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LAPTM4B enhances the stemness of CD133$^+$ liver cancer stem-like cells via WNT/β-catenin signaling

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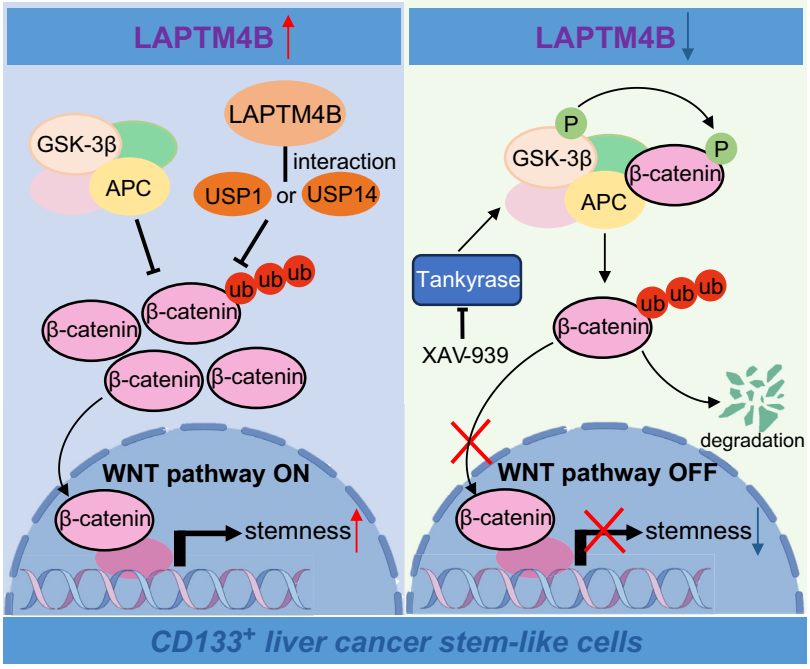
Graphical abstract



Highlights

- LAPTM4B enhances the stemness of CD133$^+$ liver CSLCs.
- LAPTM4B promotes CD133$^+$ CSLC stemness by activating WNT/β-catenin signaling.
- LAPTM4B inhibits β-catenin phosphorylation and ubiquitin-mediated degradation.
- High levels of LAPTM4B and β-catenin predict poor prognoses in patients with HCC.

Impact and implications

LAPTM4B contributes significantly to CD133$^+$ CSLC stemness and inhibits β-catenin phosphorylation and ubiquitination degradation, activating WNT/β-catenin signaling. WNT inhibitors suppress LAPTM4B-induced CD133$^+$ CSLC stemness. Thus, targeting the LAPTM4B–WNT/β-catenin axis could improve antitumor efficacy.

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LAPTM4B enhances the stemness of CD133$^+$ liver cancer stem-like cells via WNT/β-catenin signaling

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Background & Aims: Lysosome-associated protein transmembrane 4β (LAPTM4B) is an oncogene implicated in the malignant progression of hepatocellular carcinoma (HCC). Previous research established a strong association between LAPTM4B and HCC stemness. However, specific mechanisms by which LAPTM4B regulates and maintains the stemness of liver cancer stem cells remain unclear. Therefore, we investigated the effects of LAPTM4B on the stemness regulation of cluster of differentiation 133 (CD133)$^+$ liver cancer stem-like cells (CSLCs).

Methods: We used RNA interference and overexpression techniques in both *in vitro* and *in vivo* models. The involvement of LAPTM4B in wingless/integrated (WNT)/β-catenin signaling was examined through western blotting, immunofluorescence, and immunoprecipitation. The impact of LAPTM4B on β-catenin phosphorylation and ubiquitination was analyzed to elucidate its role in promoting stemness. Clinical relevance was evaluated in an in-house cohort of 105 specimens from patients with HCC through immunohistochemical and microarray analysis, enabling investigation of correlations with clinical outcomes.

Results: LAPTM4B promoted the self-renewal ability, chemoresistance, and tumorigenicity of CD133$^+$ CSLCs. Mechanistically, aberrant LAPTM4B upregulation facilitated β-catenin nuclear translocation (nucleocytoplasmic separation assay, p <0.001) and inhibited its phosphorylation (p <0.01). In addition, LAPTM4B interacts with the deubiquitinating enzymes ubiquitin carboxyl-terminal hydrolase (USP)-1 and USP14, reducing β-catenin ubiquitination. Furthermore, patients with high LAPTM4B and β-catenin expression had markedly shorter 3-year overall survival rate (42.9% *vs.* 74.4%; hazard ratio, 5.174; 95% CI 2.280–11.741, p <0.001).

Conclusions: LAPTM4B promotes CD133$^+$ CSLC stemness by activating WNT/β-catenin signaling by inhibiting β-catenin phosphorylation and ubiquitination degradation. The role of LAPTM4B in regulating WNT/β-catenin signaling suggests that LAPTM4B serves as a therapeutic target for impairing HCC stemness and progression.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most frequent malignant tumor worldwide, posing a significant threat to human life.[1] Liver cancer stem cells, a distinct subset of HCC cells with stem cell features, are a fundamental cause of tumor heterogeneity and contribute to metastasis, recurrence, and drug resistance.[2,3]

Lysosome-associated protein transmembrane-4β (LAPTM4B) is markedly upregulated in poorly differentiated HCC tissues.[4] It primarily mediates cell signal transduction, intracellular protein localization and isolation,[5] and protein degradation, especially ubiquitin degradation.[6] Overexpression of LAPTM4B can result in uncontrolled cell proliferation and malignant transformation, suggesting its role as an oncogene in HCC development.[7] Our previous single cell RNA-sequencing (RNA-seq) study identified an association between LAPTM4B and HCC stemness.[8] We sorted cluster of differentiation 133 (CD133)-positive liver cancer stem-like cells (CSLCs), which are highly heterogeneous and exhibit stem cells traits, from HCC cells. CD133, a critical surface protein marker, is widely recognized for its functional and phenotypic significance in liver cancer stem cells.[9,10] Subsequently, we further explored the functional role and regulatory mechanism of LAPTM4B in CD133$^+$ CSLCs.

