followed by 42°C (15 min) and 85°C (5 s). The qRT-PCR cycling conditions is 94°C (30 s), followed by 45 cycles of 94°C (5 s) and 60°C (30 s).

Protein extraction and western blot. Total cellular protein was extracted using RIPA lysis buffer (Solarbio) supplemented with a protease inhibitor cocktail and a phosphatase inhibitor cocktail (both from Selleck, Sugar Land, TX, USA). Protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific).

Equal amounts of protein samples were separated by 10% and 6% SDS-PAGE (Solarbio) and subsequently transferred onto polyvinylidene fluoride (PVDF) membranes (MilliporeSigma, Burlington, MA, USA). The membranes were blocked by skim milk (Beyotime) and then incubated overnight at 4°C with primary antibodies against ALDH1A1, ARHGAP19, ASF1B, CYP4F12, DMPK, DSTYK, GPNMB, NOL4L, PCMTD2, ST7, VAMP4, β -actin, and α -tubulin (Proteintech, Wuhan, PR China); mTOR, p-mTOR, and SLC31A1 (Selleck) followed by incubation with HRP-conjugated goat anti-rabbit and anti-mouse secondary antibody (Proteintech) at room temperature for 1 h. Protein bands were visualized with ELC reagent (Thermo Fisher Scientific) using GD50202 imaging system (Monad, Suzhou, PR China) and quantified with ImageJ software (version 2.14.0).

Proteomic analysis. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was employed to profile the

expression and phosphorylation landscape of HCC cells. For protein quantification, samples were sequentially reduced with dithiothreitol (Rhwan, Shanghai, PR China), alkylated with iodoacetamide (Rhwan), and digested with trypsin (Promega, Madison, WI, USA) in ammonium bicarbonate buffer (Supelco, Bellefonte, PA, USA). The digestion was quenched by adjusting the pH to 2-3 with 20% trifluoroacetic acid (Aladdin, Shanghai, PR China), and peptides were desalted using C18 ZipTips (Millipore). For phosphorylation profiling, samples were subjected to enzymatic digestion using acetone (Sinopharm), ammonium bicarbonate solution, trypsin, and Lys-C (Thermo Fisher Scientific). The resulting peptide mixtures were enriched using immobilized metal affinity chromatography materials (J&K Scientific, Beijing, PR China) and eluted with ammonia solution (Rhawn). The eluates were subsequently desalted using C18 ZipTips prior to downstream analysis.

LC separation was performed on a nanoElute instrument (Bruker, Billerica, MA, USA). Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B consisted of 0.1% formic acid in acetonitrile. Peptide samples (200 ng) were loaded onto an IonOpticks Aurora analytical column (15 cm $\times$ 75 μ m, packed with 1.6 μ m C18 resin) at 50°C. The chromatographic separation was performed at a flow rate of 500 nl/min over a 20-min gradient.

MS analysis was performed using timsTOF Pro2 mass spectrometer (Bruker). Protein quantification was operated first in data-dependent acquisition-parallel accumulation serial fragmentation (DDA-PASEF) mode to generate a reference library. The total analysis time was 20 min, and data was acquired in positive ion mode. The precursor ion scan range was set from 100 to 1700 m/z, with an ion mobility range of 0.85-1.3 Vs/cm². Ion accumulation and release time was set to 100 ms, achieving nearly 100% ion utilization. The capillary voltage was maintained at 1500 V, with a drying gas flow rate of 3 l/min and a drying temperature of 180 °C. PASEF acquisition included 4 MS/MS scans per cycle. The charge state range was set from 0 to 5, with a dynamic exclusion duration

of 0.4 min. The target ion intensity was 10,000, and the minimum intensity threshold was 1,500. Collision energy increased linearly with ion mobility, ranging from 27 eV at 0.85 Vs/cm² to 45 eV at 1.3 Vs/cm². Quadrupole isolation widths were set at 2 Th for precursor ions with $m/z < 700$ and 3 Th for those with $m/z > 800$. Data-independent acquisition (DIA)-PASEF was performed subsequently for high-throughput and highly reproducible scanning. The precursor ion scan range was set to 400-1200 m/z , with an ion mobility range of 0.85-1.3 Vs/cm². The quadrupole isolation width was 12 Th, and 24 scans were acquired per cycle with an overlap of 0.1. A total of 48 acquisition windows were defined, with an average cycle time of 1.17 s.

Phosphorylation profiling was operated in DDA-PASEF mode. The total analysis time was 60 min, and data was acquired in positive ion mode. The precursor ion scan range was set from 100 to 1,700 m/z , with an ion mobility range of 0.7-1.4 Vs/cm². Ion accumulation and release time was set to 100 ms, achieving nearly 100% ion utilization. The capillary voltage was maintained at 1500 V, with a drying gas flow rate of 3 l/min and a drying temperature of 180°C. PASEF acquisition included 10 MS/MS scans per cycle. The charge state range was set from 0 to 5, with a dynamic exclusion duration of 0.4 min. The target ion intensity was 10,000, and the minimum intensity threshold was 2,500. Collision energy increased linearly with ion mobility, ranging from 20 eV at 0.6 Vs/cm² to 59 eV at 1.6 Vs/cm². Quadrupole isolation widths were set at 2 Th for precursor ions with $m/z < 700$ and 3 Th for those with $m/z > 800$.

For protein quantification, raw MS data analysis was performed using DIA-NN software (version 1.8.1) in the LibrayFree mode. The search was conducted within uniprotkb_proteome_UP000005640_human_82493_20240528.fasta database, which contains 82,493 protein sequences. Deep learning-based parameters and Match Between Runs option were enabled for DIA data reanalysis. Protein quantification was achieved using MaxLFQ algorithm. To prevent non-physical fold-change values resulting from stable yet near-zero expression, a small pseudocount was added to the expression values prior to differential analysis. For

phosphorylation profiling, raw MS data were processed and searched using FragPipe software (version 21.1). Decoy and contaminant identifications were excluded from the final output. The minimum localization probability threshold for post-translational modification site assignment was set to 0.75.

Cell proliferation analysis. Cell proliferation was evaluated using the Cell Counting Kit-8 (CCK-8; Biosharp, Hefei, PR China) and colony formation assays. For the CCK-8 assay, 3,000 cells were seeded into each well of a 96-well plate containing 100 μ l of complete medium. At 0, 24, 48, and 72 h after cell adhesion, 10 μ l of CCK-8 reagent was added per well, followed by incubation at 37°C in a 5% CO₂ incubator for 1 h. Absorbance at 450 nm was measured using Infinite M200 PRO microplate reader (TECAN, Männedorf, Switzerland). Each time point was assayed in triplicate, and the experiment was independently repeated three times.

For colony formation assays, 5,000 cells were plated into each well of a 6-well plate and cultured for 14 days. Colonies were then fixed with 4% paraformaldehyde (Solarbio) and stained with 0.1% crystal violet (Beyotime). Colony images were captured using an X-T2 digital camera (Fujifilm, Tokyo, Japan) and analyzed using Photoshop (version 23.5.2) and ImageJ software. All experiments were performed in triplicate to ensure reproducibility.

Cell migration and invasion analysis. Cell migration was assessed using wound healing assays. Cells were seeded in 6-well plates and cultured at 37°C with 5% CO₂ until reaching over 90% confluence. A linear scratch was introduced vertically across each well using a sterile 200 μ l-pipette tip. Detached cells were removed by washing three times with phosphate-buffered saline (PBS; Solarbio), followed by the addition of serum-free medium. Images were captured at 0, 24, and 48 h using an XD microscope (SOPTOP, Ningbo, PR China), and wound closure was quantified using ImageJ software. Each experiment was independently repeated three times.

Cell invasion was evaluated using Transwell assays. The upper chambers of Transwell inserts (Corning,

Corning, NY, USA) were precoated with Matrigel (1:8 dilution; Corning) and allowed to solidify. A total of 5,000 cells suspended in medium containing 5% FBS were seeded into the upper chambers, while the lower chambers were filled with 20% FBS medium as a chemoattractant. After 48 h of incubation at 37°C and 5% CO₂, non-invading cells were removed, and the invaded cells on the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Images were acquired using an XD microscope (SOPTOP), and quantitative analysis was performed with ImageJ software. All assays were conducted in triplicate for reproducibility.

Cell apoptosis analysis. Apoptosis analysis was performed by NovoCyte Advanteon flow cytometer (Agilent, Santa Clara, CA, USA) following staining with the Annexin V-APC/PI kit (KeyGEN BioTECH, Nanjing, PR China) in accordance with the manufacturer's protocol. Prior to analysis, unstained samples were used to adjust fluorescence compensation, eliminating spectral overlap and establishing quadrant gate settings. Annexin V-APC fluorescence (Ex=633 nm, Em=660 nm) was detected via the APC-H channel (FL4), while PI red fluorescence (Ex=488 nm, Em ≥630 nm) was detected through the PE-H channel (FL2). All experiments were independently repeated three times.

Animal models. For xenograft assays, six-week-old male nude mice were purchased from the Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Lanzhou, PR China). Mice were randomly divided into experimental and control groups, with 8 mice in each group. Huh-7 cells with ALDH1A1 knockdown (experimental group) and negative control Huh-7 cells (control group) were suspended in DMEM at a concentration of 1×10^6 cells/ml. A volume of 0.1 ml of the cell suspension was subcutaneously injected into the lower left flank of each mouse. Tumor volume was measured with a vernier caliper and calculated using the formula: $0.5 \times \text{long diameter} \times (\text{short diameter})^2$. Mice that

failed to develop palpable tumors within one week after inoculation were excluded from the study. In accordance with institutional animal ethics guidelines, mice were euthanized when tumor volume reached 2,000 mm³.

