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UBC9-mediated regulation of K144 ubiquitination of Lamin A and its implications for hepatocellular carcinoma

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Abstract

Background Hepatocellular carcinoma (HCC) is a lethal malignancy with limited treatment options. UBC9, the sole E2 conjugating enzyme in the SUMOylation pathway, is frequently overexpressed in HCC, yet its specific role in hepatocarcinogenesis remains unclear. This study aims to elucidate the regulatory mechanism of UBC9 in HCC, specifically focusing on its crosstalk with Lamin A (LMNA) via SUMOylation and ubiquitination.

Methods We analyzed UBC9 and LMNA expression in HCC tissues using TCGA and GEO datasets and evaluated their correlation with clinical outcomes. A doxycycline-inducible shRNA system was established in Hep3B and Huh-7 cell lines to assess the effects of UBC9 knockdown on cell proliferation, migration, and invasion. Proteomics and ubiquitin-proteomics analyses were performed to identify downstream targets. Mechanisms of LMNA regulation were investigated using immunoprecipitation, site-directed mutagenesis (K144R), and cycloheximide chase assays. Xenograft mouse models were used to validate findings in vivo.

Results UBC9 was significantly upregulated in HCC tissues, and high levels correlated with poor prognosis. Knockdown of UBC9 suppressed HCC cell proliferation, migration, and invasion in vitro and tumor growth in vivo. Proteomic screening identified LMNA as a key downstream target. UBC9 depletion led to reduced LMNA protein stability via increased ubiquitination without affecting its mRNA levels. We identified Lysine 144 (K144) as a novel

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ubiquitination site on LMNA; the K144R mutation or enhanced SUMOylation prevented LMNA degradation. Rescue experiments demonstrated that LMNA knockdown partially reversed the oncogenic phenotype induced by UBC9 overexpression.

Conclusions UBC9 promotes HCC progression by stabilizing LMNA through SUMOylation, which prevents ubiquitin-mediated degradation at the K144 site. The UBC9-LMNA axis represents a critical regulatory mechanism and a promising therapeutic target for hepatocellular carcinoma.

Keywords Hepatocellular carcinoma, UBC9, LMNA, SUMOylation, Ubiquitination

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent and lethal malignancies worldwide, accounting for a significant proportion of cancer-related deaths annually [1, 2]. Despite advancements in diagnostic and therapeutic strategies, the prognosis for HCC patients remains rather poor due to the late diagnosis at advanced stages and the high recurrence rates [3, 4]. Hence, deciphering the molecular mechanisms underlying HCC progression is crucial for developing more effective therapeutic interventions and improving patient outcomes.

Post-translational modifications (PTMs) are essential regulatory mechanisms that modulate protein stability, localization, protein-protein interactions, thereby impacting function and various cellular processes [5–7]. Among the diverse types of PTMs, ubiquitination and SUMOylation play pivotal roles in maintaining cellular homeostasis and regulating signal transduction pathways. Ubiquitination predominantly targets proteins for degradation via the 26S proteasome system, controlling protein turnover and quality [8, 9]. In contrast, SUMOylation, involving the covalent attachment of Small Ubiquitin-like Modifier (SUMO) proteins to substrates, can alter protein function, localization, and interactions without necessarily promoting degradation [10, 11]. The interplay between ubiquitination and SUMOylation is complex and can significantly impact cellular dynamics [12]. Furthermore, cumulative evidence suggests that the dysregulation between this crosstalk may lead to tumorigenesis. These modifications can function antagonistically or synergistically, and SUMOylation can sometimes facilitate subsequent ubiquitination, a process known as SUMO-targeted ubiquitination. Dysregulation of these PTMs has been implicated in various diseases, including cancer, where they can contribute to aberrant protein expression and activity, ultimately promoting tumorigenesis and progression [13].

UBC9 (UBE2I) is the sole E2 conjugating enzyme in the SUMOylation pathway, thereby attaching SUMO to target proteins [14]. Emerging evidence suggests that UBC9 is overexpressed in several cancers [15, 16], including HCC, and thus may play a role in tumor development and progression. UBC9 overexpression has been associated with higher tumor grade and dismal patient

prognoses, indicating its potential as a prognostic biomarker and a potential therapeutic target [17]. Notably, RNF146 SUMOylation has been shown to promote Axin degradation and activate Wnt/ β -catenin signaling, a mechanism linked to HCC progression and highlighting a potential molecular axis connecting SUMOylation to hepatocarcinogenesis [18]. However, while the roles of SUMOylation in HCC are increasingly recognized, direct evidence for UBC9-mediated ubiquitination-driven effects remains limited, underscoring the need for further mechanistic investigations.

Through proteomics and ubiquitin proteomics, we have herein identified LMNA as an important downstream substrate protein of UBC9. LMNA, encoding lamin A/C proteins, is a critical component of the nuclear lamina, providing structural support to the nucleus and regulating gene expression [19]. Mutations and aberrant expression of LMNA have been linked to a range of diseases, including laminopathies [20] and cancer [21]. In HCC, altered LMNA expression may influence cell proliferation, migration, and invasion [22]. However, the regulatory mechanisms regulating LMNA levels in HCC are not fully elucidated.

Herein we investigated the roles of UBC9 and LMNA in HCC, focusing on how SUMOylation and ubiquitination regulate LMNA expression and contribute to tumor progression. By examining the expression patterns of UBC9 and LMNA in HCC tissues and analyzing their associations with clinical outcomes, we aimed to determine their prognostic and therapeutics significance. Furthermore, elucidating the molecular mechanisms underlying UBC9 and LMNA interaction may provide valuable insights into the complex PTM networks in cancer biology.

Materials and methods

Cell lines and cell culture

Human hepatocellular carcinoma cell line Hep3B and Huh-7 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂.

Plasmids and constructs

UBC9 shRNA and scrambled control shRNA sequences were cloned into the pTRIPZ lentiviral vector containing a tetracycline (doxycycline, Dox)-inducible system (Thermo Scientific, Waltham, MA, USA). For overexpression studies, full-length human UBC9 cDNA was cloned into the pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA, USA). The LMNA wild-type (WT) and mutant constructs were generated by cloning LMNA cDNA into the pcDNA3.1(+) vector. Site-directed mutagenesis was performed to create LMNA K144R (lysine 144 to arginine) using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions.

Cell transfection and establishment of stable cell lines

Transient transfections were carried out using Lipofectamine 3000 (Invitrogen) following the manufacturer's protocol. For stable knockdown or overexpression, Hep3B cells were transduced with lentiviral particles carrying UBC9 shRNA or scrambled shRNA and selected with 2 µg/mL puromycin (Sigma-Aldrich, St. Louis, MO, USA) for two weeks. Doxycycline (1 µg/mL; Sigma-Aldrich) was added to induce UBC9 knockdown.

RNA extraction and quantitative real-Time PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized using the PrimeScript RT Reagent Kit (TaKaRa, Dalian, China). Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on an ABI StepOnePlus Real-Time PCR System. The primers used were as follows: LMNA forward: 5'-AGCAGCGTGAGTTTGAGAGC-3'; LMNA reverse: 5'-AGACTGCCTGGCATTGTCC-3'; GAPDH forward: 5'-CTGGGCTACACTGAGCACC-3'; GAPDH reverse: 5'-AAGTGGTCGTTGAGGGCAATG-3'. GAPDH was used as an internal control. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method.

Western blot analysis

Cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (Roche Applied Science, Mannheim, Germany). Protein concentrations were determined using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of protein were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% non-fat milk in TBST (Tris-Buffered Saline with Tween 20) and incubated overnight at 4 °C with primary antibodies: Anti-UBC9 (1:1000 dilution); Anti-LMNA (1:5000 dilution); Anti-FLAG (1:20000 dilution) or Anti-GAPDH (1:50000 dilution).

The primary antibodies were purchased from Proteintech (Wuhan, China). Finally, IRDye® 680RD Goat anti-Mouse IgG or IRDye® 800CW Goat Anti-Rabbit IgG were used to quantify the proteins using the Odyssey® CLx Infrared Imaging System (LI-COR Biosciences).

Ubiquitination immunoprecipitation assay

Cells were first lysed on ice using an appropriate volume of pre-cooled NET lysis buffer (NaCl-EDTA-Tris lysis buffer, pH=7.5) for 30 minutes, with intermittent pipetting every 10 minutes to ensure complete cell disruption. The cell lysate was then subjected to centrifugation at 16000 ×g for 10 minutes at 4 °C, and the supernatant was carefully collected into a new 1.5 mL microcentrifuge tube. Concurrently, for each sample tube, a mixture of 200 µL of Sephadex CL-4B and 50 µL protein G were added, centrifuged at 2000 ×g for 1 minute at 4 °C to remove the solution and washed thoroughly with Co-IP buffer. This washing step was repeated ten times, with the final resuspension in half the volume of NET lysis buffer. The prepared Sephadex and protein G mixture was combined with the protein lysate and incubated on a shaker at 4 °C for 40 minutes to pre-clear the lysate. The samples were then centrifuged at 16000 ×g for 2 minutes at 4 °C, and the supernatant was transferred to a new tube for protein concentration determination using the BCA method. Based on the number of cell tubes, an equivalent of 30 µL of protein G per sample was used, centrifuged at 2000 ×g for 1 minute at 4 °C, and the supernatant discarded. A 2% BSA solution in NET lysis buffer was then added to block the protein G beads on a shaker at 4 °C for 1.5 hours. After blocking, the beads were washed and added to a combined volume of NET lysis buffer. From the measured protein concentration, 800 µg of protein (diluted in 300 µL) was incubated with 2 µg of the specific antibody and 30 µL of protein G overnight on a rotary shaker at 4 °C. Post-incubation, the mixture was centrifuged at 2000 ×g for 2 minutes at 4 °C, and the supernatant was discarded. The beads were washed three times with 500 µL of pre-cooled Co-IP buffer, discarding the supernatant after each wash. Finally, the beads were boiled in 35 µL of SDS-PAGE buffer at 95 °C for 10 minutes. The eluate was centrifuged at 16000 ×g for 5 minutes at 4 °C, and the supernatant was transferred to a new microcentrifuge tube for SDS-PAGE analysis.

Protein stability assay

To assess protein stability, cells were treated with cycloheximide (CHX; 50 µg/mL; Sigma-Aldrich) to inhibit protein synthesis. Cells were harvested at 0, 6, 12, and 18 hours after CHX treatment, and LMNA protein levels were analyzed by Western blotting. Densitometric analysis was performed using ImageJ software to quantify protein levels.

Cell proliferation assay

Cell proliferation was evaluated using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). Hep3B cells (3×10^3 cells/well) were seeded in 96-well plates. At 0, 24, 48, and 72 hours, 10 μ L of CCK-8 solution was added to each well and incubated for 2 hours at 37 °C. Absorbance was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Wound healing migration assay

Cells were seeded in 6-well plates and grown to confluence. A straight scratch was made using a sterile pipette tip. The cells were washed to remove debris and cultured in serum-free medium. Images of the wound area were captured at 0 and 48 hours using a phase-contrast microscope (Olympus, Tokyo, Japan). Migration distance was measured using ImageJ software.