Cancer stem cells maintain their stemness through multiple pathways, with wingless/integrated (WNT)/β-catenin signaling as the most commonly activated pathway in HCC.[11] With evolutionarily high conservatism, the WNT/β-catenin pathway supports tumor growth, metastasis, recurrence, and radiochemotherapy resistance.[12–14] The destruction complex comprising adenomatous polyposis coli (APC), axis inhibition protein, casein kinase 1, and glycogen synthase kinase 3β (GSK-3β) regulates β-catenin levels and nuclear translocation.[15] When the WNT/β-catenin pathway is activated, β-catenin is released from the destruction complex, and avoids being phosphorylated and targeted for ubiquitin-mediated

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proteasomal degradation. It then accumulates in the cytoplasm before moving into the nucleus to trigger the transcription and expression of target genes, such as those encoding cellular myelocytomatosis (c-MYC), Cyclin D1, and matrix metalloproteinase 7 (MMP7).[16–19]

Inhibiting the stemness of HCC cells could provide new therapeutic strategies for HCC treatment. However, the mechanisms that regulate and maintain stem cell characteristics remain unclear. Therefore, an in-depth exploration of these molecular mechanisms and their regulatory networks is essential for improving HCC treatment and prognosis. Thus, we investigated the effect of LAPTM4B on the stemness regulation of CD133+ CSLCs. These findings provide potential insight into LAPTM4B-mediated stemness, the mechanisms of action of the WNT/β-catenin pathway, and its function in cancer progression.

Material and methods

We refer readers to the supplementary data for further details of: HCC cell lines and culture; plasmids, lentiviruses, and experimental protocol for cell transfection; isolation of CD133+ CSLCs; microsphere cultures and sphere-forming assays; RNA-seq; immunofluorescence (IF) staining; construction of the xenograft tumor model; and selection of human specimens and immunohistochemical (IHC) staining.

Co-immunoprecipitation assays

According to the manufacturer's (Beyotime Biotech, Shanghai, China) instructions, cells were lysed with lysis buffer, and the whole-cell extracts were supplemented with the protease inhibitor PMSF. The lysates were then incubated with 2–5 ug IP antibodies (Table S5) overnight at 4 °C on a rotating wheel. IgG was used as the negative control in each experiment. The complexes were incubated with protein A/G magnetic beads (HY-K0202, MedChemExpress) at 4 °C. The precipitates were washed six times with ice-cold wash buffer, eluted with 0.1 M glycine-HCl (pH 3.0) buffer, and resolved by SDS-PAGE followed by western blot analysis with appropriate antibodies (Table S5).

Protein stability experiment

To measure the half-life of β-catenin, cells were treated with 100 μg/ml protein synthesis inhibitor cycloheximide (HY12320; MedChemExpress) for 0 h, 1 h, 4 h, 8 h, 12 h, and 24 h. Western blotting was performed to measure protein levels. LAPTM4B-depleted or -overexpressing cells and control cells were incubated with 10 μM MG132 (HY13259; MedChemExpress) for 12 h. Following this treatment, total protein was extracted for western blot analysis.

Ubiquitination assay

To detect ubiquitination of β-catenin, cells were incubated with MG132 (10 μM) for 8 h and then lysed by RIPA buffer. Proteins in the cell lysate were immunoprecipitated to isolate ubiquitinated β-catenin with anti-β-catenin antibodies and the ubiquitination level of β-catenin were detected through western blotting with antibodies against ubiquitin.

Statistical analysis

Statistical analysis was conducted using SPSS software version 22.0 (SPSS, Chicago, IL, USA). Data are given as mean ± SD. The Student's *t* test was used to assess differences between groups, and one-way ANOVA was used to assess variations between multiple groups. Clinical data were examined using Fisher's exact test. The Kaplan-Meier method was used to determine survival rates, with significance assessed using the Log-rank test. Pearson correlation was used to assess the relationship between gene expression levels. Regression analysis was estimated using the Cox proportional hazards model after confirmation of the proportional hazard assumption and described by hazard ratios (HR) and 95% CIs. $p<0.05$ indicated statistical significance.

Results

LAPTM4B regulates stemness in CD133+ CSLCs

Previous research demonstrated that LAPTM4B enhances invasion, metastasis, and tumor initiation in HCC.[8,20] Here, we established LAPTM4B-overexpressing Huh7 and HepG2 cells, achieving over a threefold increase in LAPTM4B expression (Fig. S1A,B). Intriguingly, IF analysis indicated that LAPTM4B overexpression increased the proportion of CD133-positive cells (a key stem cell marker in HCC[21,22]) (Fig. S1C–F), as also evidenced by flow cytometry (Fig. S1G,H). In addition, LAPTM4B expression was higher in CD133+ cells than in CD133− cells (Fig. S1I,J). IHC analysis of 105 HCC specimens with long-term clinical follow-up data revealed a positive association between LAPTM4B levels and CD133 expression (Fig. S1K,L). These findings prompted us to investigate the role of LAPTM4B in regulating CD133+ CSLC stemness. Using immunomagnetic sorting, we isolated CD133+ CSLCs from Huh7 and HepG2 cells, achieving high purity confirmed by flow cytometry (Fig. S2A,B). Sphere-formation assays demonstrated that isolated CD133+ CSLCs from both cell lines displayed excellent sphere-forming capacity (Fig. S2C–F). Western blotting revealed that expression of CD133, EpCAM, SOX9, OCT4, and NANOG was higher in CD133+ CSLCs than in CD133− tumor cells (Fig. S2G,H). These results indicated a stem-like phenotype in CD133+ CSLCs isolated from HCC cells.

Subsequently, we intervened in LAPTM4B expression in CD133+ CSLCs and observed that LAPTM4B knockdown reduced expression by over 60% (Fig. S3A,B). Reduced sphere-forming ability of CD133+ CSLCs was also observed with LAPTM4B knockdown (Fig. 1A,B). Conversely, overexpression enhanced sphere formation (Fig. S4A–D). LAPTM4B suppression significantly reduced several genes typically associated with stemness, including *SOX9*, *OCT4*, and *NANOG*, as well as a stem cell surface marker, *EpCAM* (Fig. 1C–F), whereas overexpression increased these markers (Fig. S4E–H).