Bioinformatics. The pan-cancer expression profile of ALDH1A1 was obtained from Gene Expression Profiling Interactive Analysis (GEPIA) database (<http://www.gepia.cancer-pku.cn>), which integrates data from both TCGA and GTEx projects. Differential expression was assessed using ANOVA, with a $|\log_2 \text{fold change}|$ threshold of 1 and a significance cutoff of $p < 0.01$. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed using Gene Set Enrichment Analysis software (version 4.2.3). Eukaryotic Orthologous Groups (KOG) annotation analysis was performed using ResearchEasy online platform (<http://www.researcheasy.cn>). The protein domain analysis was performed using the InterProScan software (version 5.59-91.0). The protein-protein interaction (PPI) network of differentially expressed proteins was constructed using GeneMANIA (<http://www.genemania.org>) and STRING (<http://string-db.org>) databases.

Ethical statement. All procedures involving human cDNA samples and animals were reviewed and approved by the Ethics Committee of the First Hospital of Lanzhou University (LDYYLL-2025-118, Lanzhou, China, March 6, 2025). As the cDNA samples used in this study were archived and fully anonymized, informed consent was waived by the ethics committee.

Statistical analysis. Statistical analyses were performed using GraphPad Prism software (version 10.2.0). Two-tailed p -value < 0.05 was considered statistically significant. Unpaired and paired t -tests were used to compare differences between two independent or matched groups, respectively, provided that the data met the assumptions of normality and homogeneity of variance. In proteomic analysis, proteins consistently detected across multiple replicates within the same group were selected as differentially expressed proteins

when they showed a fold change ≥ 1.5 or ≤ 0.6667 and a p -value < 0.05 . Given the exploratory nature of proteomic sequencing, the limited sample size ($n=3$), and the subsequent independent experimental validation of selected targets, results that did not reach the expected false discovery rate (FDR) threshold were retained as candidate findings.

Results

Intertumoral and intratumoral expression heterogeneity of ALDH1A1. The pan-cancer analysis of ALDH1A1 expression was performed using GEPIA database. The results showed that ALDH1A1 was significantly upregulated in diffuse large B-cell lymphoma, esophageal carcinoma, kidney renal papillary cell carcinoma, and HCC compared with adjacent normal tissues, with the most prominent upregulation observed in HCC (Figure 2A). qRT-PCR was performed on 26 HCC tissue samples and 30 adjacent normal liver tissue samples. An unpaired t -test was used to compare expression levels between the two groups. Additionally, a paired t -test was conducted on the 26 matched pairs of HCC and adjacent normal tissues derived with same patient serial number. Both unpaired and paired t -tests consistently demonstrated that ALDH1A1 expression was significantly higher in HCC tissues compared to adjacent normal liver tissues (Figure 2B, C). In contrast, ALDH1A1 expression was markedly downregulated in adrenocortical carcinoma, bladder urothelial carcinoma, breast invasive carcinoma, cervical squamous cell carcinoma and endocervical adenocarcinoma, colon adenocarcinoma, glioblastoma multiforme, head and neck squamous cell carcinoma, lung adenocarcinoma, ovarian serous cystadenocarcinoma, rectum adenocarcinoma, stomach adenocarcinoma, testicular germ cell tumor, thyroid carcinoma, uterine corpus endometrial carcinoma, and uterine carcinosarcoma (Figure 2A). Furthermore, the expression of ALDH1A1 was evaluated in HCC cell lines by quantitative qRT-PCR. Compared with the normal human liver cell line THLE-2, ALDH1A1 expression was

significantly upregulated in Huh-7 cells, downregulated in HCCLM3 cells, and remained unchanged in SK-HEP-1 cells (Figure 2D). Collectively, these results suggest that ALDH1A1 expression displays marked intertumoral and intratumoral heterogeneity. Huh-7 and HCCLM3 cell lines were selected for subsequent experiments.

ALDH1A1 enhances proliferation, migration, and tumorigenicity but suppresses apoptosis and invasion in HCC cells. The silencing efficiency of three ALDH1A1-targeting siRNAs was assessed by qRT-PCR (Figure 3A). Homo-1288 showed the most pronounced suppression of ALDH1A1 expression in Huh-7 cells, as verified by western blot analysis, and was subsequently used in further functional assays (Figure 3B). The overexpression efficiency of the lentiviral vector was verified by immunofluorescence microscopy and western blot analysis, which demonstrated a marked increase of ALDH1A1 in HCCLM3 cells (Figure 3C).

CCK-8 and colony formation assays confirmed that suppression of ALDH1A1 expression significantly reduced the proliferative capacity of HCC cells, while ALDH1A1 overexpression markedly enhanced cell proliferation (Figure 3D, E). Wound healing assays revealed that siRNA-mediated knockdown of ALDH1A1 significantly impaired the migratory ability of Huh-7 cells. Conversely, ALDH1A1 overexpression enhanced the migratory behaviors of HCCLM3 cells (Figure 3G). Annexin V-APC/PI staining combined with flow cytometry revealed that ALDH1A1 knockdown markedly increased apoptosis in Huh-7 cells, with a predominant elevation in late apoptotic populations. In contrast, ALDH1A1 overexpression significantly suppressed apoptosis in HCCLM3 cells (Figure 3F). Xenograft tumor models were established by subcutaneous injection of ALDH1A1-knockdown Huh-7 cells and negative control Huh-7 cells into nude mice. Compared with the control group, knockdown of ALDH1A1 significantly inhibited tumor growth (Figure 3I, J). Throughout the experimental period, no statistically significant differences in body weight were observed between the two groups (Figure 3K). Taken together,

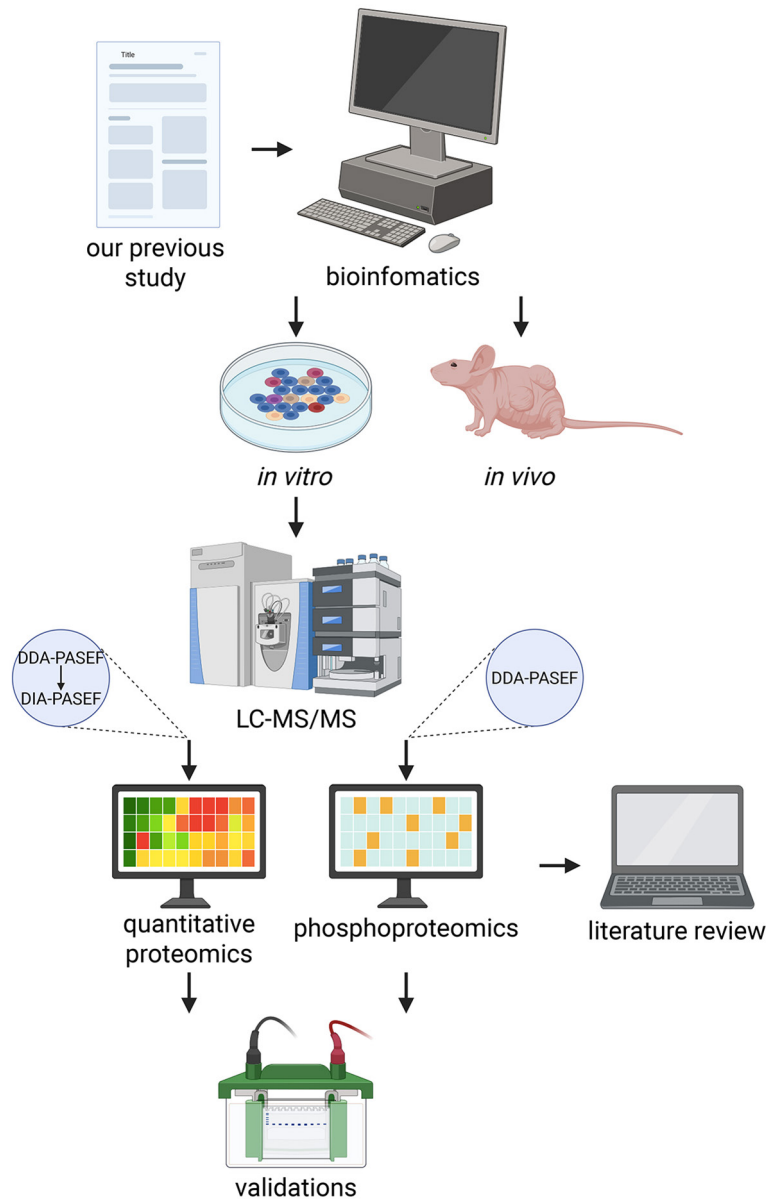


Figure 1. Work flowchart of the study.

these results indicate that ALDH1A1 primarily acts as an oncogenic driver in HCC.

However, ALDH1A1 also displayed partial tumor-suppressive characteristics in HCC. Transwell assays revealed that knockdown of ALDH1A1 enhanced the invasive potential of HCC cells, whereas ALDH1A1 overexpression modestly reduced their invasiveness

(Figure 3H). The bidirectional regulatory role of ALDH1A1 in HCC functional phenotypes implies the involvement of intricate molecular mechanisms, meriting further investigation.