Transwell invasion assay

Cell invasion was assessed using Transwell chambers with Matrigel-coated membranes (8 μ m pore size; Corning, Corning, NY, USA). Cells (1×10^5) in serum-free medium were added to the upper chamber, and medium containing 10% FBS was placed in the lower chamber as a chemoattractant. Following 24 hours of incubation, non-invading cells were removed, and invading cells on the lower surface were fixed with methanol and stained with 0.1% crystal violet. Invaded cells were counted in five random fields under a microscope at a magnification of 100x.

iTRAQ quantitative proteomics assay

The iTRAQ quantitative proteomics assay was conducted by Gene Create Company (Wuhan, China). For the iTRAQ quantitative proteomics analysis, an 8-plex labeling method was employed. Each sample was individually labeled, followed by mixing in equal amounts to ensure uniformity. The combined samples were then evenly divided into 12 fractions for further analysis. The chromatographic instrument used was the Thermo DINOEX Ultimate 3000 BioRS, with a Durashell C18 analytical column of 5 μ m, 100 Å, and 4.6 $\times$ 250 mm dimensions. The LC-MS/MS spectrometer used was the Triple TOF 5600 plus AB SCIEX nano LC-MS/MS. It featured an analytical column from AB SCIEX with specifications of a 75 μ m inner diameter, packed with 3 μ m, 120 Å ChromXP C18 material, and a length of 12 cm. The capture column was the Eksigent Chromxp Trap Column, specified as 3 μ m C18-CL, 120 Å, 350 μ m $\times$ 0.5 mm. The spray needle was a NEW objective with a 20 μ m inner diameter and 10 μ m spray orifice. Protein identification was carried out using the ProteinPilotTM software (V4.5), compatible with the AB Sciex 5600 plus system. Proteins were considered credible for inclusion if their unused score was ≥ 1.3 ,

indicating a confidence level above 95%, meaning at least one unique peptide was present in the reliable protein. Conversely, proteins not meeting this criterion were excluded. For quantification of peptides and proteins, a confidence level (conf) ≥ 95 was required, signifying a credibility above 95%. Peptides meeting this threshold were used for quantitative analysis, whereas those that did not, were excluded.

Ubiquitination proteomics assay

The ubiquitination proteomics assay was conducted by the Gene Create Company (Wuhan, China). Samples stored at -80 °C were removed and mixed with four volumes of lysis buffer containing 8M urea, 1% protease inhibitors, and 50 μ M of the broad range deubiquitination inhibitor PR-619, followed by sonication to ensure thorough lysis. The lysate was centrifuged at 12,000 xg for 10 minutes at 4 °C to remove cell debris, and the supernatant was transferred to a new centrifuge tube for protein determination of protein concentration using a BCA assay kit. Equivalent amounts of protein from each sample were subjected to proteolytic digestion with trypsin. A standard protein was added at a 1:1 mass ratio, and DTT was added to a final concentration of 5 mM for reduction at 56 °C for 30 minutes. Subsequently, iodoacetamide was added to a final concentration of 11 mM and incubated in the dark at room temperature for 15 minutes. The samples were diluted with TEAB (Tetraethylammonium bicarbonate) until the urea concentration was below 2M. Trypsin was added for digestion at a 1:50 enzyme-to-protein mass ratio overnight, followed by an additional trypsin addition at a 1:100 mass ratio for 4 hours of further digestion. The peptides were dissolved in IP buffer containing 100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, and 0.5% NP-40 at pH 8.0, and the supernatant was transferred to ubiquitination resin pre-washed as per protocol. The mixture was incubated overnight on a rotary shaker at 4 °C. Post-incubation, the resin was washed four times with IP buffer and twice with deionized water. Finally, the peptides bound to the resin were eluted with 0.1% trifluoroacetic acid, three times in total, and the eluate was collected and vacuum dried. The dried peptides were desalted using C18 ZipTips according to the manufacturer's instructions, vacuum-dried again, and prepared for liquid chromatography-tandem mass spectrometry analysis. Data from the secondary mass spectrometry were processed using MaxQuant software (v1.5.2.8).

Statistical analysis

All experiments were repeated at least three times. Data are presented as mean $\pm$ standard deviation (SD). Statistical comparisons were made using Student's t-test or one-way ANOVA followed by Tukey's post hoc test using

GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA). A p -value < 0.05 was considered statistically significant.

Results

Expression and prognostic significance of UBC9 in hepatocellular carcinoma

In this study, we first aimed to investigate the expression and prognostic significance of UBC9 in HCC. Figure 1 illustrates the correlations between UBC9 expression and various clinical parameters, as well as its possible impact on patient outcomes. This analysis revealed that

UBC9 expression is significantly higher in tumor specimens as compared to normal liver tissue, suggesting a possible role for UBC9 in HCC pathogenesis (Fig. 1A). This elevated expression of UBC9 might be contributing to tumor growth or progression as recently suggested for lung adenocarcinoma, ovarian carcinoma and malignant melanoma [17]. Notably, HCC patients under 65 exhibited significantly increased UBC9 expression compared to those over 65 (Fig. 1B), indicating that age may affect UBC9 expression in HCC patients. Remarkably, UBC9 expression significantly increased with higher grade, thereby possibly indicating its association with a more

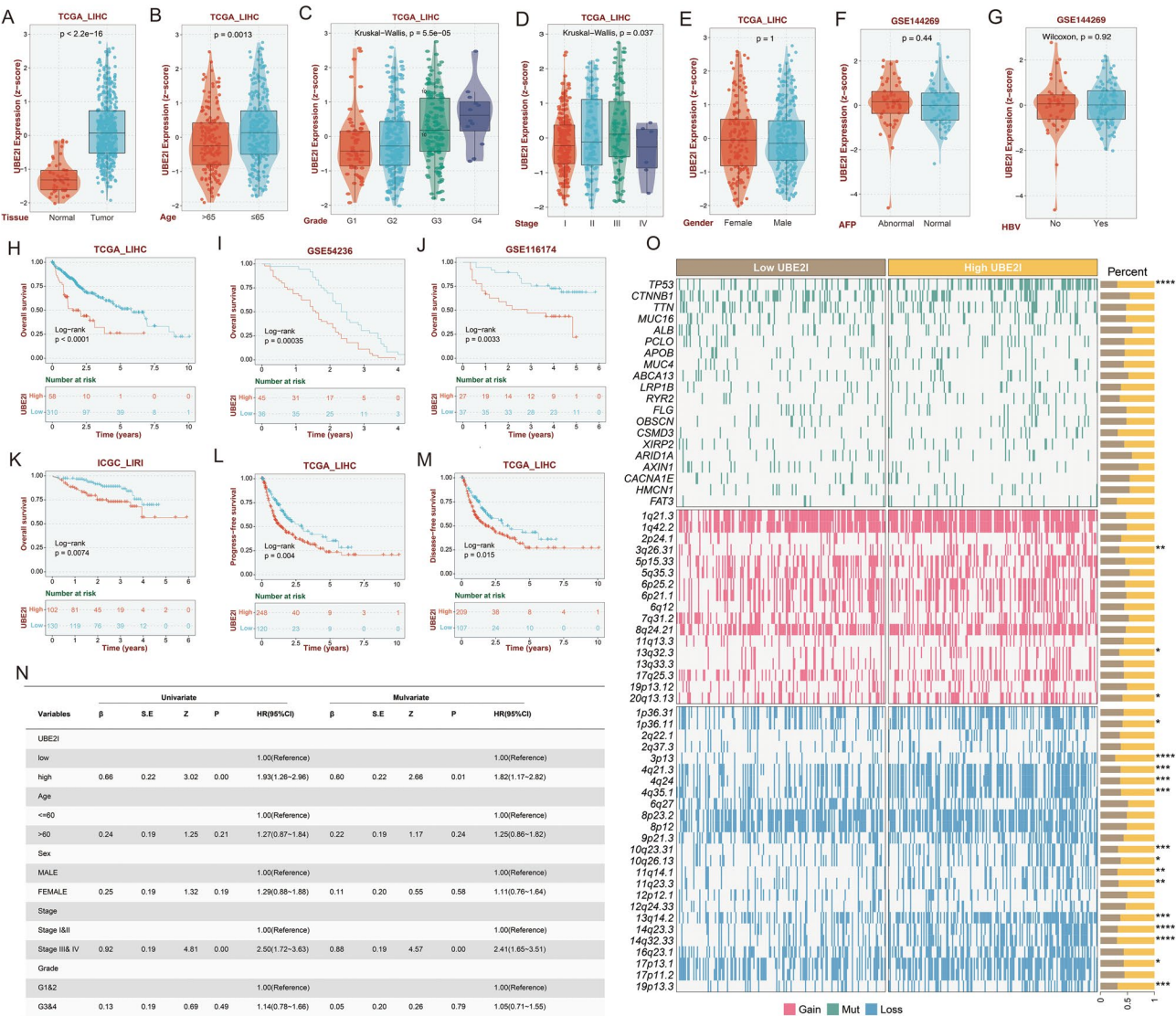


Fig. 1 Analysis of UBC9 expression in hepatocellular carcinoma. (A) UBC9 expression is significantly higher in human tumor specimens than in normal liver tissues. (B) patients aged 65 and older display significantly lower UBC9 expression that those younger than 65 years. (C) significant variation of UBC9 expression across different tumor grades, increasing with higher grades. (D-G) analysis of UBC9 expression in relation to clinical stage (D), gender (E), AFP levels (F), and HBV infection status (G). (H-K) high UBC9 expression correlates with reduced overall survival across multiple datasets (TCGA_LIHC, GSE54236, GSE116174, ICGC_LIRI). (L, M) elevated UBC9 adversely affects progression-free and disease-free survival. (N) univariate and multivariate Cox regression analyses identify UBC9 expression as an independent prognostic factor for overall survival. (O) genetic analysis using TCGA data shows higher TP53 mutation rates in patients with elevated UBC9 expression. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$

aggressive tumor phenotype (Fig. 1C). UBC9 expression levels progressively increasing from G1 to G3, indicating an association with tumor differentiation status. Interestingly, stage-stratified analysis showed significant variation across tumor stages, with Stage IV tumors displaying a paradoxical decrease in UBC9 expression compared to Stage III, possibly reflecting distinct molecular subtypes or tumor microenvironment changes in advanced disease (Fig. 1D). No significant differences in UBC9 expression were observed between male and female patients (Fig. 1E). Analysis of serum alpha-fetoprotein (AFP) levels revealed no significant difference in UBC9 expression between AFP-abnormal and AFP-normal patients (Fig. 1F). Similarly, external validation using GSE144269 showed no significant correlation between UBC9 expression and HBV infection status (Fig. 1G). Kaplan-Meier survival analysis was conducted to evaluate the prognostic value of UBC9 in HCC (Fig. 1H–K). High UBC9 expression levels were correlated with HCC recurrence and metastasis (Fig. 1L and M). To determine whether UBC9 expression represents an independent prognostic factor in HCC, we performed multivariate Cox regression analysis adjusting for key clinicopathological variables including age, sex, tumor stage, and tumor grade using the TCGA dataset (Fig. 1N). In univariate analysis, high UBC9 expression was significantly associated with poor overall survival. Importantly, multivariate Cox regression analysis revealed that UBC9 expression remained an independent prognostic factor after adjustment for confounding clinical variables. Tumor stage (Stage III&IV vs I&II) also emerged as an independent predictor of overall survival, whereas age, sex, and tumor grade showed no significant independent prognostic value.