Given that cancer stem cells often exhibit drug resistance and evade treatments through mechanisms such as the epithelial-to-mesenchymal transition (EMT),[23,24] we investigated EMT markers in LAPTM4B-altered CSLCs. LAPTM4B knockdown upregulated the epithelial marker E-cadherin and reduced the mesenchymal markers N-cadherin, vimentin, and snail2 (Fig. 1G,H), whereas overexpressed LAPTM4B induced EMT in CD133+ CSLCs (Fig. S4I,J). To determine the impact of

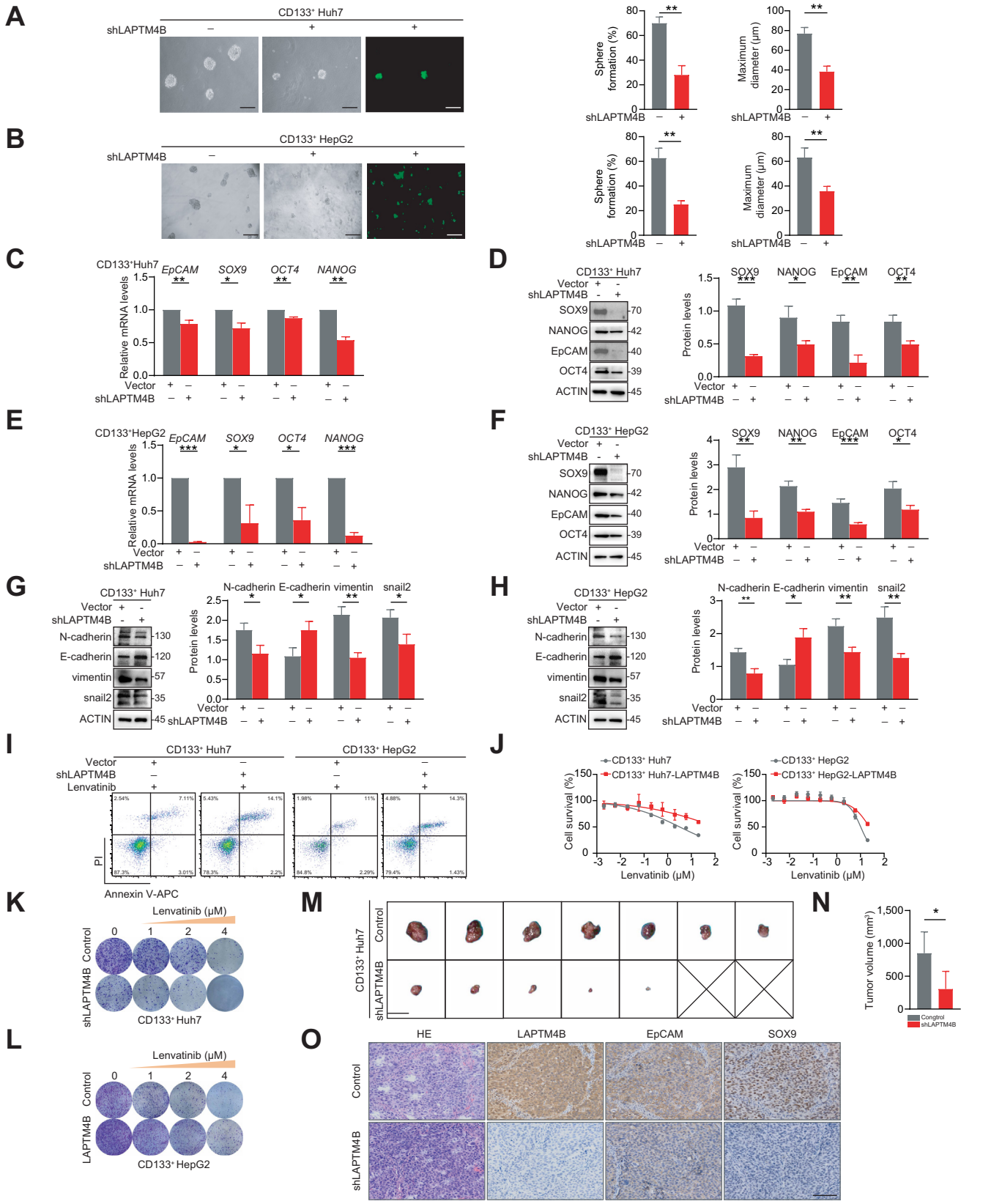

Fig. 1. LAPTM4B enhances CD133$^+$ CSLC stemness. (A,B) Effect of LAPTM4B-knockdown on sphere-forming ability of CD133$^+$ Huh7 and CD133$^+$ HepG2 cells on day 5. (C–F) qPCR and western blot analysis of *EpCAM*, *SOX9*, *OCT4* and *NANOG* expression in CD133$^+$ Huh7 and CD133$^+$ HepG2 cells after LAPTM4B knockdown. (G,H) Effect of LAPTM4B knockdown on EMT in CD133$^+$ Huh7 and CD133$^+$ HepG2 cells analyzed by western blotting. (I) Effect of silencing LAPTM4B on CD133$^+$ Huh7 and CD133$^+$ HepG2 cells apoptosis in presence of lenvatinib (10 μM, 72 h). (J) Survival curves of CD133$^+$ Huh7/CD133$^+$ HepG2 LAPTM4B-overexpressing and control cells treated with lenvatinib for 72 h. (K,L) Colony formation assay to detect CD133$^+$ Huh7/CD133$^+$ HepG2 proliferation with LAPTM4B knockdown or overexpression in

LAPTM4B on the drug resistance of CD133+ CSLCs, we examined the apoptosis and proliferation of CD133+ CSLCs in the presence of lenvatinib. LAPTM4B knockdown increased sensitivity to lenvatinib-induced apoptosis (Fig. 1I; Fig. S3C), whereas overexpression conferred resistance (Fig. S4K,L) and protected against lenvatinib-induced growth inhibition (Fig. 1J–L). Furthermore, we explored whether LAPTM4B promotes the tumor-forming ability of cells by injecting CD133+ CSLCs subcutaneously with or without LAPTM4B knockdown in nude mice. The tumor volume of mice injected with LAPTM4B-knockdown CD133+ Huh7 cells was significantly reduced compared with that in the control group (Fig. 1M,N). Limiting dilution assays further showed that LAPTM4B overexpression enhanced tumor formation *in vivo* (Fig. S4M). Correspondingly, IHC staining of the tumor tissues revealed lower expression of stemness markers in LAPTM4B-knockdown tumors (Fig. 1O). The above results revealed that LAPTM4B has an essential role in promoting CD133+ CSLC stemness in HCC.