ALDH1A1 significantly influences protein expression landscape in HCC cells. To further explore the molecular

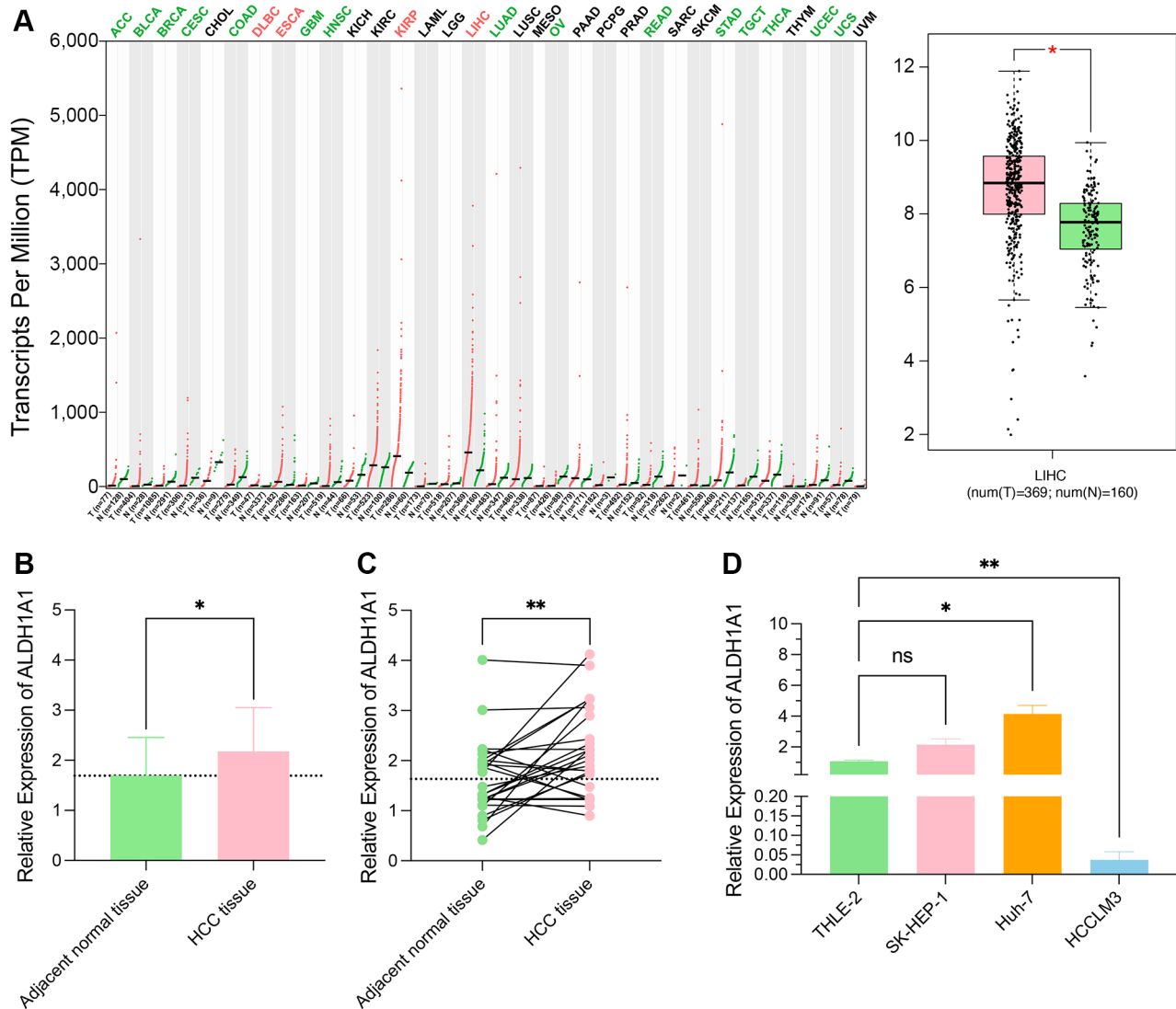


Figure 2. Expression patterns of ALDH1A1. (A) Pan-cancer expression analysis of ALDH1A1 in GEPIA; (B) Unpaired t-test of samples; (C) Paired test of samples; (D) Heterogeneous expression of ALDH1A1 in HCC cell lines. * $p < 0.05$; ** $p < 0.01$. GEPIA: Gene Expression Profiling Interactive Analysis; HCC: hepatocellular carcinoma.

mechanisms by which ALDH1A1 regulates HCC, LC-MS/MS-based quantitative proteomic profiling was conducted in HCCLM3 cells. Principal component analysis (PCA) and 3D-PCA analyses demonstrated distinct clustering between ALDH1A1-overexpressing and control groups, indicating significant alterations in protein expression patterns (Figure 4A). The volcano plot showed that a total of 57 proteins were differentially expressed

in response to ALDH1A1 overexpression, including 21 upregulated and 36 downregulated proteins (Figure 4B). GO enrichment analysis of ALDH1A1-associated differentially expressed proteins (DEPs) revealed significant enrichment across diverse biological processes (BP), cellular components (CC), and molecular functions (MF). The majority of enriched terms were dominated by downregulated proteins, with exclusive

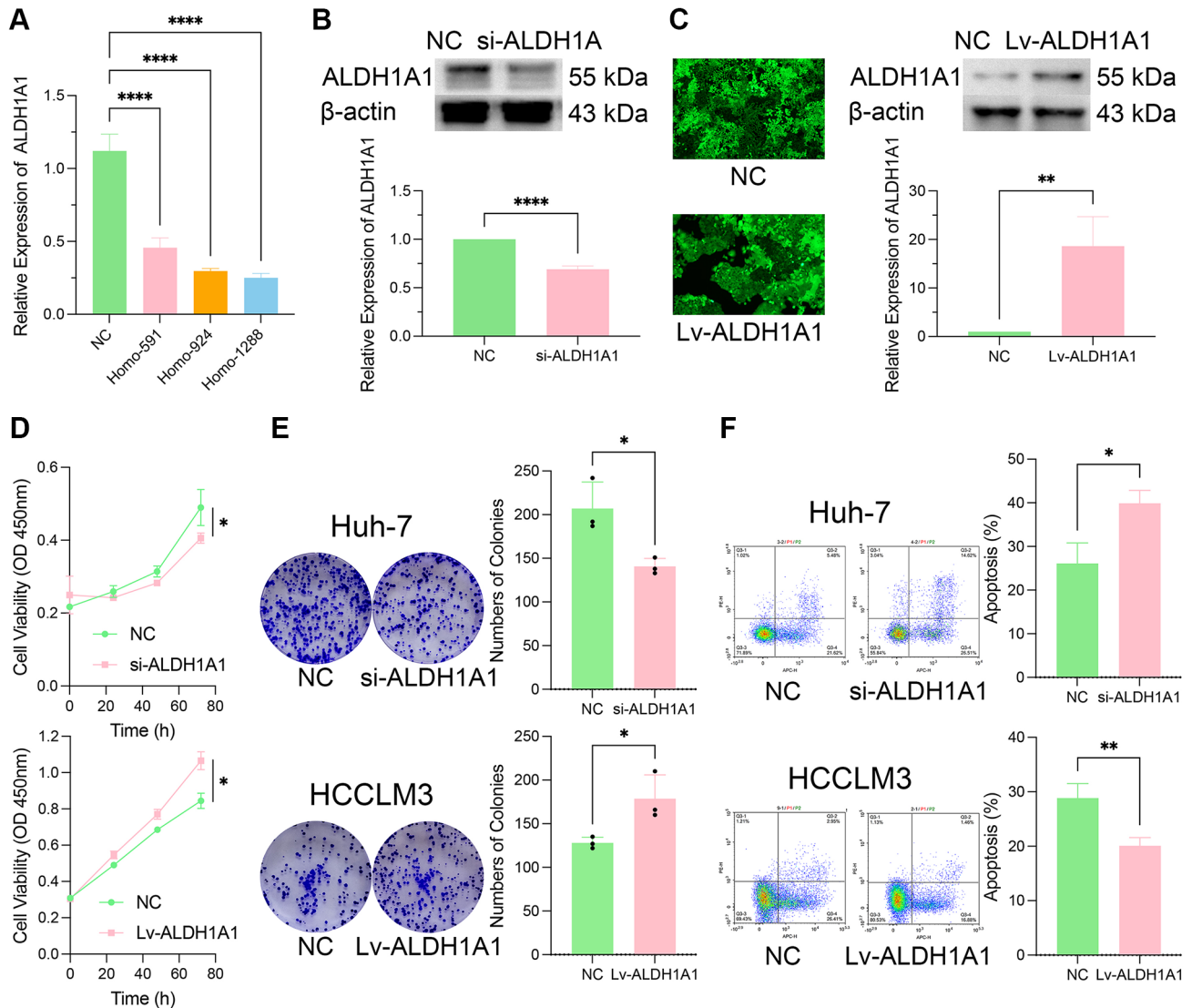


Figure 3. Continued

enrichment of downregulated proteins observed in pathways related to immune response, cell motility, cell growth, biomineralization, and viral infection, as well as in structural molecule, antioxidant, and molecular carrier activities (Figure 4C). KEGG analysis was also performed in ALDH1A1-associated DEPs. Among the top 20 enriched biological pathways, both upregulated and downregulated proteins were enriched in pathways such as cytochrome P450 (CYP)-mediated xenobiotic metabolism, calcium signaling, MAPK signaling, and

PI3K/AKT signaling (Figure 4D). These pathways are closely associated with malignant tumors development, and the bidirectional differential expression of the related proteins may underlie the complex mechanisms by which ALDH1A1 modulates HCC progression. KOG annotation of DEPs indicated that proteins upregulated upon ALDH1A1 overexpression were mainly enriched in secondary metabolic processes, implying that ALDH1A1 may exert a notable impact on the diversity and activity of intratumoral microorganism in HCC (Figure 4E). The