We also explored genetic mutations in various genes that may be linked to UBC9 expression using TCGA data. Patients with elevated UBC9 expression exhibited higher rates of TP53 mutations, suggesting a possible interaction between UBC9 activity and genomic instability in HCC (Fig. 1O). Our findings indicate that elevated UBC9 expression is associated with more aggressive clinicopathological features in HCC and co-occurs with higher TP53 mutation rates, suggesting a potential link between UBC9 activity and tumorigenic pathways. Indeed, previous studies have shown that oncogenesis of colorectal cancer driven by the mutant Ras/Raf pathway requires the activity of UBC9 [23].

Functional consequences of UBC9 modulation on hepatocellular carcinoma cells

To further investigate the impact of UBC9 on HCC cell functionality, we employed a tetracycline (Dox)-inducible gene expression system to knockdown UBC9 gene expression via shRNA. This system was crucial due to the embryonic lethality resulting from UBC9 knockout [24],

and hence allowed us to modulate UBC9 expression specifically in Hep3B, a childhood HCC cell line (Fig. 2A).

We thus first validated the system's effectiveness in downregulating UBC9 expression in an inducible manner. Western blot analysis showed a 90% reduction in UBC9 protein levels in cells treated with Dox for 72 hours compared to untreated controls, confirming the system's efficiency (Fig. 2B). We then assessed the impact of UBC9 knockdown on cell proliferation using the colorimetric CCK8 assay. The results demonstrated a significant decrease in the optical density (OD) values over 72 hours in UBC9-depleted cells compared to controls, indicating that reduced UBC9 expression significantly impairs Hep3B cell growth and proliferation (Fig. 2C). Beyond proliferation, we further investigated UBC9's role in cell migration using a scratch assay. The migration ability of Hep3B cells upon substantial UBC9 knockdown was significantly diminished 48 hours after Dox induction, whereas scrambled shRNA controls showed no notable change. These results underscore the involvement of UBC9 in promoting cell migration (Fig. 2D). Finally, we evaluated cell invasion ability using a Matrigel invasion assay. Consistent with the migration results, UBC9 knockdown led to a pronounced reduction in the invasive capacity, as evidenced by fewer cells invading through the Matrigel matrix compared to DOX-negative controls (Fig. 2E).

To validate the generalizability of these findings across different HCC cell models, we extended our investigations to the Huh-7 cell line. Consistent with the observations in Hep3B cells, Western blot analysis confirmed efficient UBC9 knockdown in Huh-7 cells upon Dox induction (Fig. 2F). The CCK-8 proliferation assay demonstrated that UBC9 depletion significantly reduced cell proliferation in Huh-7 cells (Fig. 2G). Similarly, the scratch wound healing assay revealed that UBC9 knockdown markedly impaired the migratory capacity of Huh-7 cells (Fig. 2H). Furthermore, the Matrigel invasion assay showed a pronounced reduction in the invasive ability of Huh-7 cells upon UBC9 depletion (Fig. 2I).

To further validate the oncogenic role of UBC9 in vivo, we performed xenograft tumor experiments using Hep3B cells with UBC9 knockdown. Nude mice were subcutaneously injected with either scrambled control or UBC9 shRNA-expressing Hep3B cells. Tumor growth was monitored over 13 days, and both tumor volume and weight were measured at the endpoint. Consistent with our in vitro findings, UBC9 knockdown significantly suppressed tumor growth, as evidenced by reduced tumor volume and decreased tumor weight (Fig. 2J). These in vivo results provide compelling evidence that UBC9 promotes HCC tumor progression in a physiologically relevant context.

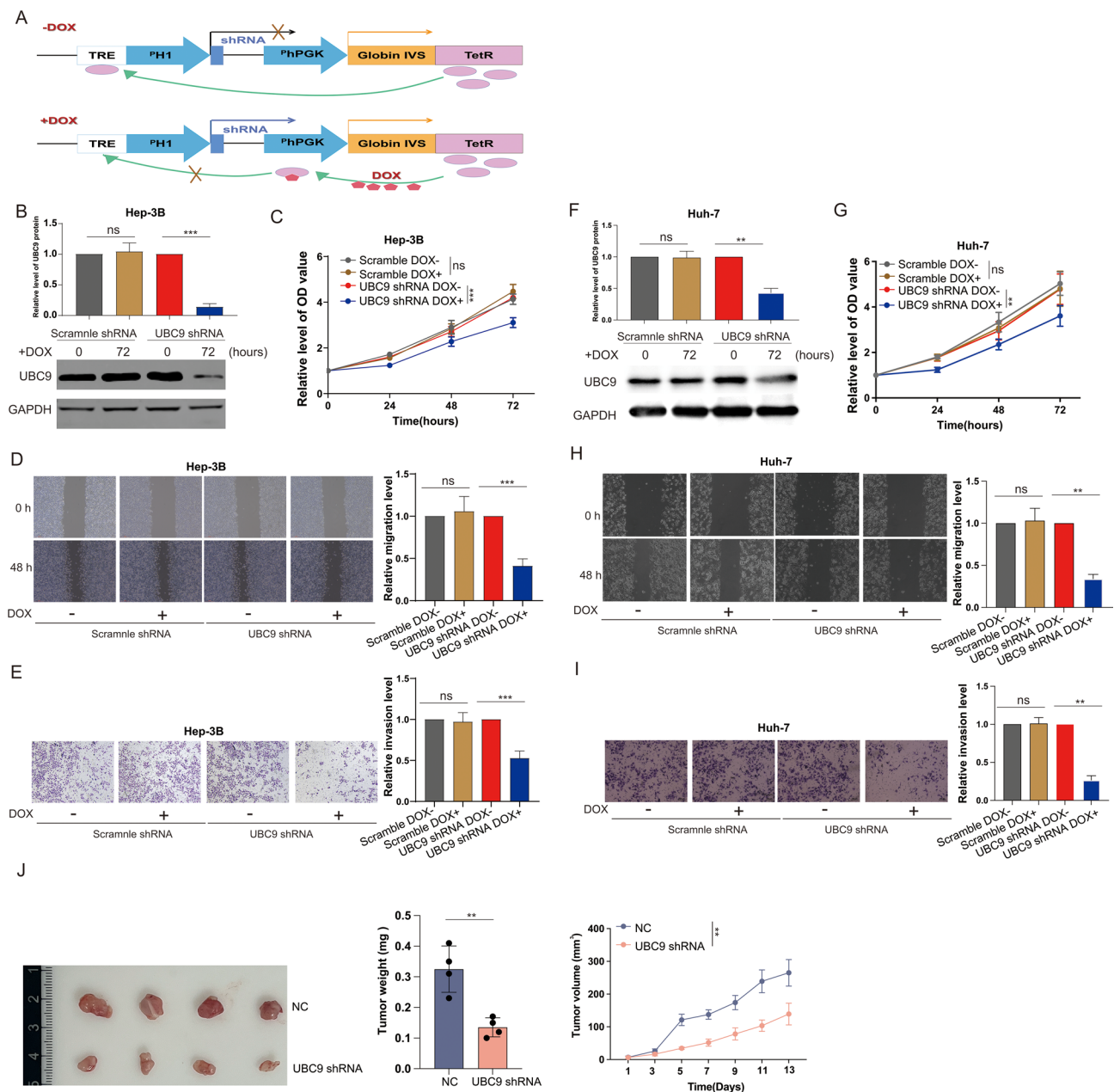


Fig. 2 Impact of UBC9 knockdown on HCC cells using a doxycycline-inducible system. **(A)** schematic of the doxycycline (Dox)-inducible gene repression system used to downregulate UBC9 expression. **(B)** Western blot analysis shows reduced UBC9 protein levels after 72-hour of Dox treatment. **(C)** colorimetric CCK8 proliferation assay reveals significantly decreased cell proliferation in UBC9-depleted cells over 72 hours. **(D)** scratch assay shows reduced cell migration in UBC9 knockdown cells, 48 hours post-Dox induction. **(E)** Matrigel invasion assay demonstrates a marked decrease in the invasive capacity of HCC cells upon UBC9 knockdown. **(F)** Western blot analysis and quantification confirming UBC9 knockdown efficiency in Huh-7 cells. **(G)** CCK-8 assay revealing the effect of UBC9 depletion on Huh-7 cell proliferation. **(H)** wound healing assay showing the impact of UBC9 knockdown on the migration of Huh-7 cells. **(I)** Transwell invasion assay demonstrating the effect of UBC9 knockdown on the invasion of Huh-7 cells. **(J)** in vivo xenograft tumor assay. Images of dissected tumors, tumor weights, and tumor volume growth curves from nude mice injected with control (NC) or UBC9-shRNA Hep3B cells. ns, $p > 0.05$; **, $p < 0.01$; ***, $p < 0.001$

Proteomic characterization of downstream targets of UBC9

To further investigate the downstream proteins regulated by UBC9, we conducted a proteomics analysis to elucidate the differential protein expression in Hep3B cells before and after UBC9 knockdown. This analysis

revealed that in the scrambled shRNA group treated with DOX, 299 proteins exhibited altered expression compared to the untreated group (Fig. 3A). In contrast, the UBC9 shRNA group subjected to DOX induction displayed 225 proteins with changed expression levels