LAPTM4B activates the WNT/β-catenin pathway in CD133+ CSLCs

We analyzed the expression profiles of LAPTM4B-silence or control CD133+ Huh7 cells to gain a broader understanding of the changes in LAPTM4B-mediated genes and their effects on the CD133+ CSLC phenotype. Kyoto Encyclopedia of Genes and Genomes pathway enrichment and Gene Ontology functional category enrichment analyses showed that the regulation of CD133+ Huh7 cell stemness by LAPTM4B involved multiple biological processes, including G2/M transition of the mitotic cell cycle, positive regulation related to cell morphology differentiation, WNT signaling pathway, and protein dephosphorylation (Fig. 2A). Given that hyperactivation of WNT/β-catenin signaling significantly contributes to liver cancer stem cell stemness,[25,26] we focused on the regulation of WNT/β-catenin signaling by LAPTM4B. We observed increased mRNA levels of *GSK-3β* and *APC* in CD133+ Huh7 and CD133+ HepG2 cells following LAPTM4B knockdown, whereas c-Myc and Cyclin D1 decreased (Fig. 2B,C). The protein level of GSK-3β was elevated, whereas the expression of p-GSK-3β, c-Myc, and Cyclin D1 declined when LAPTM4B was silenced (Fig. 2D,E). Conversely, overexpressing LAPTM4B resulted in opposite effects (Fig. S5A–D). Nuclear–cytoplasmic shuttling of β-catenin is a pivotal step in WNT/β-catenin pathway activation.[27] As expected, nucleocytoplasmic separation assays and IF indicated enrichment of β-catenin nuclear localization in LAPTM4B-overexpressing CD133+ CSLCs (Fig. 2F–I) and also at the organizational level (Fig. 2J,K). These observations demonstrated that LAPTM4B activated the WNT/β-catenin pathway in CD133+ CSLCs.

LAPTM4B stabilizes β-catenin by inhibiting its degradation

Phosphorylated β-catenin (Thr41/Ser45) undergoes ubiquitination degradation, resulting in a closed WNT/β-catenin pathway.[28] We observed that β-catenin decreased at both the mRNA and protein levels in LAPTM4B-deficient CD133+ CSLCs, whereas the phosphorylated β-catenin (Thr41/Ser45) protein level increased (Fig. 3A–D). The opposite results were achieved by overexpressing LAPTM4B (Fig. S5E–H). This result validated the robustness of the RNA-seq results (Fig. 2A) and further suggested that LAPTM4B promotes WNT signaling activation by modulating phosphorylation of β-catenin.

Previous studies have shown that LAPTM4B inhibits ubiquitination, which prevents protein degradation,[6,8] and that ubiquitinated degradation causes poor stability of β-catenin in the cytoplasm.[15] Thus, we hypothesized that LAPTM4B regulates β-catenin protein stability by affecting its ubiquitinated degradation. Cycloheximide (CHX) chase assays were conducted to identify the underlying molecular mechanism of the role of LAPTM4B in β-catenin stability. With increased CHX treatment time, the degradation of β-catenin increased and the half-life of β-catenin decreased in LAPTM4B-depleted cells (Fig. 3E,F). In addition, LAPTM4B knockdown attenuated the inhibitory effects of the proteasome inhibitor MG132 on β-catenin degradation (Fig. 3G). MG132 was administered to CD133+ Huh7 cells to further validate the role of LAPTM4B in inhibiting the ubiquitination and subsequent degradation of β-catenin. Ubiquitination assays demonstrated that LAPTM4B knockdown increased ubiquitinated β-catenin in CD133+ Huh7 cells (Fig. 3H). By contrast, LAPTM4B overexpression prolonged the half-life of β-catenin in CD133+ HepG2 cells (Fig. 3I,J). Furthermore, LAPTM4B overexpression enhanced the inhibitory effect of MG132 on β-catenin degradation (Fig. 3K). The ubiquitination assays confirmed that β-catenin protein ubiquitination was significantly reduced in LAPTM4B-overexpressing cells (Fig. 3L). These outcomes showed that LAPTM4B promotes β-catenin stability by inhibiting its ubiquitin–proteasome pathway degradation.

LAPTM4B inhibits β-catenin degradation by binding to USP1 and USP14

We demonstrated that LAPTM4B promotes β-catenin stability by inhibiting its ubiquitination. Here we used three side-by-side datasets: the deubiquitinating enzyme dataset, the gene set co-expressed with LAPTM4B and β-catenin from the TCGA database, and the gene set interacting with CTNNB1 from the STRING database. We found that the deubiquitinating enzymes ubiquitin carboxyl-terminal hydrolase (USP)-1 and USP14 are involved in the inhibition of β-catenin ubiquitination degradation by LAPTM4B (Fig. 4A). The Gene Expression Profiling Interactive Analysis (GEPIA) platform online analysis identified a significant positive correlation between LAPTM4B expression and both USP1 and USP14 in HCC (Fig. 4B). The UbiBrowser database highlighted USP1 as a predicted and USP14 as a known deubiquitinating enzyme for β-catenin (Fig. 4C). Co-immunoprecipitation (Co-IP) assays confirmed the interaction between LAPTM4B and USP1 (Fig. 4D). In ubiquitination

presence of lenvatinib. (M,N) Subcutaneous xenograft experiment evaluating tumor-formation capability of LAPTM4B-knockdown CD133+ Huh7 cells (n = 7 per group). (O) Immunohistochemical staining of LAPTM4B, EpCAM, and SOX9 in LAPTM4B-knockdown and control cells from xenografted tumors. Data are means ± SD for at least 2 independent experiments, analyzed with unpaired Student's *t* tests: **p* <0.05; ***p* <0.01; ****p* <0.001. Scale bars: 200 μm (A,B), 100 μm (O), 1.5 cm (M,N). See also Figs S1–S4. CD133, cluster of differentiation 133; CSLC, cancer stem-like cell; EMT, epithelial–mesenchymal transition; LAPTM4B, lysosome-associated protein transmembrane-4β; qPCR, quantitative real-time PCR.