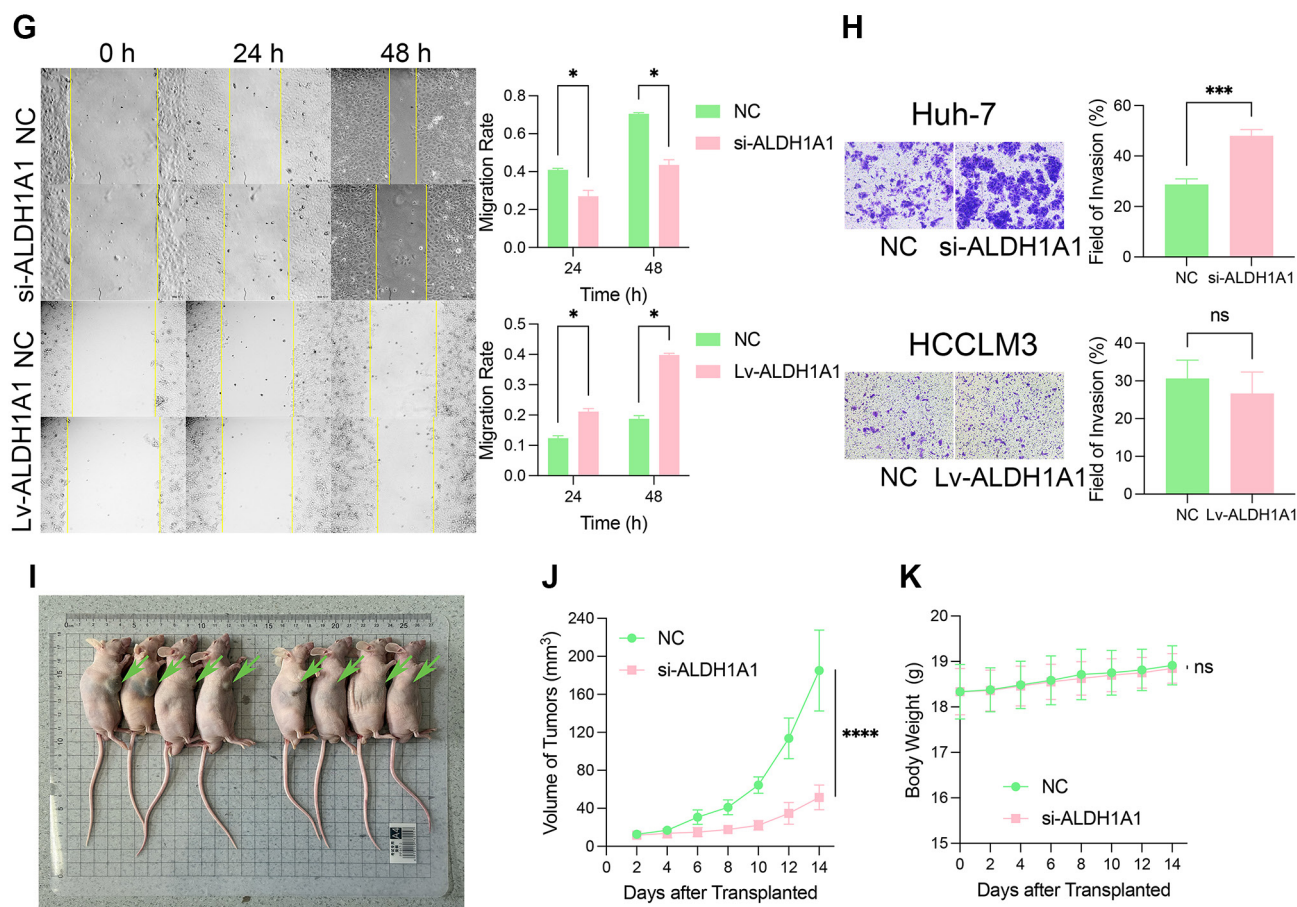


Figure 3. Functional assays of ALDH1A1 in HCC cells. (A) Selection of siRNAs; (B) Validation of siRNA knockdown efficiency; (C) Validation of lentiviral transduction efficiency; (D) CCK-8 assays; (E) Colony formation assays; (F) Apoptosis detection by flow cytometry; (G) Wound healing assays; (H) Transwell assays; (I) Representative images of tumor-bearing mice; (J) Tumor growth curves; (K) Body weight of tumor-bearing mice. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. HCC: Hepatocellular carcinoma; siRNA: small interfering RNA; CCK-8: Cell Count Kit-8.

downregulated proteins associated with ALDH1A1 overexpression were predominantly enriched in cellular motility and cytoskeletal organization, suggesting that these alterations may constitute the molecular mechanisms for the attenuated invasive potential of HCC cells induced by ALDH1A1 (Figure 4F). Domain enrichment analysis showed that CYP-related structural domains were significantly overrepresented among DEPs, indicating that CYP family members serve as critical downstream components of the ALDH1A1-mediated regulatory network in HCC (Figure 4H). Interestingly, the subcellular distribution of DEPs was not predominantly

in the cytoplasm, where ALDH1A1 is mainly localized, but rather in nucleus, extracellular space, and plasma membrane. Moreover, only downregulated proteins were found to be localized in mitochondria and peroxisomes (Figure 4G).

Using GeneMANIA database, a protein-protein interaction network was generated based on the identified DEPs, showing that ALDH1A1 shared the strongest functional connections with NPR3, FGD6, MICAL2, RBPJ, TBC1D2B, GPNMB, SLC31A1, ABCB1, EIF4G3, NT5E, SIAE, DPY30, MDFIC, MXRA7, PCMTD2, COL5A1, ARHGAP19, GSTT1, and PRR3 (Figure 5A). The