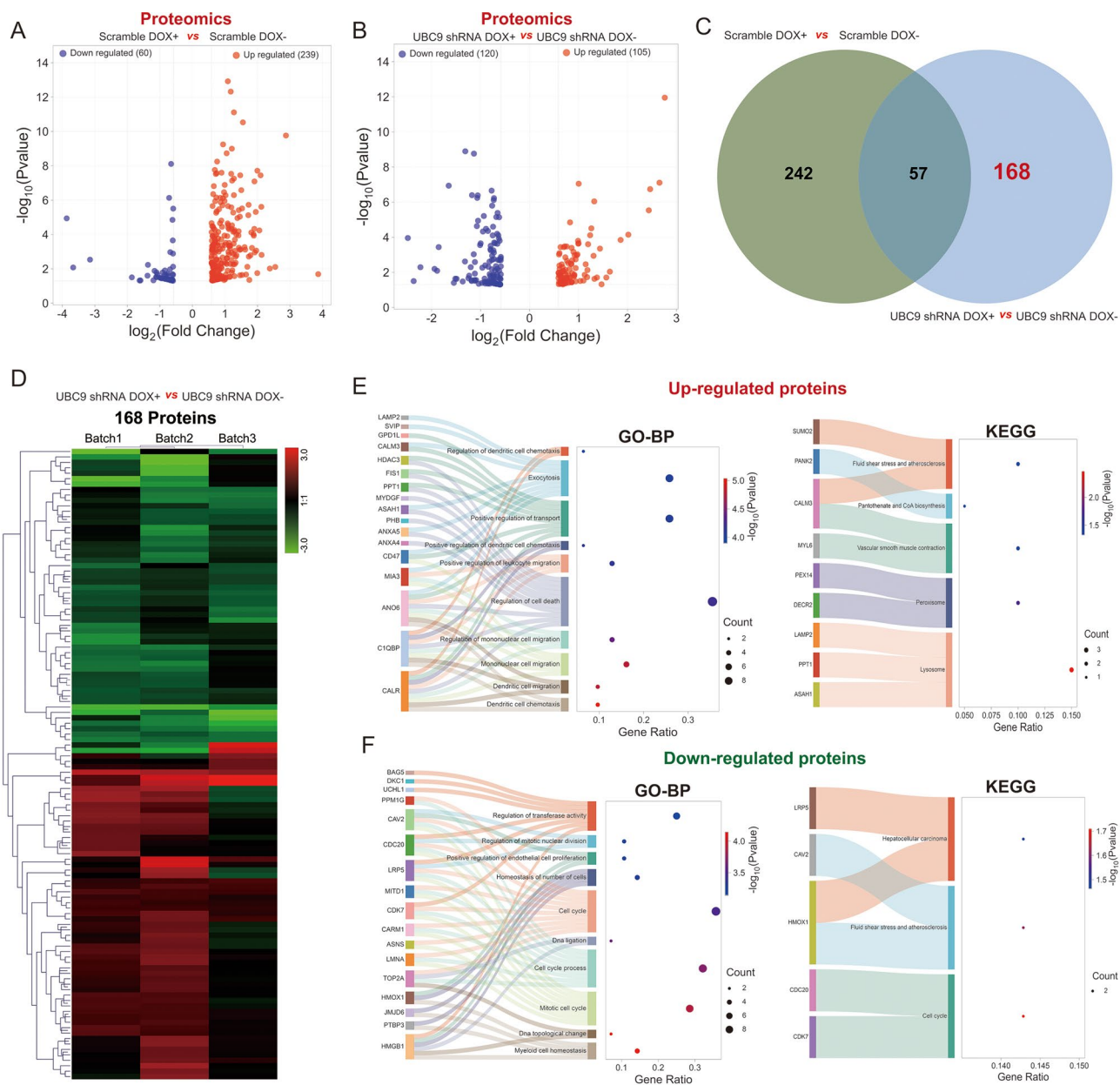


Fig. 3 Proteomics analysis of differential protein expression in Hep3B cells upon UBC9 knockdown. **(A)** volcano plot depicting differentially expressed proteins in the scrambled shRNA group induced by DOX treatment vs untreated controls. **(B)** volcano plot showing differentially expressed proteins in UBC9 shRNA group with DOX induction vs untreated control. **(C)** venn diagram illustrating 57 overlapping proteins between scrambled and UBC9 shRNA groups, with 168 unique proteins found in the UBC9 shRNA group. **(D)** Heatmap of 168 uniquely expressed proteins in UBC9 shRNA group upon DOX treatment. **(E)** GO-BP and KEGG analyses of upregulated proteins displayed roles in various cellular processes and pathways. **(F)** GO-BP and KEGG analyses of downregulated proteins associated with certain cellular functions and pathways

relative to the untreated samples (Fig. 3B). A comparative analysis between the two groups identified 57 overlapping proteins, suggesting common pathways affected by DOX induction in both scrambled and UBC9 knock-down conditions (Fig. 3C). Notably, the UBC9 shRNA group also displayed 168 unique proteins with differential expression upon DOX induction, suggesting specific target candidates impacted by UBC9 depletion (Fig. 3C and D, Table S1).

Further enrichment analyses were conducted on these 168 differentially expressed candidate proteins. We performed KEGG and GO analyses separately on the upregulated and downregulated proteins to explore their potential functional significance. The GO-BP analysis of upregulated proteins highlighted their roles in Regulation of dendritic cell chemotaxis, Exocytosis, Positive regulation of transport, Positive regulation of leukocyte migration,

Regulation of cell death, Regulation of mononuclear cell migration, Mononuclear cell migration, Dendritic cell migration and Dendritic cell chemotaxis. KEGG pathways suggested involvement in Fluid shear stress and atherosclerosis, Pantothenate and CoA biosynthesis, Vascular smooth muscle contraction, Peroxisome and Lysosome (Fig. 3E). Conversely, the GO-BP analysis of downregulated proteins indicated associations with the Regulation of transferase activity, Regulation of mitotic nuclear division, Positive regulation of endothelial cell proliferation, Homeostasis of number of cells, cell cycle, DNA ligation, Cell cycle process and Mitotic cell cycle. KEGG pathway analysis revealed downregulation linked to HCC, Fluid shear stress and atherosclerosis and Cell cycle (Fig. 3F).

In summary, this proteomics analysis suggests the multifaceted roles of UBC9 in regulating HCC cell functionality. The unique protein changes observed in response to UBC9 knockdown underscore its plausible involvement in various cellular pathways and key biological processes.

Ubiquitin proteomics unveil the UBC9 regulatory network

Previous studies have shown that SUMOylation of the E3 ubiquitin ligase RNF146 promotes its association with Axin and accelerates Axin degradation, enhancing β -catenin signaling, thereby promoting HCC progression [18]. It should be noted that Axin is a key scaffold protein and an important negative regulator of the Wnt/ β -catenin signaling pathway which forms a “destruction complex” that targets β -catenin for degradation; hence it has a key role as a tumor suppressor gene in HCC and hepatoblastoma [25–27]. We hence focused our further experiments on the possible impact of UBC9-regulated ubiquitination on HCC progression. To explore the ubiquitination level of proteins possibly regulated by UBC9, we conducted a comprehensive ubiquitin proteomics analysis. Upon DOX induction of the scrambled shRNA group, ubiquitin proteomics analysis identified changes in the ubiquitination status of 115 proteins compared to the untreated controls (Fig. 4A). In contrast, the UBC9 shRNA group subjected to DOX induction, exhibited changes in the ubiquitination of only 60 proteins (Fig. 4B). A comparative analysis between these groups (Scrambled DOX⁺ vs Scrambled DOX⁻ and UBC9 shRNA DOX⁺ vs UBC9 shRNA DOX⁻) revealed an overlap of 13 proteins, outlining common effects by DOX induction irrespective of UBC9 downregulation (Fig. 4C). Additionally, we identified 47 unique proteins with 52 distinct ubiquitination sites altered specifically in the UBC9 shRNA group upon DOX induction, emphasizing the unique regulatory pathways modulated by UBC9 (Fig. 4C, Table S2).

Subsequent KEGG and GO analyses focused on these 47 unique proteins, distinguishing those with increased and decreased ubiquitination levels. The GO-BP analysis

of upregulated proteins indicated their involvement in processes like Microvillus organization, Endomembrane system organization, Lipid metabolic process, Microvillus assembly, Sterol biosynthetic process, Regulation of microvillus organization, Steroid biosynthetic process, Steroid metabolic process and Regulation of microvillus assembly. KEGG pathway enrichment suggested these proteins are linked with pathways such as Jak-STAT signaling pathway, Phospholipase D signaling pathway, HIF-1 signaling pathway, Choline metabolism in cancer, GnRH signaling pathway, Gap junction, Endocytosis, Pancreatic cancer, Parathyroid hormone synthesis, Secretion and action, and Steroid biosynthesis (Fig. 4D).

Conversely, for proteins with decreased ubiquitination, GO-BP analysis highlighted processes related to Intracellular protein transport, Coagulation, Protein localization to membrane, DNA replication, Cellular macromolecule localization, Regulation of lipid catabolic process, Viral gene expression, Establishment of protein localization, Protein localization to organelle and Establishment of protein localization to organelle. KEGG analysis associated these proteins with pathways related to Influenza A, Tight junction, Cellular senescence, Cushing syndrome, Ribosome, Pancreatic secretion, Pancreatic cancer, and Human cytomegalovirus infection (Fig. 4E).

In summary, this ubiquitin proteomics analysis suggested some key proteins and pathways under UBC9 regulation at the ubiquitination level.

LMNA as a downstream target of UBC9 in HCC

Our comprehensive analysis of both proteomics and ubiquitin proteomics revealed that LMNA is the sole protein among the 168 proteins identified in the proteomics screen and the 47 proteins identified in the ubiquitination proteomics screen. Upon UBC9 knockdown, LMNA protein expression was markedly decreased whereas its ubiquitination level was increased, suggesting differential regulation at the post-translational modification level (Fig. 5A). Western blot analysis confirmed that UBC9 knockdown significantly reduced LMNA protein levels (Fig. 5B). Interestingly, RT-PCR results indicated that UBC9 knockdown did not affect LMNA mRNA expression, implying that UBC9 regulates LMNA at the protein level rather than the transcript level (Fig. 5C). These results suggest that LMNA is a key downstream gene regulated by UBC9.