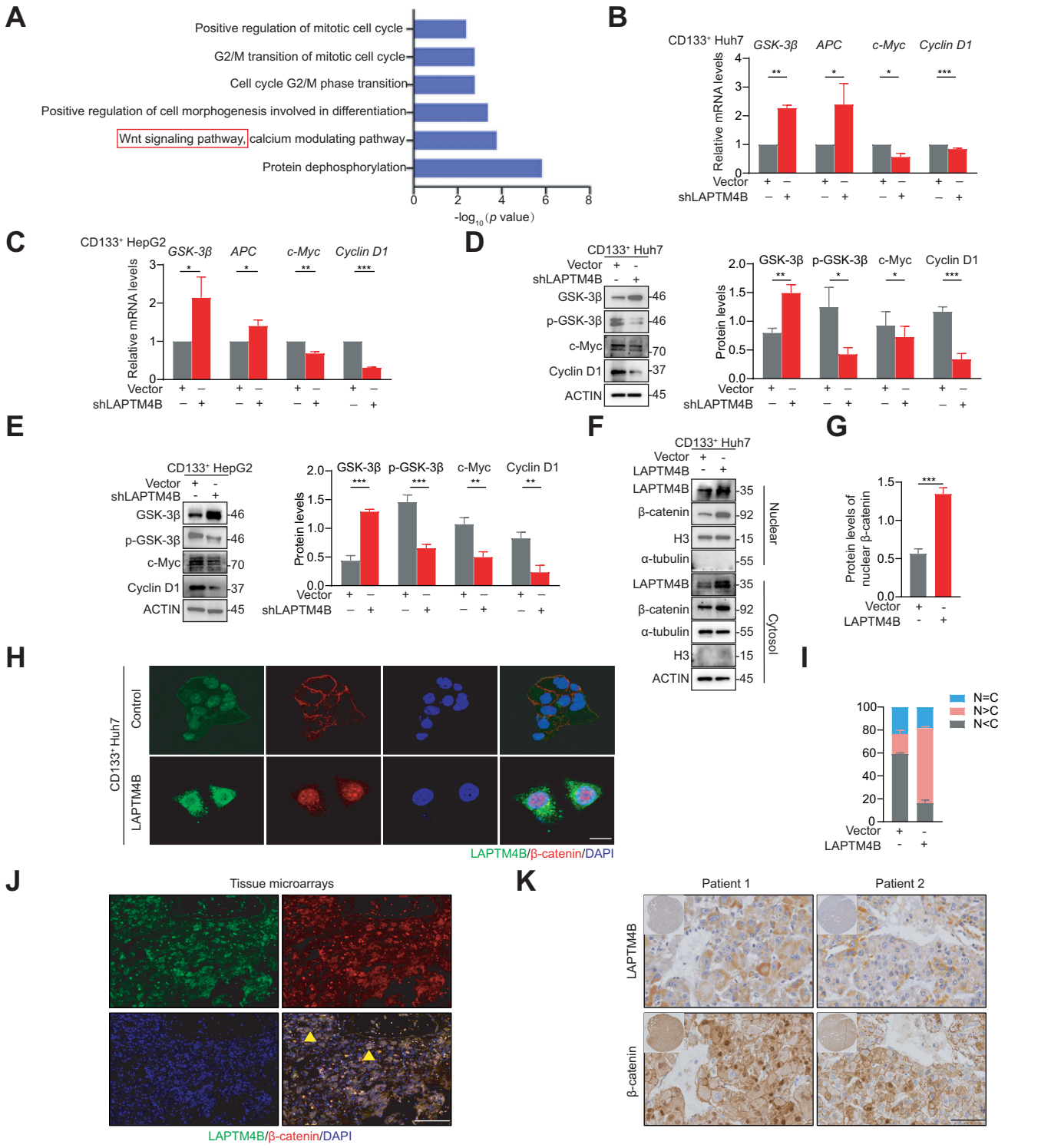

Fig. 2. LAPTM4B affects WNT/β-catenin pathway activation. (A) Pathway enrichment analysis in LAPTM4B-knockdown CD133$^+$ Huh7 or control cells identified by RNA sequencing. (B,C) GSK-3β, APC, c-Myc and Cyclin D1 expression in CD133$^+$ Huh7 and CD133$^+$ HepG2 cells with LAPTM4B knockdown. (D,E) Western blots of GSK-3β, p-GSK-3β, c-Myc, and Cyclin D1 expression in CD133$^+$ Huh7 and CD133$^+$ HepG2 cells when silencing LAPTM4B. (F) Western blots of nucleocytoplasmic distribution of β-catenin in LAPTM4B-overexpressing CD133$^+$ Huh7 cells. (G) Relative protein levels of nuclear β-catenin in CD133$^+$ Huh7 cells overexpressing LAPTM4B or not. (H,I) Immunofluorescence of nuclear translocation of β-catenin. (J,K) Colocalization of LAPTM4B and β-catenin in HCC examined by immunofluorescence and immunohistochemistry. Data are means ± SD for at least 2 independent experiments, analyzed with unpaired Student's *t* tests: **p* <0.05; ***p* <0.01; ****p* <0.001. Scale bars: 100 μm (J,K); 25 μm (H,I). See also Fig. S5. APC, adenomatous polyposis coli; c-MYC, cellular myelocytomatosis; CD133, cluster of differentiation 133; GSK-3β, glycogen synthase 3β; HCC, hepatocellular carcinoma; LAPTM4B, lysosome-associated protein transmembrane-4β; p-GSK-3β, phosphorylated glycogen synthase 3β; qPCR, quantitative real-time PCR.