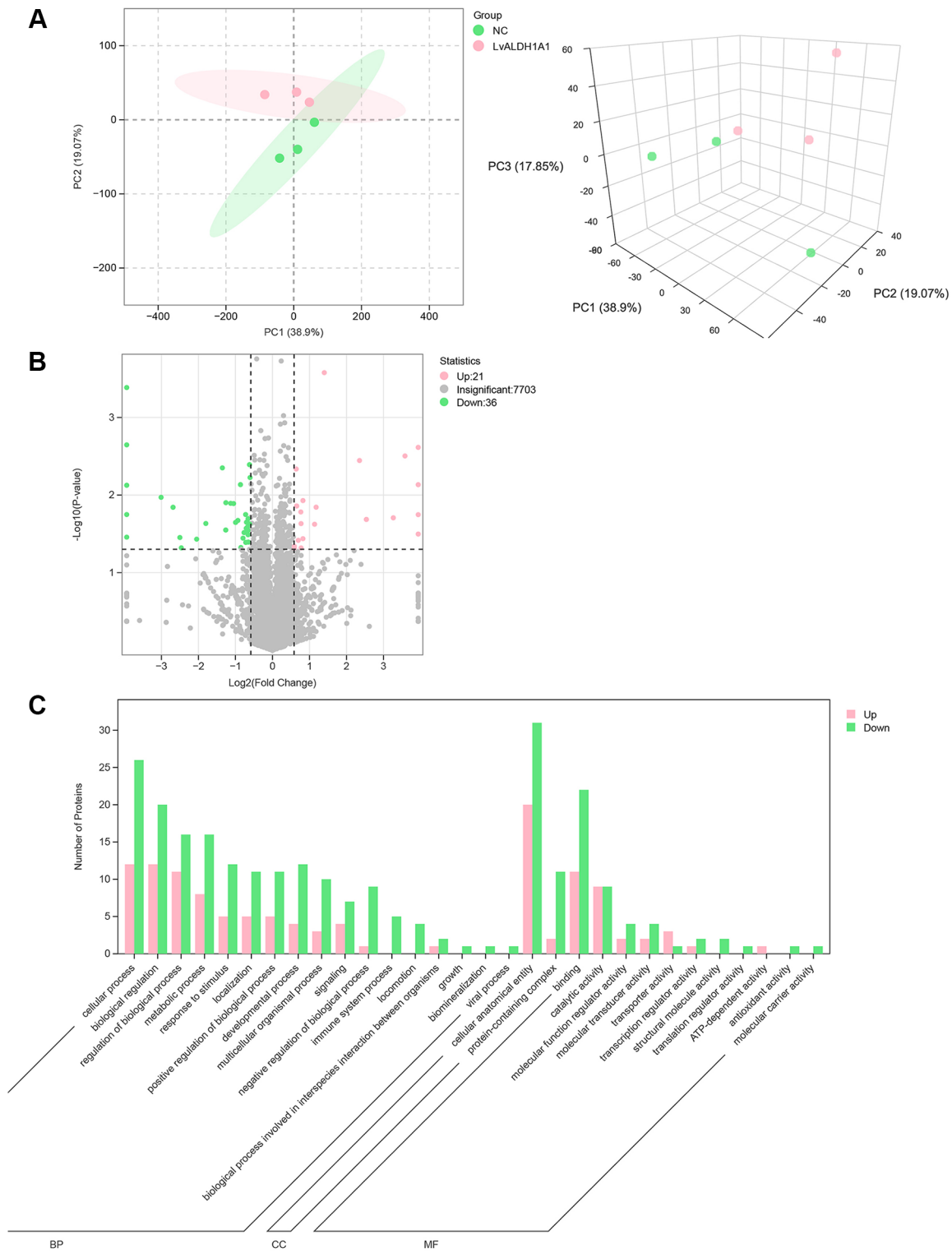


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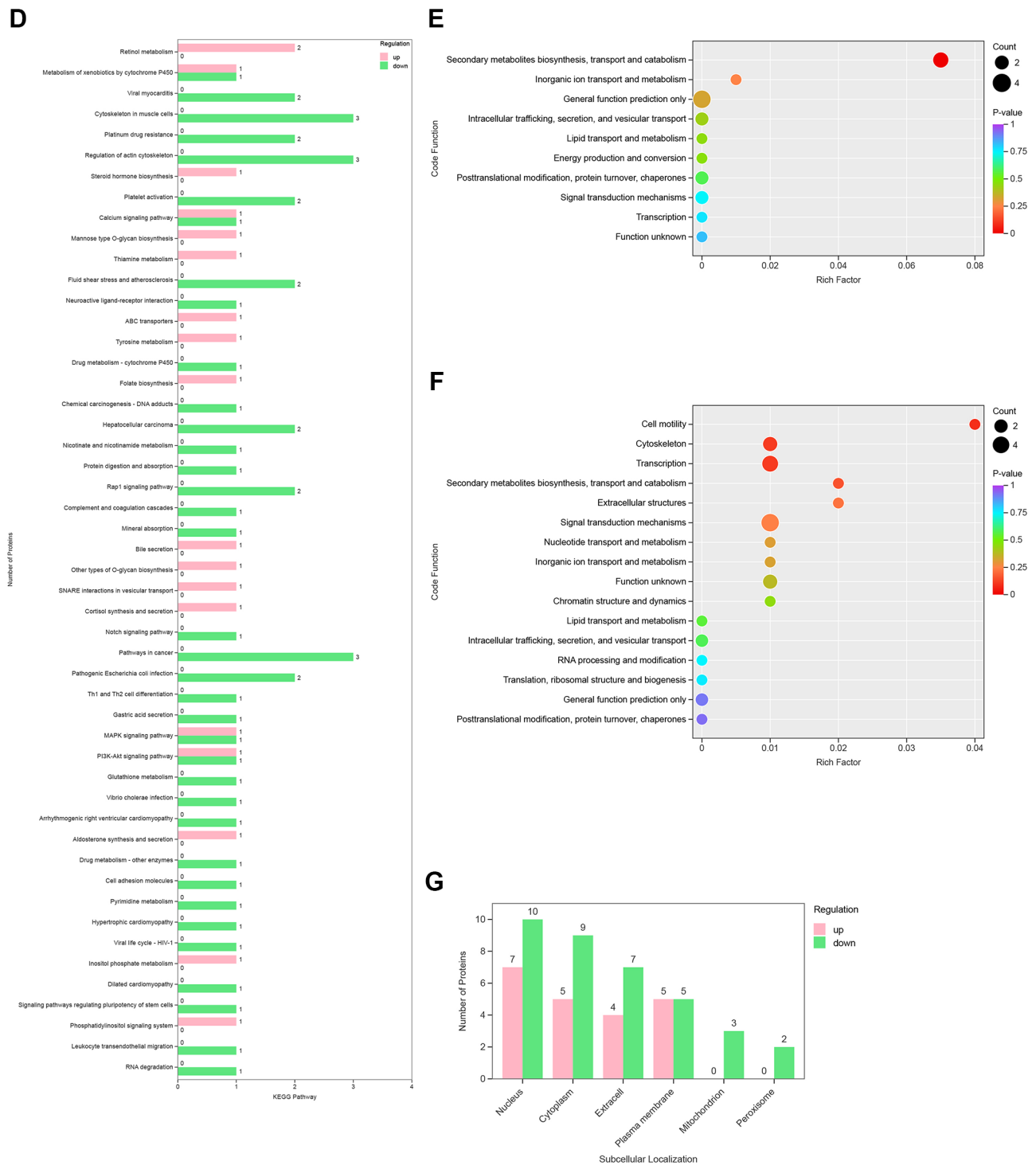


Figure 4. Continued

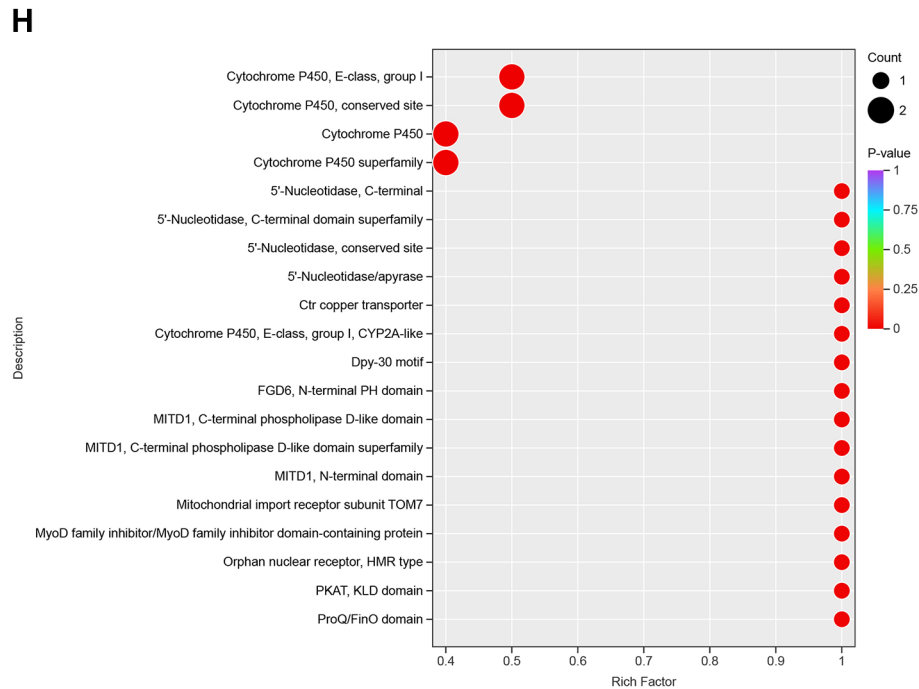


Figure 4. Quantitative proteomic analysis. (A) PCA and 3D-PCA of samples; (B) Volcano plot of proteins; (C) GO enrichment of DEPs; (D) KEGG enrichment of DEPs; (E) KOG annotation of upregulated proteins; (F) KOG annotation of downregulated proteins; (G) Subcellular localization of DEPs; (H) Domain enrichment of DEPs. PCA: Principal component analysis; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; DEP: differentially expressed protein.

DEPs were further analyzed using STRING database, which showed that only MCU1, CYP2S1, ABCB1, and STRA6 had experimentally determined interactions with ALDH1A1 (Figure 5B). To validate the proteomic results, western blot analysis was performed on the DEPs that exhibited the most significant variations and the largest fold change following ALDH1A1 upregulation. The results showed that siRNA-mediated knockdown of ALDH1A1 in Huh-7 cells markedly decreased the expression of DMPK, PCMTD2, VAMP4, ARHGAP19, NOL4L, and ST7, while increasing the expression of SLC31A1, DSTYK, CYP4F12, GPNMB, and ASF1B (Table I and Figure 5C). Consistently, lentiviral vector-mediated overexpression of ALDH1A1 in HCCLM3 cells, followed by western blot validation, confirmed the same expression patterns (Figure 5D).

ALDH1A1 markedly alters phosphorylation profile in HCC cells. To elucidate the impact of ALDH1A1 on the phosphorylation dynamics of proteins in HCC cells,

phosphoproteomic profiling of the Huh-7 cell line was conducted using LC-MS/MS. PCA and 3D-PCA demonstrated strong intragroup consistency among ALDH1A1-knockdown HCC cell samples, while showing a distinct segregation from the control group (Figure 6A). Volcano plot indicated that ALDH1A1 knockdown significantly altered the phosphorylation levels of 3,151 sites, with 1,966 showing positive regulation and 1,185 exhibiting negative regulation by ALDH1A1 (Figure 6B). The proteins whose phosphorylation levels were positively influenced by ALDH1A1 were subjected to GO and KEGG enrichment analyses. GO enrichment analysis revealed that ALDH1A1 knockdown decreased the phosphorylation of proteins involved in GTPase activation, cytoskeletal organization, glycolytic metabolism, and transcriptional regulation (Figure 6C). And the proteins whose phosphorylation levels were negatively affected by ALDH1A1 may influence genomic instability and proteomic diversity in HCC