We subsequently explored the expression and prognostic implications of LMNA in HCC. Our analysis demonstrated that LMNA expression is significantly higher in tumor specimens compared to normal tissues, based on the TCGA_LIHC dataset (Fig. 5D). HCC patients under 65 exhibited significantly increased LMNA expression compared to those over 65 (Fig. 5E). No significant differences in LMNA expression were observed between male

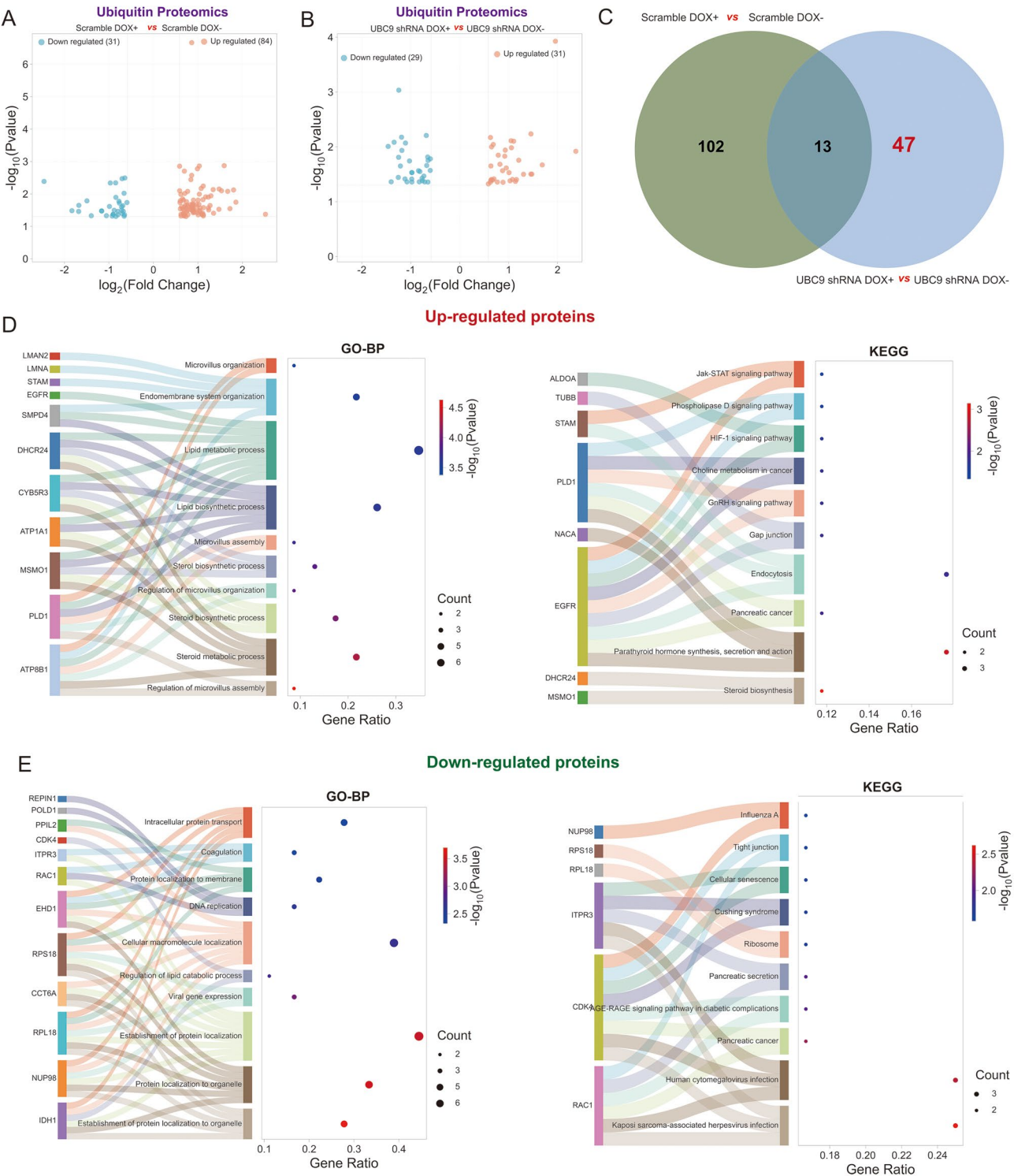


Fig. 4 Ubiquitin proteomics analysis in Hep3B cells with UBC9 knockdown. **(A)** volcano plot of ubiquitination changes in the scrambled shRNA group upon DOX induction. **(B)** volcano plot shows changes in ubiquitination in the UBC9 shRNA group after DOX induction. **(C)** venn diagram illustrating 13 overlapping proteins and 47 unique proteins with altered ubiquitination in the UBC9 shRNA group. **(D)** GO-BP and KEGG analyses of proteins with increased ubiquitination highlight involvement in cellular processes and pathways such as microvillus organization and steroid biosynthesis. **(E)** GO-BP and KEGG analyses of proteins with decreased ubiquitination emphasize roles in processes like intracellular protein transport and viral gene expression

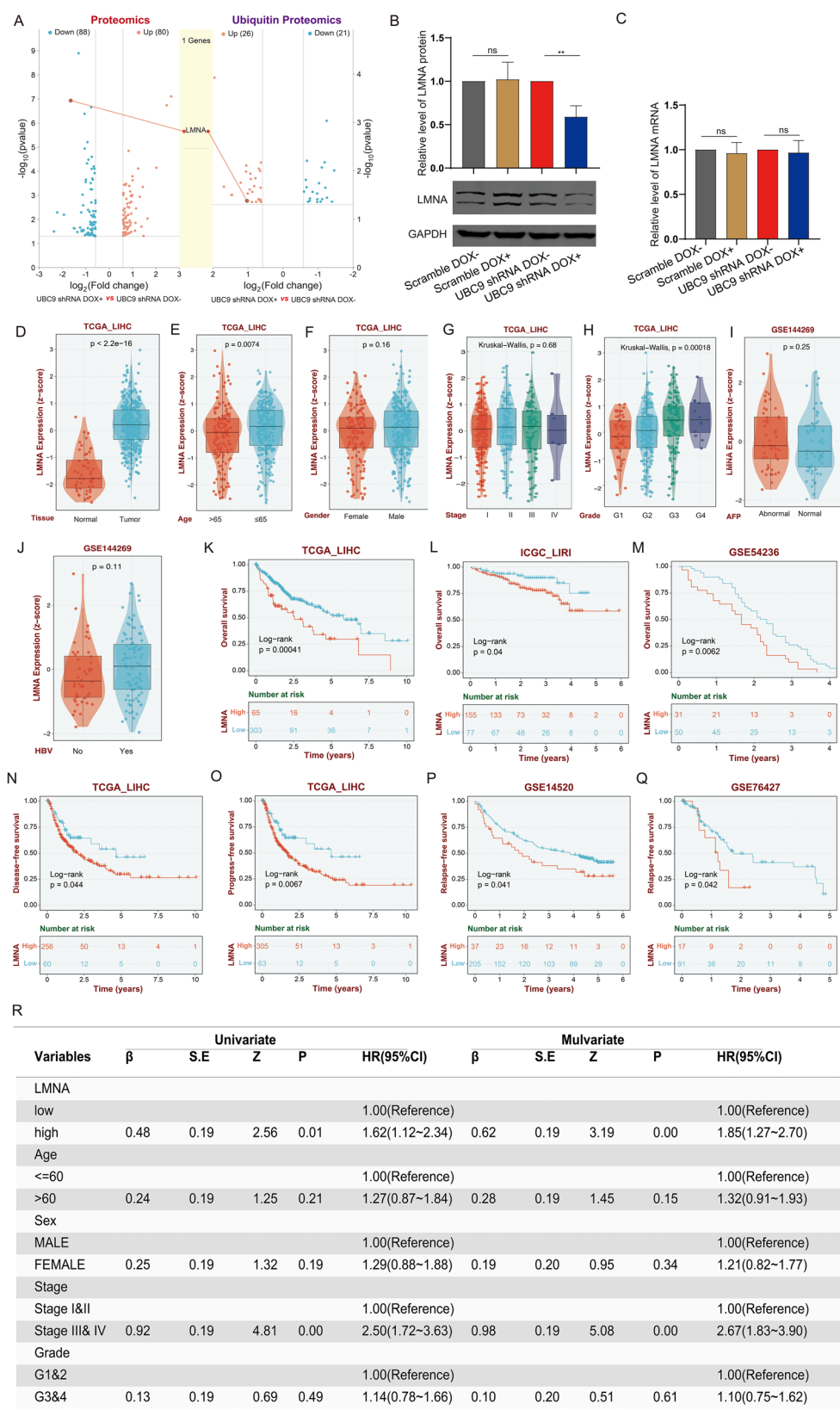


Fig. 5 (See legend on next page.)

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Fig. 5 Analysis of LMNA as a downstream target of UBC9 in hepatocellular carcinoma. **(A)** overlap of LMNA in proteomics and ubiquitin proteomics, with decreased LMNA expression and increased ubiquitination upon UBC9 knockdown. **(B)** Western blot analysis showing reduced LMNA protein levels after UBC9 knockdown. **(C)** RT-PCR indicates unchanged LMNA mRNA levels, suggesting post-transcriptional regulation of LMNA protein levels. **(D)** TCGA_LIHC data show higher LMNA expression in tumor tissues compared to normal tissues. **(E)** correlation between LMNA expression and patient age in the TCGA_LIHC cohort. **(F)** comparison of LMNA expression between male and female patients in the TCGA_LIHC cohort. **(G)** analysis of LMNA expression across different tumor stages in the TCGA_LIHC cohort. **(H)** analysis of LMNA expression across different tumor grades in the TCGA_LIHC cohort. **(I)** correlation between LMNA expression and serum AFP levels (normal vs. Abnormal). **(J)** validation of LMNA expression relative to HBV infection status. **(K–M)** higher LMNA expression correlates with poorer overall survival across various datasets TCGA_LIHC, ICGC_LIRI, and GSE54236. **(N–Q)** elevated LMNA levels are associated with reduced disease-free survival, progression-free survival, and relapse-free survival. **(R)** univariate and multivariate Cox regression analysis of clinical parameters and LMNA expression associated with overall survival in the TCGA_LIHC cohort. ns, $p > 0.05$; **, $p < 0.01$

and female patients (Fig. 5F). LMNA expression shows no significant differences among stages (Fig. 5G). Tumor grade analysis demonstrated a significant positive correlation between LMNA expression and histological grade, with expression levels progressively increasing from G1 to G4 (Fig. 5H). Analysis of serum AFP levels revealed no significant difference in LMNA expression between AFP-abnormal and AFP-normal patients (Fig. 5I). Similarly, external validation using GSE144269 showed no significant correlation between LMNA expression and HBV infection status (Fig. 5J).

Kaplan-Meier survival analysis was performed to assess LMNA's prognostic value. Higher LMNA expression correlated with a poorer overall survival, as demonstrated across several datasets, including TCGA_LIHC, ICGC_LIRI, and GSE54236 (Fig. 5K–M). This analysis also indicated that high LMNA levels are associated with reduced disease-free survival (Fig. 5N), progression-free survival (Fig. 5O), and relapse-free survival, further emphasizing its presumed role in HCC (Fig. 5P–Q). Our findings suggest that LMNA appears to be a crucial downstream target of UBC9 and is associated with adverse outcomes in HCC.

To validate LMNA as an independent prognostic biomarker in HCC, we conducted multivariate Cox regression analysis incorporating major clinicopathological confounders including age, sex, tumor stage, and tumor grade using the TCGA_LIHC cohort (Fig. 5R). Univariate analysis demonstrated that high LMNA expression was significantly associated with poor overall survival. Critically, multivariate Cox regression analysis confirmed that LMNA expression retained its independent prognostic significance after adjusting for potential confounding factors. Notably, tumor stage (Stage III&IV vs I&II) also demonstrated strong independent prognostic value, while age, sex, and tumor grade did not reach statistical significance as independent predictors. These results establish LMNA as an independent prognostic factor in HCC and underscore its clinical relevance as both a downstream target of UBC9 and a potential biomarker for risk stratification in HCC patients.