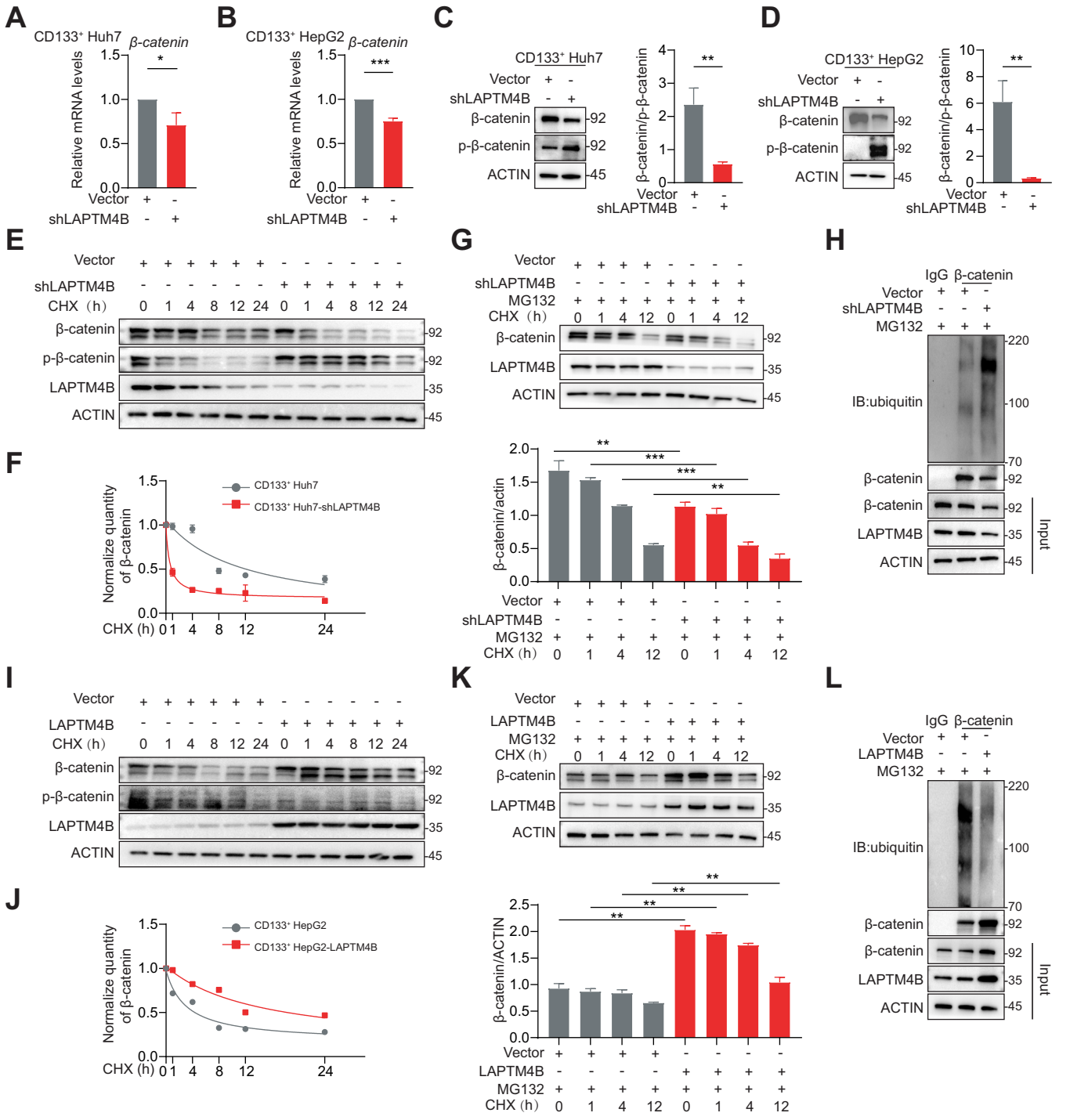

Fig. 3. Effect of LAPTM4B on β-catenin degradation. (A,B) qPCR detection of β-catenin expression in CD133⁺ Huh7 and CD133⁺ HepG2 cells following silencing of LAPTM4B. (C,D) Western blots of β-catenin and p-β-catenin expression in CD133⁺ Huh7 and CD133⁺ HepG2 cells following LAPTM4B knockdown. (E) β-catenin and p-β-catenin protein levels in LAPTM4B-knockdown CD133⁺ Huh7 cells after CHX treatment. (F) Half-life of β-catenin in LAPTM4B-knockdown CD133⁺ Huh7 cells after CHX treatment. (G) Western blots of β-catenin levels in CD133⁺ Huh7 cells with LAPTM4B knockdown after treatment with CHX and MG132. (H) Western blot *in vitro* ubiquitination assay of β-catenin in CD133⁺ Huh7 cells with LAPTM4B knockdown after MG132 treatment. (I) β-catenin and p-β-catenin levels in LAPTM4B-overexpressing CD133⁺ HepG2 cells after CHX treatment. (J) Half-life of β-catenin in LAPTM4B-overexpressing CD133⁺ HepG2 cells after CHX treatment. (K) Western blots of β-catenin levels in LAPTM4B-overexpressing CD133⁺ HepG2 cells after CHX and MG132 treatment. (L) Western blot *in vitro* ubiquitination assay of β-catenin in LAPTM4B-overexpressing CD133⁺ HepG2 cells after MG132 treatment. Data are means ± SD for at least two independent experiments, analyzed with unpaired Student's *t* tests: **p* <0.05; ***p* <0.01; ****p* <0.001. CD133, cluster of differentiation 133; CHX, cycloheximide; LAPTM4B, lysosome-associated protein transmembrane-4β; qPCR, quantitative real-time PCR.

assays, LAPTM4B overexpression decreased β-catenin ubiquitination, whereas knockdown of USP1 reversed the inhibitory effect of LAPTM4B on β-catenin ubiquitinated-degradation (Fig. 4E). Similar results were observed between LAPTM4B and USP14 (Fig. 4F,G). Meanwhile, Co-IP assays using anti-β-catenin antibodies reconfirmed that LAPTM4B interacted with β-catenin, USP1, and USP14.