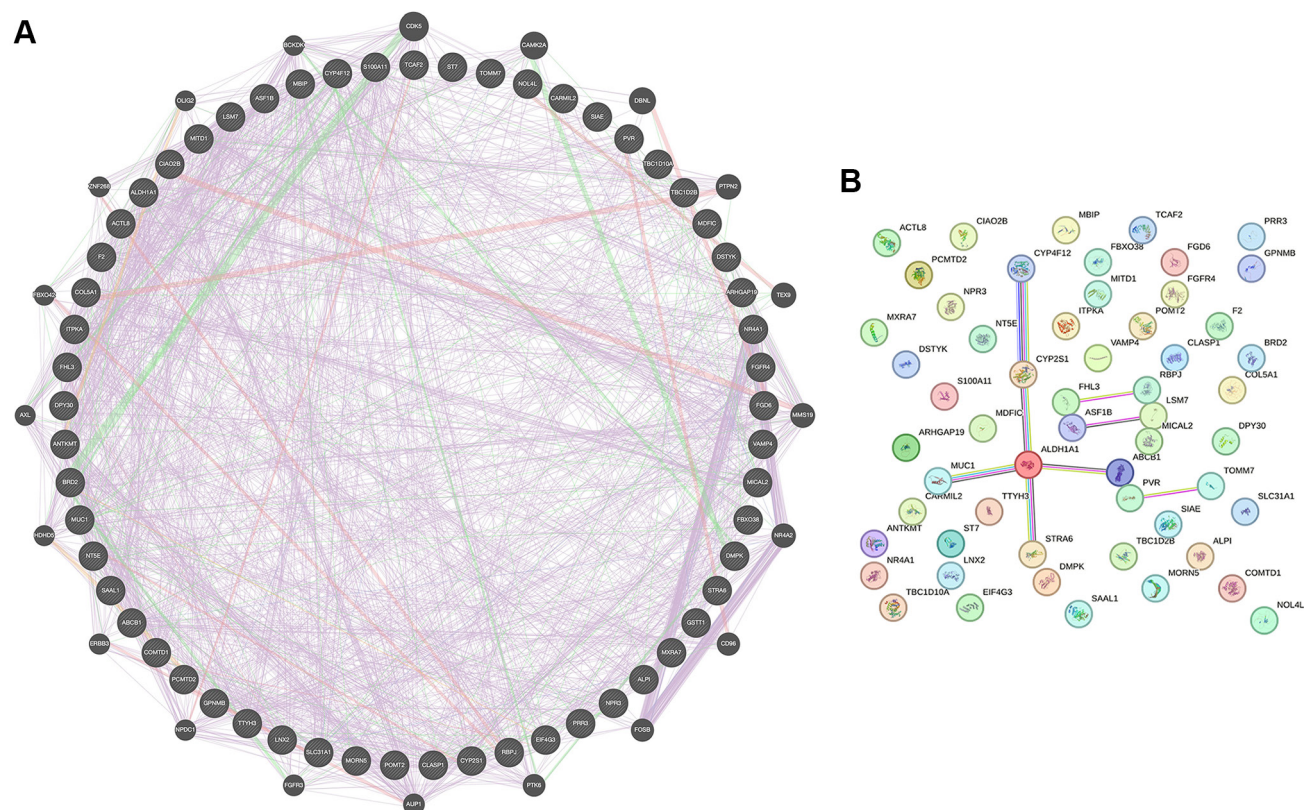


Figure 5. Continued

cells by modulating transcriptional regulation, post-transcriptional modification, and mRNA splicing. In addition, they participate in processes related to cell-cell adhesion, transmembrane transport, and signal transduction. Notably, the phosphorylation of spectrin exhibited a bidirectional response to ALDH1A1 modulation. The influence of ALDH1A1 on cell fate specification may also be linked to its previously reported role in maintaining stem cell properties. The altered phosphorylation of migration-related proteins may involve in the mechanism by which ALDH1A1 promotes HCC cell migration (Figure 6D). KEGG enrichment analysis revealed that ALDH1A1 may promote phosphorylation-dependent regulation of multiple tumor-related signaling pathways in HCC cells, including the ErbB, insulin, MAPK, mTOR, phospholipase D, GnRH, AMPK, and NOD-like receptor signaling pathways. Additionally, ALDH1A1

appeared to influence the synthesis, secretion, and action of peptide- and aldosterone-type hormones. Noteworthy, enhanced phosphorylation of proteins involved in EGFR tyrosine kinase inhibitor resistance and natural killer (NK) cells mediated cytotoxicity may constitute the pathway through which ALDH1A1 promotes the chemoresistance of tumors (Figure 6E). Phosphoproteins with elevated phosphorylation following ALDH1A1 knockdown were significantly enriched in the Notch, Rap1, and cAMP signaling pathways. KEGG analysis also showed that ALDH1A1 may modulate the differentiation potential of stem cells by suppressing associated proteins phosphorylation (Figure 6F).

Activation of small GTPases and promotion of mTOR phosphorylation underlie the oncogenic potential of ALDH1A1 in HCC. Phosphoproteomic profiling indicated

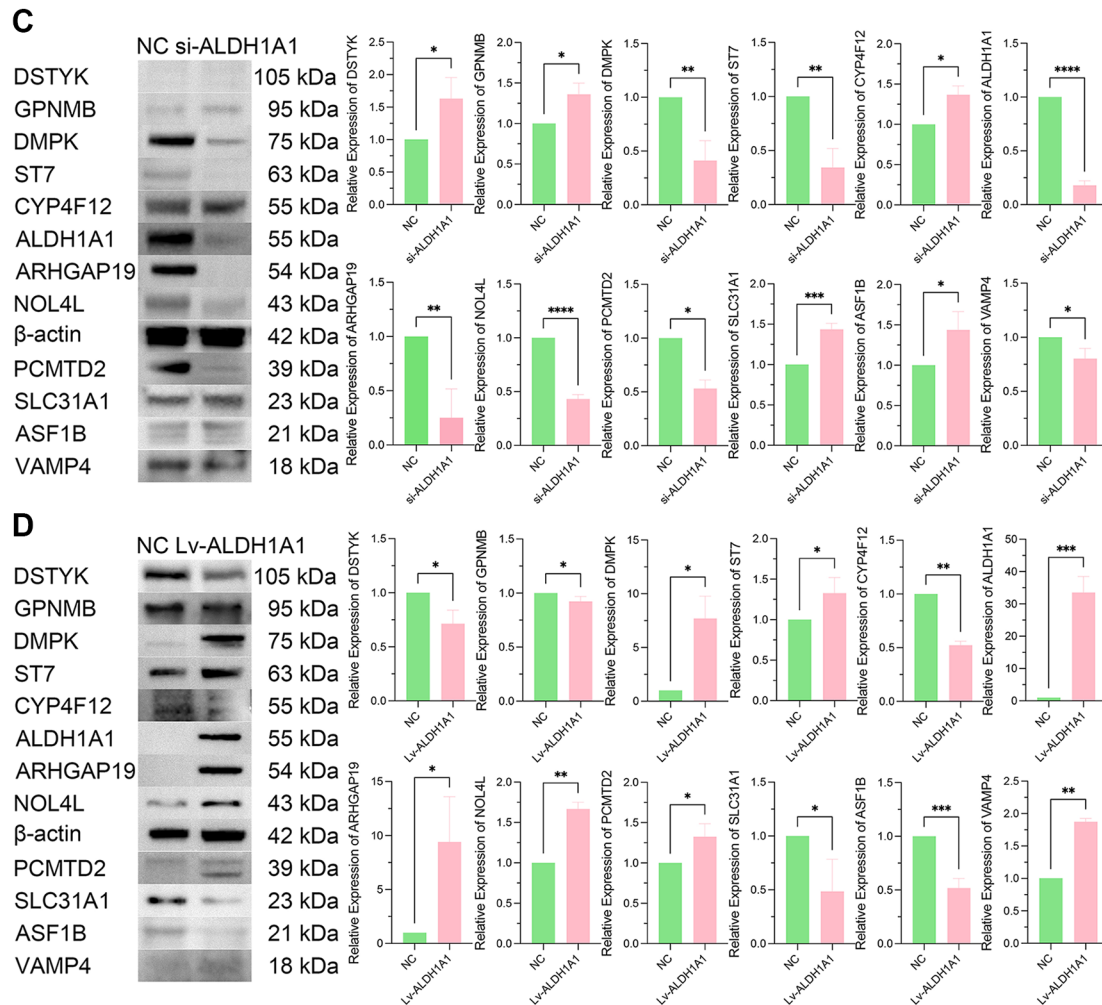


Figure 5. Interactive networks and experimental validation of DEPs. (A) Network of DEPs in GeneMANIA database; (B) Network of DEPs in STRING database; (C) Expression of DEPs in Huh-7 cells; (D) Expression of DEPs in HCCLM3 cells. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. DEP: Differentially expressed proteins.

Table I. ALDH1A1-associated significantly differentially expressed proteins.

Protein	Gene ID	Fold change	p-Value	Alteration
DMPK	1760	8.485304829	0.007346048	Upregulation
PCMTD2	55251	15.51333319	0.017876445	Upregulation
VAMP4	8674	87.92810938	0.031803547	Upregulation
ARHGAP19	84986	11.96007537	0.003131252	Upregulation
NOL4L	140688	9.614273132	0.019630368	Upregulation
ST7	7982	5.806318467	0.020603347	Upregulation
SLC31A1	1317	0.124247236	0.010686965	Downregulation
DSTYK	25778	0.040004646	0.002253037	Downregulation
CYP4F12	66002	0.065597203	0.007476813	Downregulation
GPNMB	10457	0.064949729	0.017811997	Downregulation
ASF1B	55723	0.08393602	0.034863126	Downregulation