The role of SUMOylation and ubiquitination in regulating LMNA expression

To investigate the possible impact of SUMOylation on LMNA protein expression, we co-transfected Hep3B cells with Flag-LMNA, V5–UBC9 along with either His-SUMO1, His-SUMO2, or His-SUMO3. Western blot analysis revealed that ectopic overexpression of SUMO1, SUMO2, or SUMO3 markedly increased LMNA protein levels by 2.5–5-fold (Fig. 6A). SENPs are specific proteases that mediate the de-SUMOylation of substrates, maintaining a dynamic balance in SUMO modifications [28]. To assess the impact of SENPs on LMNA expression, we transfected Hep3B cells with Flag-LMNA along with either SENP1, SENP3, SENP5, SENP6, or SENP7. Western blot analysis revealed that only SENP7 significantly decreased LMNA protein levels (Fig. 6B). These results reveal that SUMOylation markedly enhances LMNA protein levels, whereas SENP7 removes SUMO modifications, thereby decreasing LMNA protein levels. Furthermore, ubiquitination proteomic analysis revealed an increased ubiquitination of LMNA at the K144 site upon UBC9 knock down (Table S2). To further investigate the role of the K144 site in regulating LMNA protein levels, we constructed a K144R LMNA mutant plasmid. Co-immunoprecipitation of ubiquitinated products indicated that the K144R point mutation resulted in a significantly decreased ubiquitination of LMNA, establishing K144 as a *bona fide* ubiquitination site for LMNA (Fig. 6C).

Moreover, half-life studies via cycloheximide-based translational inhibition revealed that both SUMO2 and the K144R mutation markedly increased the half-life of LMNA protein (Fig. 6D). These findings underscore the critical relationship between SUMOylation and ubiquitination in regulating the levels and stability of LMNA.

To directly address the functional impact of the K144 ubiquitination site beyond protein stability, we evaluated the biological behavior of HCC cells expressing the ubiquitination-deficient mutant LMNA (K144R). CCK-8 proliferation assays demonstrated that overexpression of the K144R mutant significantly accelerated cell growth in both Hep3B and Huh-7 cell lines compared to the control group (Supplementary Figure 1A, D). Wound healing assays revealed that cells expressing the K144R

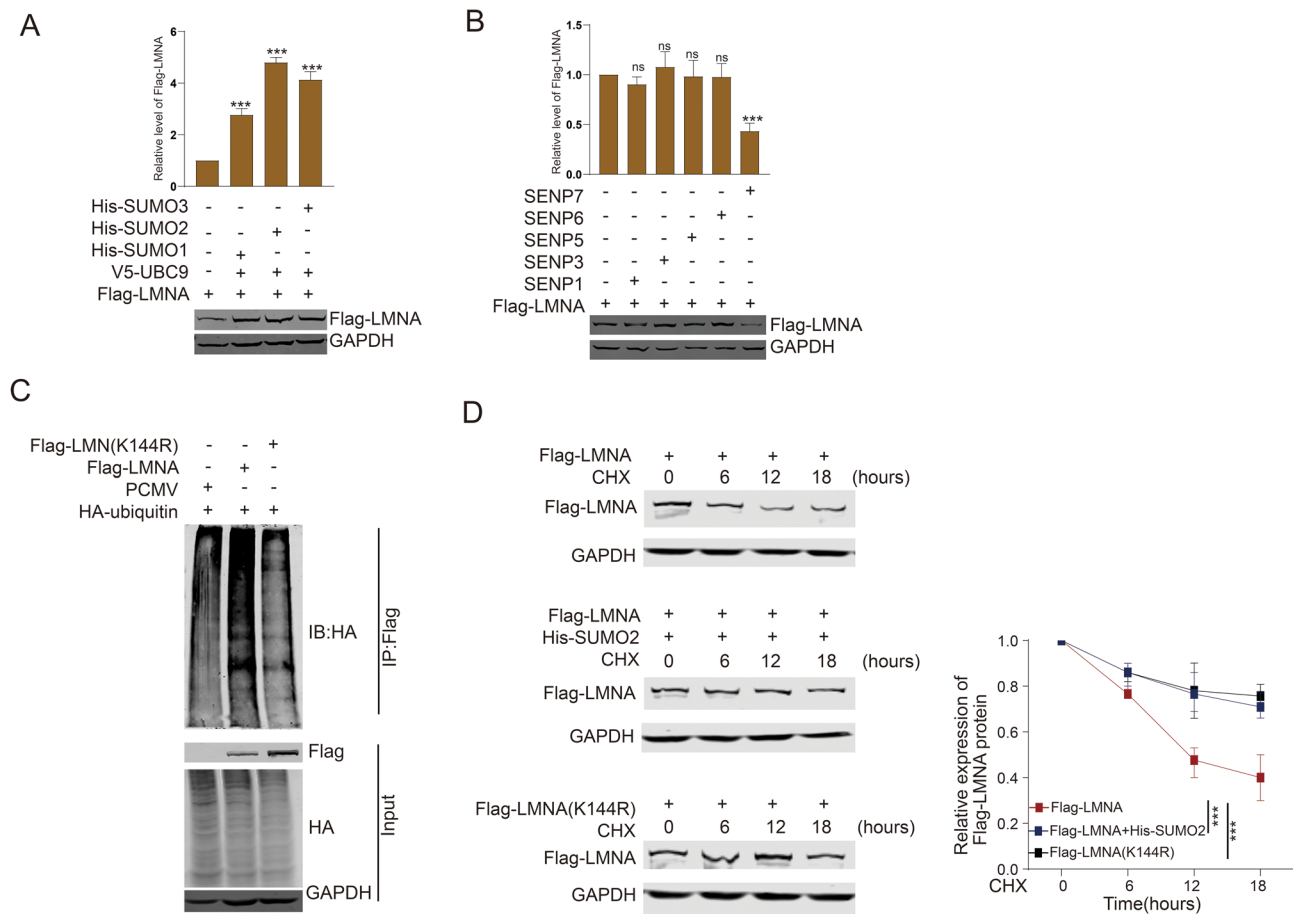


Fig. 6 Impact of SUMOylation and ubiquitination on LMNA levels. **(A)** Western blot analysis showing increased LMNA expression with SUMO1, SUMO2, and SUMO3 in Hep3B cells. **(B)** SENP7 decreases LMNA expression as opposed to other transfected SENPs. **(C)** Co-immunoprecipitation reveals reduced ubiquitination of LMNA in cells transfected with the K144R mutation. **(D)** cycloheximide chase assays for half-life protein determination reveal LMNA protein stability differences, showing protection and stabilization by His-SUMO2 and destabilization by K144R mutation. ns, $p > 0.05$; ***, $p < 0.001$

mutant exhibited a significantly faster wound closure rate compared to control cells (Supplementary Figure 1B, E). Furthermore, Transwell Matrigel invasion assays showed that the number of invasive cells was significantly increased in the K144R overexpression group (Supplementary Figure 1C, F).

Collectively, these findings provide compelling evidence that preventing K144 ubiquitination not only stabilizes LMNA protein but also functionally promotes the malignant phenotype of HCC cells, including proliferation, migration, and invasion.

Role of LMNA in UBC9-mediated hepatocellular carcinoma cell functionality

To explore whether the impact of UBC9 on Hep3B cell functionality involves LMNA, we conducted functional recovery experiments. Overexpression of UBC9 in Hep3B cells significantly enhanced tumor cell proliferation (Fig. 7A). However, when LMNA was knocked down in UBC9-overexpressing cells, this proliferative effect was partially attenuated, indicating that the role of UBC9 in

promoting cell growth is, at least in part, dependent on LMNA.

We also examined the migratory ability of Hep3B cells using a scratch wound healing assay. UBC9 overexpressing cells demonstrated a significantly higher migration capacity, rapidly closing the wound gap when compared to control cells (Fig. 7B). Knockdown of LMNA in these UBC9-overexpressing cells resulted in a noticeable reduction in migration ability, underscoring the partial reliance of UBC9-enhanced migration on LMNA (Fig. 7B). This result suggests that while UBC9 promotes migration, LMNA is a critical component of this process. Further investigation into the invasive capacities of the tumor cells using a Matrigel invasion assay revealed that UBC9 overexpression leads to a significantly increased invasion capacity compared to control cells (Fig. 7C).

We performed parallel rescue experiments in Huh-7 cells. Consistent with the findings in Hep3B cells, UBC9 overexpression in Huh-7 cells significantly enhanced cell proliferation, and this proliferative advantage was partially abrogated upon LMNA knockdown (Fig. 7D). The

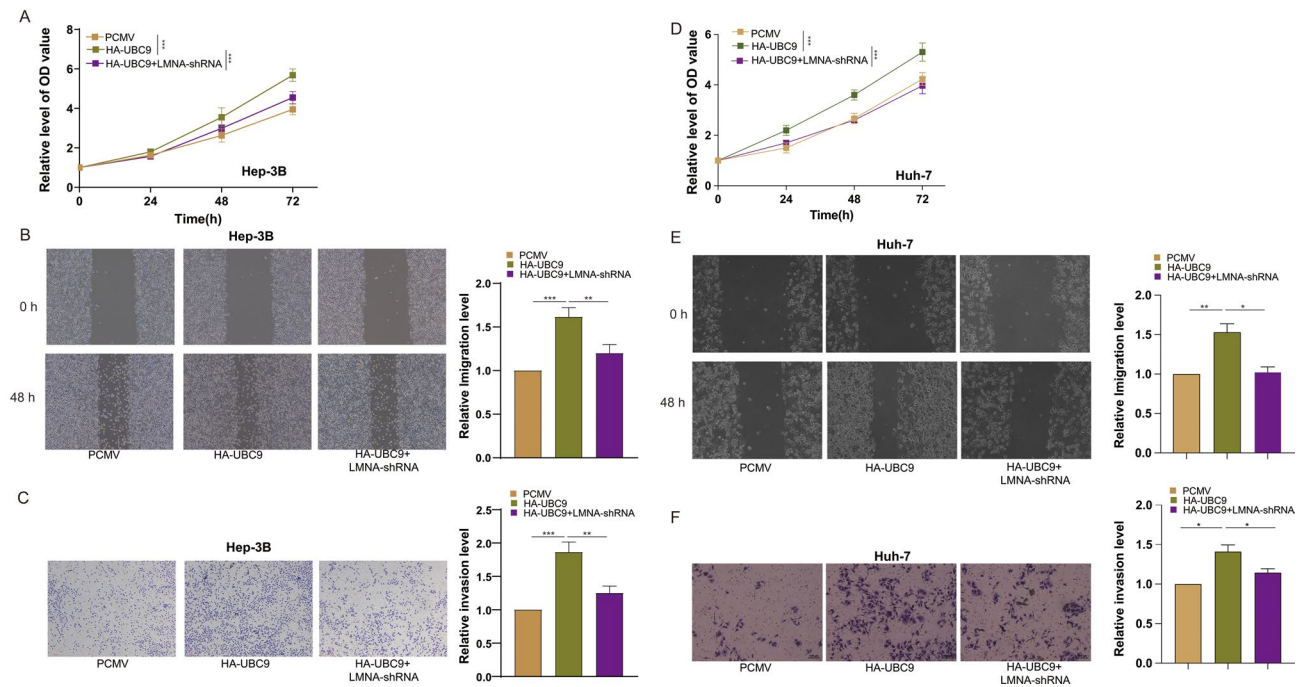


Fig. 7 Role of LMNA in UBC9-mediated Hep3B cell functionality. **(A)** overexpression of UBC9 significantly enhances cell proliferation in Hep3B cells, while LMNA knockdown partially alleviates this effect. **(B)** scratch wound healing assay shows increased migration in UBC9-overexpressing cells, and reduced migration upon LMNA knockdown. **(C)** Matrigel invasion assay indicates that UBC9 overexpression increases cell invasion, which is diminished by LMNA knockdown, highlighting the role of LMNA in UBC9-induced cell aggressiveness. **(D)** CCK-8 assay demonstrating the rescue effect of LMNA knockdown on proliferation in UBC9-overexpressing Huh-7 cells. **(E)** wound healing assay revealing the impact of LMNA knockdown on the migratory capacity of UBC9-overexpressing Huh-7 cells. **(F)** Transwell invasion assay showing the effect of LMNA knockdown on the invasion of UBC9-overexpressing Huh-7 cells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