Given that LAPTM4B might inhibit β-catenin ubiquitination degradation by assisting USP1 and USP14 in binding to β-catenin (Fig. S6A), we overexpressed various LAPTM4B

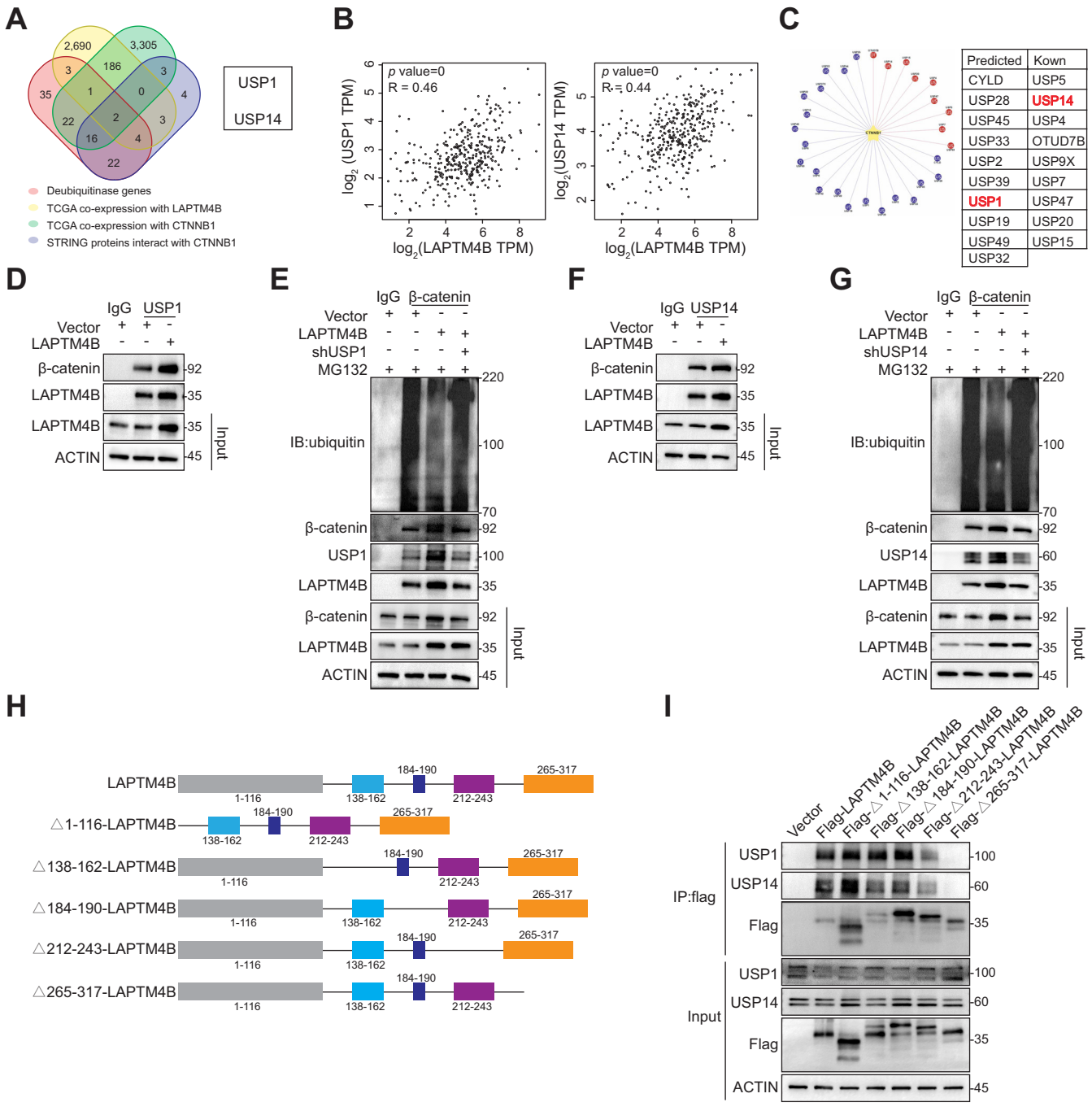

Fig. 4. LAPTM4B and USP1/USP14 interaction inhibit β-catenin ubiquitinated degradation. (A) Comprehensive analysis combining the deubiquitinating enzyme dataset, genes co-expressed with LAPTM4B/β-catenin from the TCGA database, and genes interacting with *CTNNB1* in the STRING database. (B) Pearson's correlation analysis of the association between LAPTM4B and USP1/USP14 expression in patients with HCC from the TCGA database. (C) Predicted and known deubiquitinating enzymes of β-catenin from the UbiBrowser database. (D) Co-IP of the interaction between LAPTM4B and USP1. (E) β-catenin in CD133^{+} Huh7 cells with LAPTM4B-overexpression and USP1-knockdown after MG132 treatment used western blotting in vitro ubiquitination assay. (F) Co-IP of the interaction between LAPTM4B and USP14. (G) Western blot *in vitro* ubiquitination assay of β-catenin in CD133^{+} Huh7 cells with LAPTM4B overexpression and USP14 knockdown after MG132 treatment. (H) Schematic of LAPTM4B protein truncation. (I) Co-IP assay using anti-Flag antibodies with truncated LAPTM4B overexpression. See also Fig. S6. Co-IP, co-immunoprecipitation; LAPTM4B, lysosome-associated protein transmembrane-4β; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; TCGA, The Cancer Genome Atlas; USP, ubiquitin carboxyl-terminal hydrolase.

truncations in CD133+ Huh7 cells to further clarify the interaction domain of LAPTM4B with USP1 and USP14. The interaction was disrupted only in cells overexpressing the FlagΔ265-317-LAPTM4B truncation (Fig. 4H,I). These findings suggest that LAPTM4B inhibits β-catenin ubiquitination and stabilizes it through interactions with USP1 or USP14 via its 265–317 structural domain.