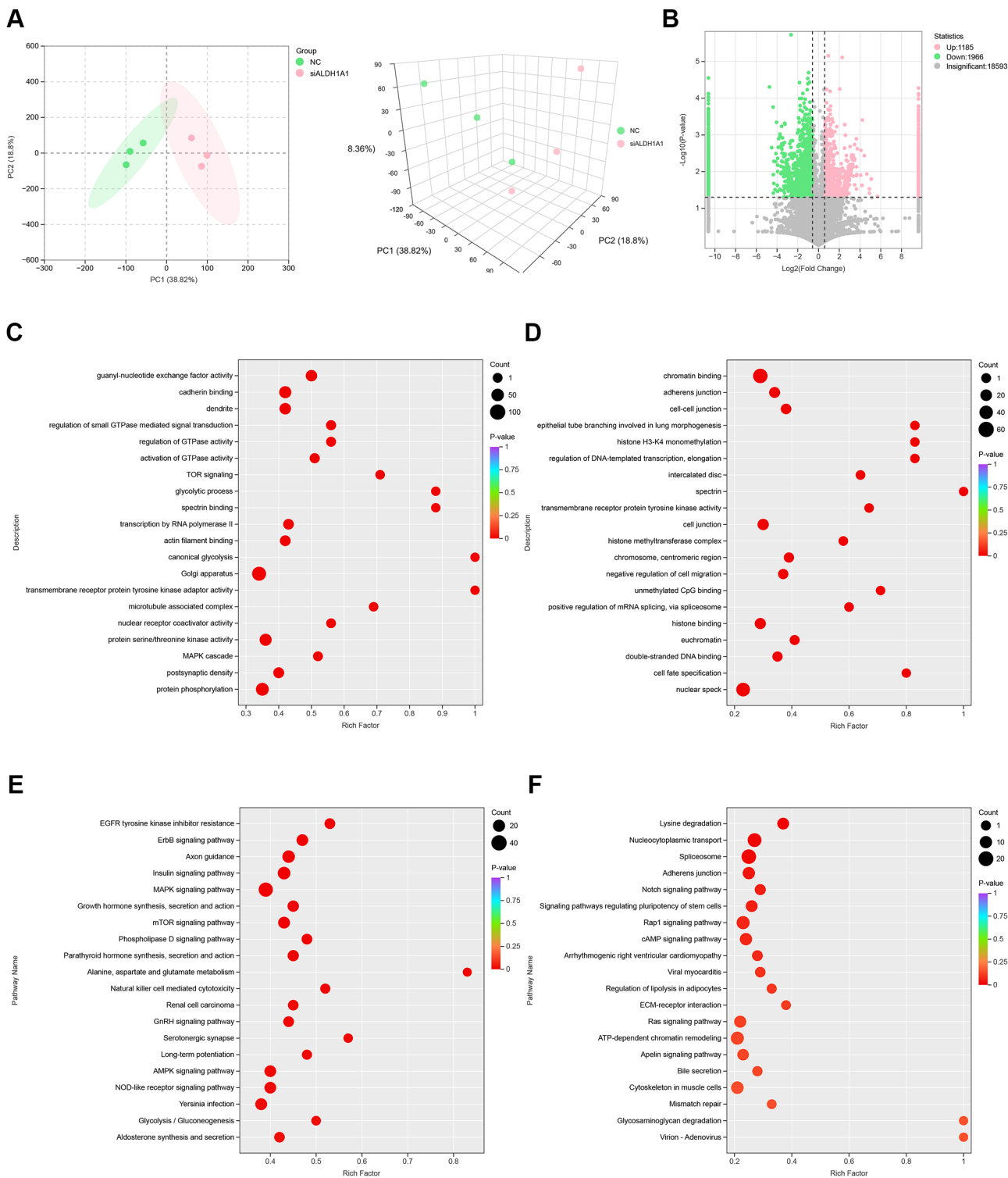


Figure 6. Continued

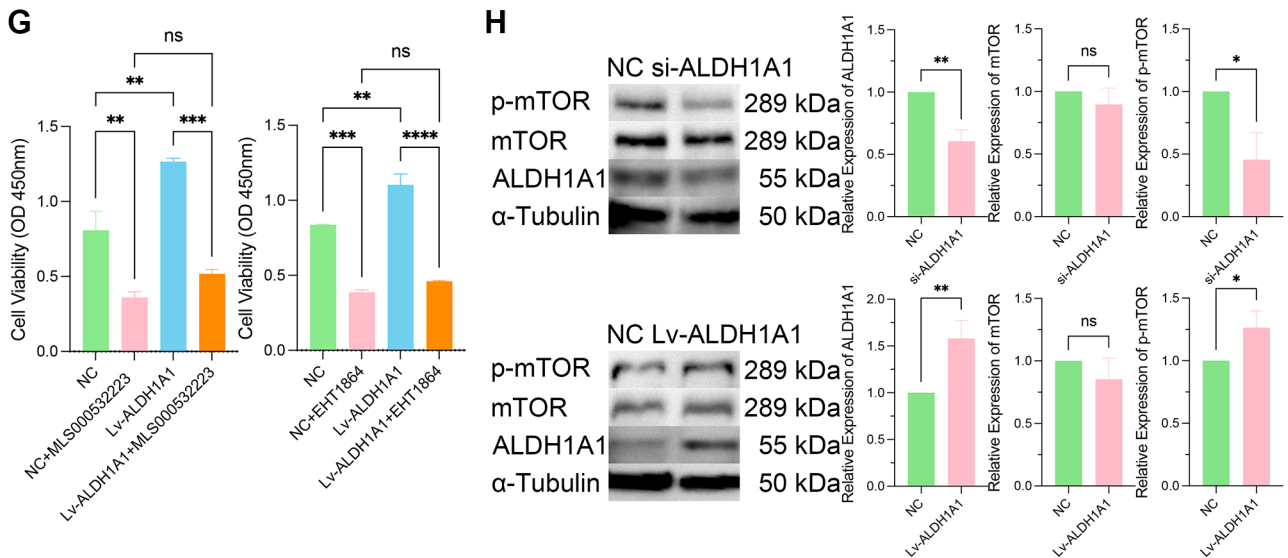


Figure 6. Phosphoproteomic analysis. (A) PCA and 3D-PCA of samples; (B) Volcano plot of phosphorylation site; (C) GO enrichment of downregulated phosphorylation site; (D) GO enrichment of upregulated phosphorylation site; (E) KEGG enrichment of downregulated phosphorylation site; (F) GO enrichment of upregulated phosphorylation site; (G) Reversal effect of small GTPase inhibitor on ALDH1A1-induced enhancement of cell activity; (H) Validation of ALDH1A1 promoting mTOR phosphorylation. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. PCA: Principal component analysis; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

that ALDH1A1 markedly enhances the activation of small GTPases in HCC cells. To validate these findings, HCCLM3 cells were treated with MLS000532223, a Rho family small GTPase-specific high-affinity inhibitor, and EHT1864, a Rac subfamily small GTPase-specific inhibitor. Both inhibitors were dissolved in DMSO and co-incubated with cells for 72 h, after which cell viability was measured using the CCK-8 assay. Based on the preliminary results, the final concentrations of MLS000532223 and EHT1864 were 10 μ M and 20 μ M, respectively, and the control group was treated with an equivalent volume of DMSO. Both inhibitors fully abrogated the ALDH1A1 overexpression-induced cell viability increase, indicating that ALDH1A1 promotes HCC progression through activation of Rho family small GTPases, especially Rac subfamily (Figure 6G).

Consistent with the phosphoproteomic data, western blot validation confirmed that ALDH1A1 positively regulates mTOR phosphorylation. ALDH1A1 knockdown in Huh-7 cells markedly reduced mTOR phosphorylation,

whereas ALDH1A1 overexpression in HCCLM3 cells significantly enhanced mTOR phosphorylation, suggesting that ALDH1A1 promotes HCC progression through activation of the mTOR signaling pathway (Figure 6H).

Discussion

In this study, we characterized the expression pattern of ALDH1A1 in HCC and systematically investigated its impact on HCC cell phenotypes. ALDH1A1 expression was significantly higher in HCC tissues than in adjacent normal liver tissues. Notably, ALDH1A1 exhibited marked heterogeneity among different HCC cell lines: compared with normal hepatocytes (THLE-2), its expression was markedly upregulated in well-differentiated Huh-7 cells, downregulated in poorly differentiated HCCLM3 cells, and remained relatively unchanged in SK-HEP-1 cells. Functional manipulation through siRNA-mediated knockdown and lentiviral overexpression demonstrated that ALDH1A1 promotes HCC cell proliferation, migration,

and tumorigenicity while suppressing apoptosis and, paradoxically, invasion. To further delineate the underlying molecular mechanisms, we performed quantitative proteomic and phosphoproteomic profiling *via* LC-MS/MS, revealing widespread ALDH1A1-dependent alterations in protein expression and phosphorylation.

Quantitative proteomic analysis revealed that ALDH1A1 overexpression markedly altered the protein expression landscape of HCC cells, resulting in 21 upregulated and 36 downregulated proteins. GO enrichment analysis showed that most terms encompassed both upregulated and downregulated DEPs, implying that ALDH1A1-mediated regulation in HCC is inherently complex and may operate in a context-dependent and dynamically balanced manner. Among these pathways, CYP-mediated xenobiotic metabolism is closely associated with drug action, drug-drug interaction, and therapeutic response (10). Calcium signaling pathway has been reported to regulate oncogenes and tumor suppressor genes, modulates immune signaling and checkpoint activity, and contributes to the remodeling of the tumor microenvironment (11). MAPK signaling contributes to the progression of nonalcoholic steatohepatitis (NASH) to HCC and regulates HCC development *via* autophagy and apoptosis (12, 13). PI3K/AKT signaling cascade not only modulates autophagy and apoptosis in HCC cells but also enhances glucose uptake and glycolysis, facilitates epithelial–mesenchymal transition, induces matrix metalloproteinase expression and angiogenesis, and ultimately contributes to increased chemoresistance and decreased radiosensitivity (14). KOG annotation linked ALDH1A1 to secondary metabolism, suggesting its potential influence on tumor-associated microbial activities. Beyond hepatitis action, recent studies have shown that intratumoral microbe may influence HCC progression by regulating of DNA methylation (15, 16). We performed western blot analysis to validate the proteomic results, focusing on the DEPs that showed the most significant variations and largest fold changes following ALDH1A1 upregulation. Our findings confirmed that ALDH1A1 positively regulated