scratch wound healing assay demonstrated that UBC9 overexpression markedly accelerated wound closure in Huh-7 cells, whereas LMNA depletion in UBC9-overexpressing cells reduced this migratory capacity (Fig. 7E). Similarly, the Matrigel invasion assay revealed that UBC9 overexpression promoted cell invasion in Huh-7 cells, and this invasive phenotype was significantly attenuated by LMNA knockdown (Fig. 7F).

This increase highlights the importance of LMNA in mediating an invasive phenotype promoted by UBC9, indicating that UBC9-induced cell aggressiveness is partly facilitated through LMNA. Taken *in toto*, these findings reveal that UBC9 substantially promotes proliferation, migration, and invasion of HCC cells. Our data also demonstrate that LMNA plays a critical role in facilitating UBC9's effects on malignant HCC cell behavior. Reducing LMNA levels in UBC9-overexpressing cells mitigates the aggressive properties of these tumor cells, pointing to a potential synergistic mechanism between UBC9 and LMNA. This mutual relationship opens up further avenues for possible therapeutic strategies targeting both UBC9 and LMNA in HCC treatment.

Discussion

PTMs are crucial in regulating protein function, stability, and interactions, thereby influencing key cellular pathways and processes [29–31]. Among these modifications, ubiquitination and SUMOylation are particularly significant due to their profound impact on protein turnover and signaling pathways [32]. Among its multiple functions, ubiquitination predominantly tags proteins for degradation via the 26S proteasome, maintaining protein homeostasis within the cell. Conversely, SUMOylation involves the covalent attachment of SUMO proteins to target substrates, influencing their subcellular localization, stability, activity, and interactions without necessarily marking them for degradation [33–35]. The interplay between these two PTMs is complex and can manifest as antagonistic, synergistic, or SUMOylation-dependent ubiquitination pathways, collectively shaping cellular dynamics [12, 36]. In the context of HCC, our current study sheds light on the critical role of UBC9, the sole E2 conjugating enzyme in the SUMOylation pathway. We specifically showed that UBC9 is significantly

overexpressed in HCC compared to normal liver tissues, implicating its involvement in liver tumorigenesis as recently suggested [18]. Remarkably, this overexpression correlated with higher tumor grade and poorer patient prognosis, including reduced overall survival, progression-free survival, and disease-free survival. Since a plethora of proteins involved in cell cycle regulation and progression, cell proliferation, apoptosis and DNA repair are *bona fide* SUMOylation substrates, alterations in SUMOylation could ultimately have an impact on cancer cell growth, proliferation, cancer progression as well as therapy and radiotherapy response [37].

Given that UBC9 is the sole E2-conjugating enzyme required for SUMOylation, and since we and others have shown that UBC9 is upregulated in a wide range of human cancers, UBC9 emerges as a potential druggable target for cancer therapeutics including papillary thyroid cancer [38]. In strong support of the UBC9 inhibition concept for targeted cancer therapy, it has been recently discovered that UBC9 inhibition using a small molecule 2-D08 or UBC9 knockdown, attenuated double minute (DM) chromosome formation and decreased double minute-carried gene expression [39]. This resulted in repression of tumor growth and the malignant phenotype, via micronuclei expulsion of DM and decreased non-homologous end joining activity, thereby increasing DNA damage. These novel findings uncover a tight relationship between elevated UBC9 activity, increased DM formation, and tumor progression, providing a plausible modality for targeted cancer therapy via UBC9 inhibition [39]. The functional consequences of UBC9 modulation were further explored using a tetracycline (Dox)-inducible downregulation system in Hep3B HCC cells. Knockdown of UBC9 resulted in a marked reduction in cell proliferation, migration, and invasion, indicating that UBC9 promotes an aggressive cancer cell phenotype.

To delve deeper into the molecular mechanisms underlying the oncogenic functions of UBC9, we performed proteomics analysis to identify downstream targets affected by UBC9 depletion. The analysis revealed a significant number of differentially expressed proteins upon UBC9 knockdown, highlighting UBC9's influence on various cellular pathways. GO and KEGG enrichment analyses indicated that these proteins are involved in an assortment of critical processes including cell cycle regulation, DNA replication, cellular metabolism and apoptosis. Notably, many of these pathways are intimately linked to cancer cell proliferation and progression. A key finding of our current study is the identification of LMNA as a critical downstream target of UBC9. LMNA,

a key component of the nuclear lamina, plays a fundamental role in maintaining nuclear integrity, regulating gene expression, and influencing cell proliferation and differentiation [40, 41]. Our results demonstrate that LMNA expression is significantly elevated in HCC tissues and is associated with dismal clinical outcomes, including decreased overall survival and increased recurrence rates. This association positions LMNA as a potential prognostic marker and therapeutic target in HCC. Indeed, based on LMNA knockout in HepG2 cells, recent studies suggested that LMNA might function as an oncogene in HCC, thereby constituting a potential target for HCC treatment [22]. Mechanistically, a recent discovery revealed that crotonylation of LMNA at K265/270 promotes liver cancer cell proliferation and prevents cellular senescence [42]. These novel findings highlight the role of LMNA crotonylation in liver cancer progression, thereby filling the current research gap in non-histone protein crotonylation. Beyond these modifications, LMNA is also known to undergo phosphorylation and acetylation, which are critical for nuclear lamina assembly and stability. It is plausible that a complex interplay exists between these diverse PTMs and the UBC9-mediated SUMOylation/ubiquitination axis described herein. Future studies should therefore investigate whether UBC9 activity influences this broader "PTM code," thereby fine-tuning LMNA function in a context-dependent manner during HCC progression.

The regulatory relationship between UBC9 and LMNA involves intricate mechanisms mediated by SUMOylation and ubiquitination. We discovered that UBC9 knockdown leads to decreased LMNA levels, suggesting that UBC9 positively regulates LMNA levels. Further experiments revealed that SUMOylation stabilizes LMNA. Specifically, overexpression of SUMO isoforms (SUMO1, SUMO2, and SUMO3) resulted in increased LMNA protein levels. Conversely, co-expression of SENP7, a SUMO-specific protease, led to a significant reduction in LMNA levels. These observations highlight the dynamic regulation of LMNA by SUMOylation. However, the precise molecular nature of the UBC9-LMNA interaction requires further elucidation. While UBC9, as an E2 conjugating enzyme, can directly recognize and SUMOylate substrates containing the consensus motif ψ -K-x-E (where ψ is a hydrophobic residue), efficient modification *in vivo* often necessitates E3 SUMO ligases to enhance specificity and kinetics. It remains to be determined whether UBC9 directly engages LMNA or acts in concert with specific E3 ligases, such as the PIAS family or RanBP2, to catalyze this modification.

Future co-immunoprecipitation assays using purified proteins or in vitro SUMOylation reconstitution systems are warranted to distinguish between these direct and indirect mechanisms. In mechanistic support of these findings, a previous study reported that SUMO1 conjugation of the Retinoblastoma protein (RB) and Lamin A/C is modulated by the SUMO protease SENP1 and that SUMOylation of both proteins is crucial for their protein-protein interaction [43]. Consistent with our current findings, this SUMO1-dependent complex protected both RB and Lamin A/C from proteasomal degradation hence stabilizing them.

Our results indicate a reciprocal relationship between SUMOylation and ubiquitination in regulating LMNA expression. SUMOylation appears to shield LMNA from ubiquitination and subsequent proteasomal degradation, thereby stabilizing the protein. These insights uncover a novel regulatory mechanism by which UBC9-mediated SUMOylation enhances LMNA stability, impacting HCC behavior. It is pertinent to speculate on potential candidates known to participate in SUMO-ubiquitin crosstalk, which could contextualize our findings and guide future research. For instance, a class of E3 ligases known as SUMO-targeted ubiquitin ligases (STUbLs), such as RNF4, are specialized in recognizing SUMOylated proteins and targeting them for ubiquitination. It is conceivable that under certain cellular contexts, STUbLs could compete with the protective effect of SUMOylation to regulate LMNA turnover. Conversely, on the deubiquitination side, enzymes like USP7 and SENP family members, which are known to be involved in de-SUMOylating or deubiquitinating nuclear proteins, could play a role. Indeed, our finding that SENP7 expression reduces LMNA levels supports the idea that de-SUMOylation exposes LMNA to the ubiquitin-proteasome system. Structurally, K144 is situated within the coil 1B segment of the central α -helical rod domain of LMNA. We postulate that the spatial positioning of this residue on the helical surface renders it accessible for recognition by ubiquitin ligases, whereas UBC9-mediated interaction or proximal SUMOylation may induce steric hindrance or conformational changes that mask this site, thereby preventing degradation. Although our current study identifies K144 as a critical ubiquitination site, the specific upstream enzymes remain to be characterized. Therefore, future studies focused on screening for E3 ligase and DUB candidates that specifically target this site could definitively pinpoint the enzymatic machinery responsible for this crosstalk and may reveal new therapeutic targets for HCC.

Functionally, the interplay between UBC9 and LMNA significantly affects HCC cell proliferation, migration, and invasion. Overexpression of UBC9 in Hep3B cells enhanced their malignant phenotype, whereas knockdown of LMNA partially abrogated the pro-tumorigenic effects of UBC9 overexpression. These findings suggest that LMNA mediates, at least in part, the oncogenic functions of UBC9 in HCC. The partial inhibition of the malignant phenotype upon LMNA knockdown indicates that UBC9 may exert additional effects through other downstream targets.

Beyond LMNA, several classes of UBC9-regulated substrates may cooperate to drive HCC progression. First, additional nuclear lamina components such as lamin B1/B2 and emerin could be SUMOylated in a manner that complements LMNA to reshape nuclear architecture, mechanotransduction, and chromatin organization, thereby influencing transcriptional programs relevant to malignancy. Second, transcriptional regulators are plausible mediators. In HCC, pathway-level interactions with p53, c-Myc, and NF- κ B are particularly noteworthy, consistent with our observation that UBC9-high tumors harbor elevated TP53 mutation rates, suggesting potential convergence on genome surveillance and stress-response circuits. Third, signaling intermediates within oncogenic cascades—especially Wnt/ β -catenin—may constitute additional UBC9 targets beyond the RNF146-Axin axis already implicated in HCC progression, aligning with the centrality of aberrant Wnt signaling in liver tumor biology.

The clinical implications of our study are substantial. The overexpression of UBC9 and LMNA in HCC and their association with poor patient outcomes, highlight their potential as biomarkers for prognosis as well as potential targets for therapeutic interventions. Targeting the UBC9-LMNA axis could disrupt critical pathways that facilitate tumor growth and metastasis. Inhibitors of UBC9 or modulators of SUMOylation processes may offer novel avenues for cancer treatment. However, given the essential roles of UBC9 and SUMOylation in normal cellular functions, therapeutic strategies must be designed to selectively target cancer cells, while minimizing adverse effects to healthy tissues.

Several mechanisms could potentially enable selective therapeutic targeting of UBC9 in HCC while sparing normal hepatocytes. First, our data demonstrate that UBC9 is significantly overexpressed in HCC tissues compared to normal liver tissue, suggesting a wider therapeutic window wherein cancer cells may be more dependent on elevated UBC9 activity for survival and proliferation than

healthy hepatocytes. This differential expression pattern implies that cancer cells may exhibit heightened vulnerability to UBC9 inhibition. Second, the differential metabolic state between rapidly proliferating cancer cells and quiescent normal hepatocytes may render cancer cells more susceptible to perturbations in protein homeostasis, including SUMOylation-ubiquitination crosstalk.

Beyond monotherapy approaches, combination strategies pairing UBC9 inhibition with established HCC-targeted therapies could significantly enhance therapeutic efficacy while minimizing toxicity. For instance, combining UBC9 inhibitors with sorafenib or lenvatinib, the current first-line multi-kinase inhibitors for advanced HCC, may produce synergistic anti-tumor effects by simultaneously disrupting multiple oncogenic pathways. Given that UBC9 inhibition impairs cell proliferation, migration, and invasion as demonstrated in our study, such combinations could overcome resistance mechanisms that frequently emerge during single-agent therapy. Additionally, combining UBC9 inhibition with immune checkpoint inhibitors such as atezolizumab plus bevacizumab represents another promising strategy. Recent evidence suggests that SUMOylation regulates immune checkpoint expression and immune cell function; hence, UBC9 inhibition might potentiate anti-tumor immunity by modulating the tumor micro-environment and enhancing T cell-mediated cancer cell killing. Furthermore, given the role of UBC9 in DNA damage response and genomic stability combining UBC9 inhibitors with DNA-damaging agents or PARP inhibitors could exploit synthetic lethality in HCC cells with compromised DNA repair mechanisms. Importantly, such combination approaches may allow for dose reduction of individual agents, thereby minimizing off-target toxicities while maintaining or enhancing therapeutic efficacy. Future preclinical and clinical studies should systematically evaluate these combination strategies, establish optimal dosing schedules, and identify predictive biomarkers to guide patient selection for UBC9-targeted therapies in HCC.

Our study also contributes to the broader understanding of PTMs in cancer. The interplay between SUMOylation and ubiquitination represents a critical regulatory mechanism that balances protein stability and function. Dysregulation of this balance can lead to aberrant protein expression, contributing to oncogenesis and tumor progression. Elucidating these

mechanisms provides valuable insights into cancer pathophysiology and identifies potential targets for therapeutic development.

To translate these mechanistic insights into clinical practice, future efforts must prioritize prospective clinical validation. While our retrospective analysis establishes a strong prognostic link, large-scale, multi-center prospective cohorts are required to validate UBC9 and LMNA as independent biomarkers and to establish standardized immunohistochemical cut-off values. Additionally, longitudinal monitoring of these markers via liquid biopsy could offer dynamic insights into disease progression. Clinically, these biomarkers offer a compelling framework for patient stratification to guide therapeutic decisions. We propose a risk-adapted treatment strategy where patients with dual high expression of UBC9 and LMNA are classified as a high-risk subgroup. These patients may benefit from more aggressive adjuvant therapies or earlier consideration for combination regimens, such as pairing UBC9 inhibitors with sorafenib or immune checkpoint inhibitors, to overcome potential resistance. Conversely, patients with low expression profiles might be managed with standard-of-care surveillance, thereby sparing them from unnecessary toxicity. Integrating UBC9/LMNA profiling with existing staging systems could thus refine prognostic accuracy and enable truly personalized precision medicine in HCC.

Conclusion

Our current study highlights the crucial role of UBC9 in HCC progression through its regulation of LMNA expression via SUMOylation and ubiquitination pathways. In HCC cells, elevated UBC9 expression promotes SUMOylation of LMNA, which blocks its ubiquitination at K144, leading to markedly increased LMNA stability. This UBC9-mediated regulation of LMNA contributes to enhanced cell proliferation, migration, and invasion in HCC, ultimately promoting tumor progression (Fig. 8). The overexpression of UBC9 and LMNA in HCC correlates with dismal clinical outcomes, emphasizing their significance as prognostic markers and therapeutic targets. The interplay between SUMOylation and ubiquitination in controlling LMNA stability underscores the complexity of PTM networks in cancer biology. Future studies should focus on developing targeted therapies that disrupt the UBC9-LMNA axis and explore the therapeutic potential of modulating SUMOylation in HCC.

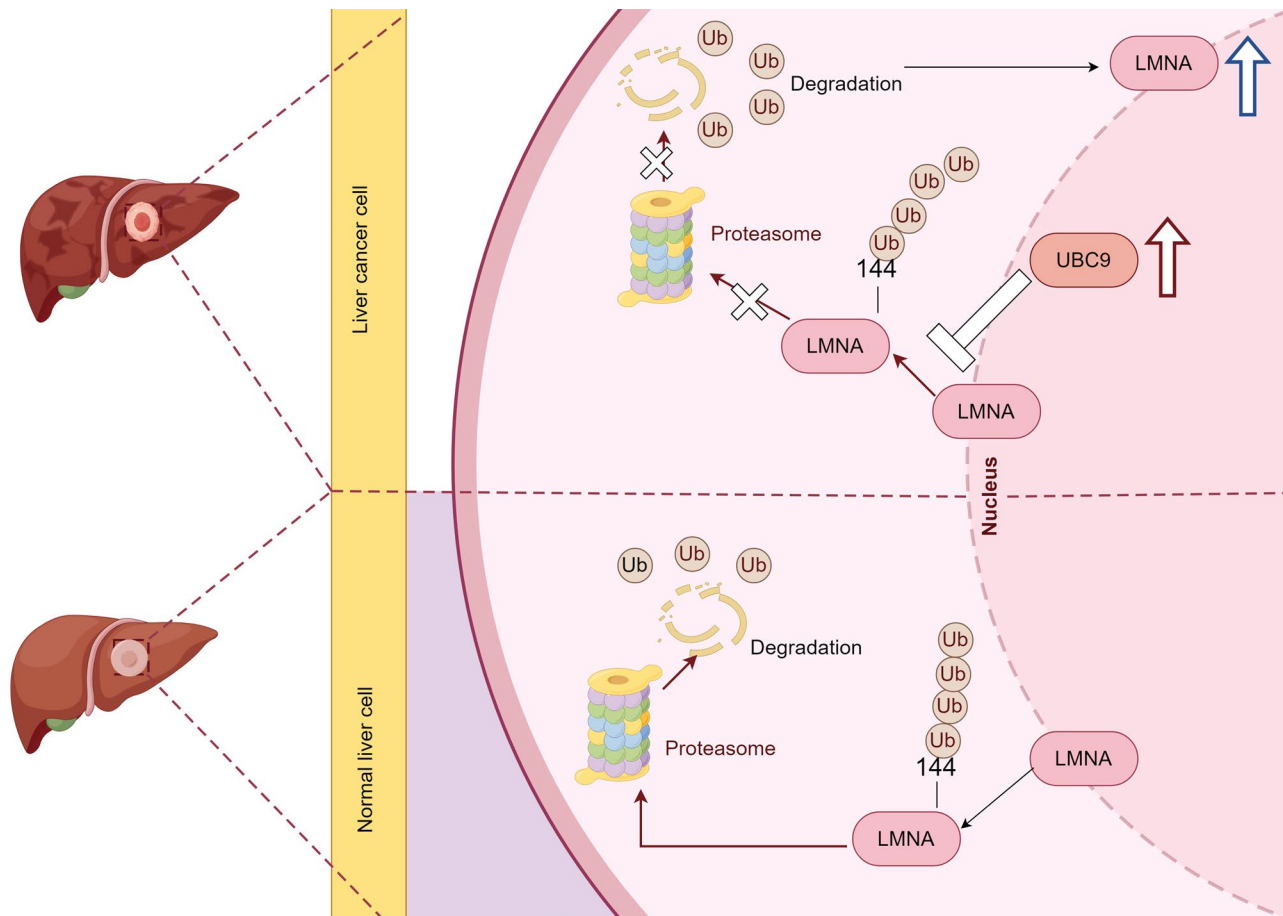


Fig. 8 Schematic illustration of the UBC9-LMNA regulatory axis in hepatocellular cell carcinoma. In normal liver cells (bottom), LMNA undergoes regular ubiquitination at K144 and subsequent proteasomal degradation. In liver cancer cells (top), elevated UBC9 expression promotes SUMOylation of LMNA, which inhibits its ubiquitination at K144, leading to increased LMNA stability and expression. This UBC9-mediated regulation of LMNA contributes to enhanced cell proliferation, migration, and invasion in HCC, ultimately promoting tumor progression and metastasis

Supplementary information

The online version contains supplementary material available at <https://doi.org/10.1186/s12967-026-07722-0>.

Supplementary material 1

Supplementary material 2

Supplementary material 3

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Author contributions

QingShui Wang, Ziqiang Liao, Zhuoqin Zhong, Hao Zhang, Ziyi Jin, Xinji Gao, and YiMin Huang carried out the bioinformatic analyses. Menghua Qiu, Wudan Ren, Yunjia Liu, Liyan Zhang, Runjing Fan and Yaqian Feng conducted the experiments. QingShui Wang, Kunmu Zhang, FangQin Xue, Yehuda G. Assaraf, and Yao Lin supervised the work, wrote the manuscript and revised it.

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Data availability

The results of the differential analysis of the Proteomics and Ubiquitin Proteomics are in the supplementary table.

Declarations

Ethics approval and consent to participate

NA.

Conflict of interest

The authors declare no conflict of interest for this article.

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