the expression of DMPK, PCMTD2, VAMP4, ARHGAP19, NOL4L, and ST7, while negatively regulating SLC31A1, DSTYK, CYP4F12, GPNMB, and ASF1B. Most of the genes has been reported to be related to cancers. PCMTD2 interacts with CUL5, thereby blocking the T-cell receptor and interleukin-2 signaling pathways, which in turn suppresses the cytotoxic activity of CD8⁺ T cells against tumor cells (17). VAMP4, as a member of the SNARE family, participates in the release of cytotoxic granules and facilitates plasma membrane localization in cells (18, 19). VAMP4 has also been reported to participate in the formation of invadopodia in breast cancer cells, thereby promoting tumor metastasis (20). In addition, VAMP4 may synergy with ATP11B to mediate the vesicular transport of cisplatin from Golgi apparatus to plasma membrane, thereby contributing to cisplatin resistance in ovarian cancer (21). ARHGAP19 has been identified as a pro-metastatic factor in triple-negative breast cancer, where its expression is post-transcriptionally regulated by miR-192 (22). NOL4L has been shown to promote ovarian cancer cell proliferation and metastasis *via* activating PI3K/AKT signaling pathway, and to enhance the proliferation and invasion of neonatal neuroblastoma cells (23, 24). ST7 is a highly conserved tumor suppressor gene whose ubiquitination markedly inhibits colorectal cancer progression and also exerts tumor-suppressive effects in pancreatic, breast, and ovarian cancers (25-32). SLC31A1, a copper ion transporter, promotes copper uptake of HCC cells. Copper chelators induce cuproptosis to suppress HCC progression, and SLC31A1 enhances the sensitivity of HCC cells to copper chelation therapy (33). SLC31A1 can be activated by paclitaxel, leading to cuproptosis and thereby suppressing HCC (34). SLC31A1 also inhibits the progression from NASH to HCC by inducing cuproptosis (35). In animal models, high DSTYK expression predicts increased HCC risk (36). In a systematic analysis of the CYP family, CYP4F12 was recognized as a marker associated with favorable prognosis in HCC (37). GPNMB plays a crucial role in the tumor immunity of HCC. It is highly expressed in tumor-associated macrophages, particularly in immunosuppressive subsets. GPNMB-positive

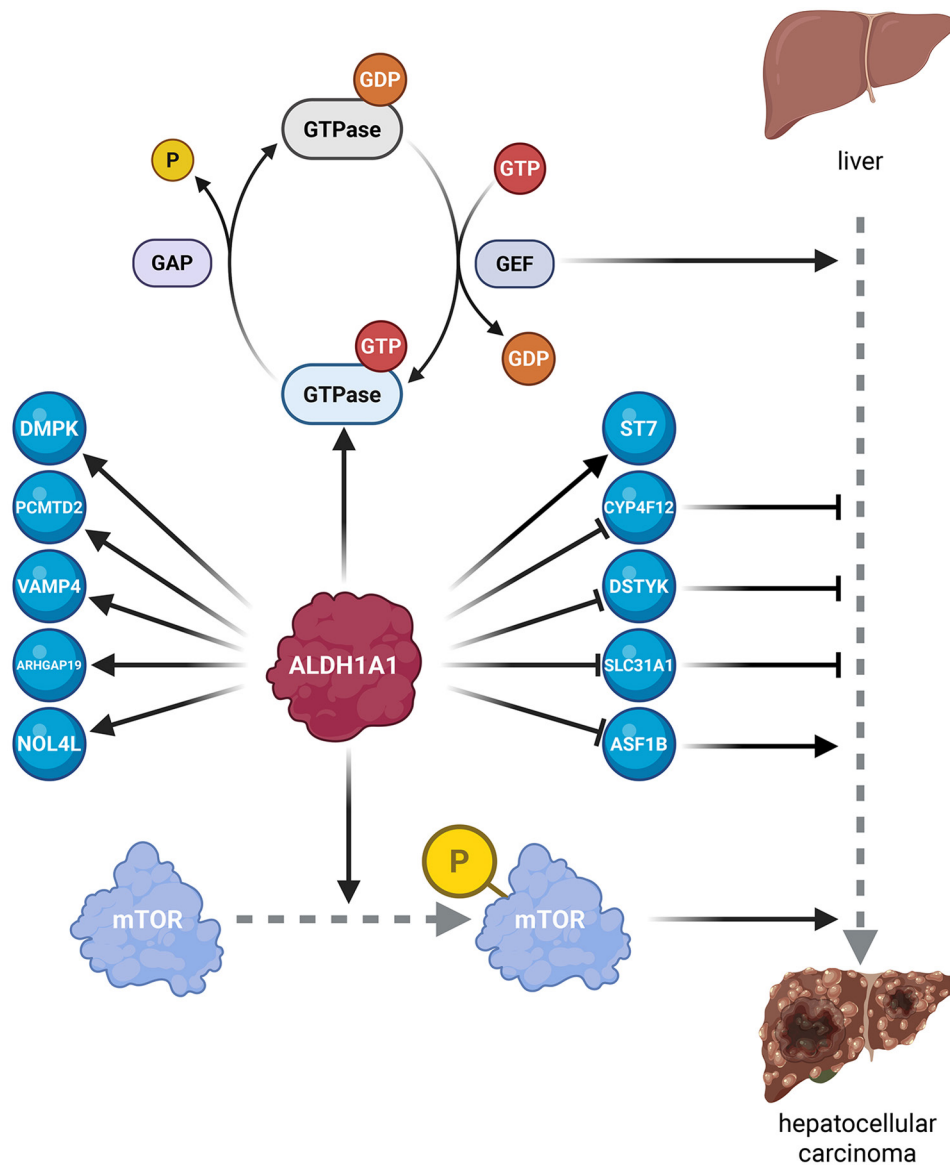


Figure 7. Schematic diagram of the ALDH1A1-centered regulatory network in HCC. HCC: Hepatocellular carcinoma.

macrophages suppress CD8⁺ T-cell activity and mediate immune evasion in HCC. Therefore, GPNMB represents a promising therapeutic target, as its blockade can enhance tumor sensitivity to immune checkpoint inhibitors (38-40). Previous studies have demonstrated that targeted inhibition of the FTO/m6A/GPNMB axis suppresses HCC growth and metastasis while enhancing antitumor immunity (41). Iodine-125 have been shown to markedly

inhibit GPNMB expression, providing a novel therapeutic strategy for radiotherapy in HCC (42). ASF1B promotes tumor cell proliferation, influences cell cycle regulation and immune infiltration, and has been recognized as a biomarker of poor prognosis in HCC (43-47).

Phosphorylation is one of the most extensively studied post translational modifications, which regulates a number of cellular functions (48). Phosphoproteomic

analysis revealed that ALDH1A1 markedly altered the phosphorylation profile of HCC cells, increasing the phosphorylation of 1,966 sites and decreasing that of 1,185 sites. GO and KEGG enrichments showed that ALDH1A1-mediated phosphorylation significantly influenced the regulation of small GTPases. Rho family small GTPases contribute to tumor initiation and progression by regulation proliferation, metabolism, senescence, metastasis, and stemness. They also influence tumor microenvironment and inflammation (49). Within Rho family of small GTPases, Rho, Rac, and Cdc42 subfamilies are well-studied. Rac subfamily plays important role in HCC progression (50). Treatment with MLS000532223, a high-affinity inhibitor of Rho family small GTPases, effectively reversed the ALDH1A1 overexpression-induced enhancement of HCC cell viability. Consistent results were obtained with EHT1864, a specific inhibitor targeting Rac subfamily small GTPases. These findings indicate that ALDH1A1 promotes HCC progression primarily through activation of Rho family small GTPases, especially Rac subfamily. ALDH1A1-mediated phosphorylation abnormalities may interfere with transcription, post-transcriptional modification, and RNA splicing, thereby affecting genomic instability and modulating HCC progression (51). ALDH1A1-mediated phosphorylation abnormalities also significantly impact on cytoskeleton, which may dictate the morphology, migration, motility, adhesion, cytokinesis, and phagocytosis of HCC cells (52). Among cytoskeletal proteins, ALDH1A1 exerts a significant influence on spectrin, with the potential to both enhance and suppress its phosphorylation. The ALDH1A1-spectrin interaction may regulate HCC cell behavior in context-dependent and dynamically adaptive manner (53). According to KEGG enrichment analysis, several canonical pathways affected by ALDH1A1, such as the ErbB, MAPK, phospholipase D, AMPK, Notch, and Rap1 signaling, have been reported to be associated with HCC progression (54-58). Activation of mTOR is widely recognized to promote tumor growth and metastasis (59, 60). Phosphorylation is one of the primary mechanisms for mTOR activation (61). We confirmed that ALDH1A1 significantly promotes

mTOR phosphorylation, which may contribute to HCC progression. This mechanism warrants further investigation and validation. Furthermore, ALDH1A1 may participate in NK cell-mediated cytotoxicity, suggesting its potential involvement in tumor-immune interactions and offering a novel perspective for the development of HCC immunotherapies.

Conclusion

ALDH1A1 is overexpressed in HCC and displays expression heterogeneity among different HCC cell lines. ALDH1A1 enhances tumor cell proliferation, migration, and tumorigenicity while inhibiting apoptosis and modestly suppressing invasion. Integrative proteomic and phosphoproteomic analyses revealed that ALDH1A1 reprograms both the protein expression and phosphorylation landscapes in HCC cells, particularly affecting small GTPase activation and mTOR signaling. In conclusion, ALDH1A1 is an oncogenic driver and regulatory hub in HCC (Figure 7).

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Hanxun Yue: Writing – original draft, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization; Zenan Hu: Writing – review & editing, Conceptualization, Funding acquisition, Resources, Supervision; Guozhi Wu: Writing – original draft, Data curation, Formal analysis, Investigation, Methodology, Validation; Na Jiang: Writing – original draft, Data curation, Investigation; Renpeng Li: Writing – original draft, Investigation, Methodology, Validation; Ya Zheng: Writing – review & editing, Resources, Supervision; YW: Writing – review & editing, Resources, Supervision; Yongning Zhou: Writing – review & editing, Conceptualization, Resources, Supervision.

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Artificial Intelligence (AI) Disclosure

No artificial intelligence (AI) tools, including large language models or machine learning software, were used in the preparation, analysis, or presentation of this manuscript.

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