LAPTM4B promotes the stemness of CD133+ CSLCs via WNT/β-catenin signaling

The above results indicated that LAPTM4B enhances the stability of β-catenin by suppressing its phosphorylation and ubiquitination-mediated degradation. Therefore, we investigated the crucial involvement of WNT/β-catenin signaling in the modulation of CD133+ CSLC stemness by LAPTM4B. Sphere-forming assays showed that LAPTM4B deficiency reduced the formation of spheres in CD133+ Huh7 cells, which was partially rescued by β-catenin overexpression (Fig. 5A,B). Conversely, LAPTM4B upregulation facilitated the forming of spheres, whereas β-catenin suppression prevented LAPTM4B-induced sphere formation in CD133+ Huh7 cells (Fig. S7A,B). Similarly, the sphericity of CD133+ HepG2 cells decreased after knocking down LAPTM4B, but improved when using LiCl (a WNT/β-catenin pathway activator[29,30]) (Fig. 5C,D). The expression of stemness-related genes, *EpCAM*, *SOX9*, *OCT4*, and *NANOG* decreased when LAPTM4B was silenced, whereas β-catenin overexpression reversed this effect in CD133+ Huh7 cells (Fig. 5E,F). However, overexpressing LAPTM4B increased the expression of these genes, which was suppressed by depleting β-catenin (Fig. S7C,D). Likewise, LiCl prevented the downregulation of *EpCAM*, *SOX9*, *OCT4*, and *NANOG* caused by LAPTM4B knockdown in CD133+ HepG2 cells (Fig. 5G,H). Moreover, overexpression of LAPTM4B reduced apoptosis in response to lenvatinib treatment, whereas subsequent β-catenin knockdown significantly increased apoptosis in CD133+ Huh7 and CD133+ HepG2 cells (Fig. 5I,J). Consistent with the above findings, LAPTM4B overexpression led to CD133+ Huh7 cells resistant to lenvatinib-induced growth inhibition. However, this resistance was substantially diminished following β-catenin knockdown post-LAPTM4B overexpression (Fig. 5K).

We further investigated the effects of WNT/β-catenin signaling on the regulation of HCC stemness by LAPTM4B *in vivo*. Mice were inoculated subcutaneously with CD133+ Huh7-Vector, CD133+ Huh7-shLAPTM4B, or CD133+ Huh7-shLAPTM4B-CTNNB1 cells (5×10^6 cells per mouse). The tumor volume in the mice injected with the CD133+ Huh7-shLAPTM4B cells was dramatically reduced compared with that in the vector group. However, restoration of β-catenin expression led to an increase in tumorigenicity (Fig. 5L). IHC staining of the tumor tissues verified the correlation between LAPTM4B, β-catenin, and the levels of stemness-related genes *in vivo* (Fig. 5M). In addition, limiting dilution subcutaneous xenografts indicated that LAPTM4B overexpression enhanced the capacity of tumor formation in CD133+ Huh7 cells, whereas silencing β-catenin counteracted the tumor-promoting effects of LAPTM4B (Fig. S7E). These observations indicated that LAPTM4B enhances the stemness of CD133+ CSLCs through WNT/β-catenin signaling.

WNT inhibition suppresses LAPTM4B-induced CD133+ CSLC stemness activation

XAV-939 (a WNT/β-catenin signaling inhibitor[30–32]) was added during the suspension culture of CD133+ CSLCs overexpressing LAPTM4B to preliminarily investigate the potential therapeutic effectiveness of targeting the WNT/β-catenin pathway in inhibiting LAPTM4B-induced HCC with high stemness characteristics. As previously described, LAPTM4B overexpression improved the sphere-forming ability of CD133+ CSLCs, whereas XAV-939 attenuated it. The sphere-forming capacity of CD133+ CSLCs overexpressing LAPTM4B was reduced but not significantly so after XAV-939 treatment (Fig. 6A,B). The expression of β-catenin decreased under the action of XAV-939, whereas the addition of XAV-939 to the LAPTM4B-overexpressing group facilitated higher levels of β-catenin compared with the non-LAPTM4B-overexpressing group (Fig. 6C,D). Moreover, the expression of the stemness-related genes *EpCAM*, *SOX9*, *OCT4*, and *NANOG* was downregulated under XAV-939, although overexpression of LAPTM4B prevented this inhibitory effect to some extent (Fig. 6E–G). These results suggest that the WNT/β-catenin pathway is crucial for LAPTM4B-regulated stemness in CD133+ CSLCs and lays the foundation for considering the LAPTM4B-WNT/β-catenin axis as a potential therapeutic target for the treatment of HCC.

Abnormally high expression of LAPTM4B and β-catenin in patients with HCC confers a poor prognosis

According to the above results, LAPTM4B overexpression increases β-catenin stability by suppressing its degradation. Furthermore, the activity of WNT/β-catenin signaling is required for LAPTM4B-induced CD133+ CSLC stemness. Thus, we analyzed their expression patterns in surgical samples obtained from patients with HCC to additionally investigate the association between LAPTM4B and β-catenin in HCC and their potential implications for clinical prognosis.

The TCGA database was used to identify the mRNA levels of LAPTM4B and β-catenin, between which there was a positive correlation ($R = 0.18$; $p = 0.0004$) (Fig. 7A). IHC analysis revealed that elevated LAPTM4B protein levels were associated with the accumulation of β-catenin in the nucleus in tissue microarrays (TMAs) comprising 105 HCC specimens from a local cohort with long-term clinical follow-up data (Fig. 7B,C). A positive correlation between LAPTM4B and β-catenin was observed ($p < 0.05$; Fig. 7D; Table S1).

In addition, we obtained and extracted eight clinical factors from clinical follow-up records relating to the clinical background of the patients (sex, age, presence of HBV, and cirrhosis) and information about cancer stage (tumor size, microvascular invasion, Edmondson classification, and microsatellites). We found that increased LAPTM4B was only associated with tumor size and Edmondson classification ($p < 0.05$, Table S2).

Subsequently, a Kaplan-Meier analysis was conducted to assess the correlation between LAPTM4B and β-catenin expression levels and the overall survival of patients. The findings indicated a significant relationship between elevated LAPTM4B/β-catenin levels and an unfavorable prognosis ($p = 0.0026$ and $p = 0.0388$, respectively) (Fig. 7E,F). Furthermore, individuals with heightened LAPTM4B and β-catenin expression exhibited reduced survival time, whereas those with lower

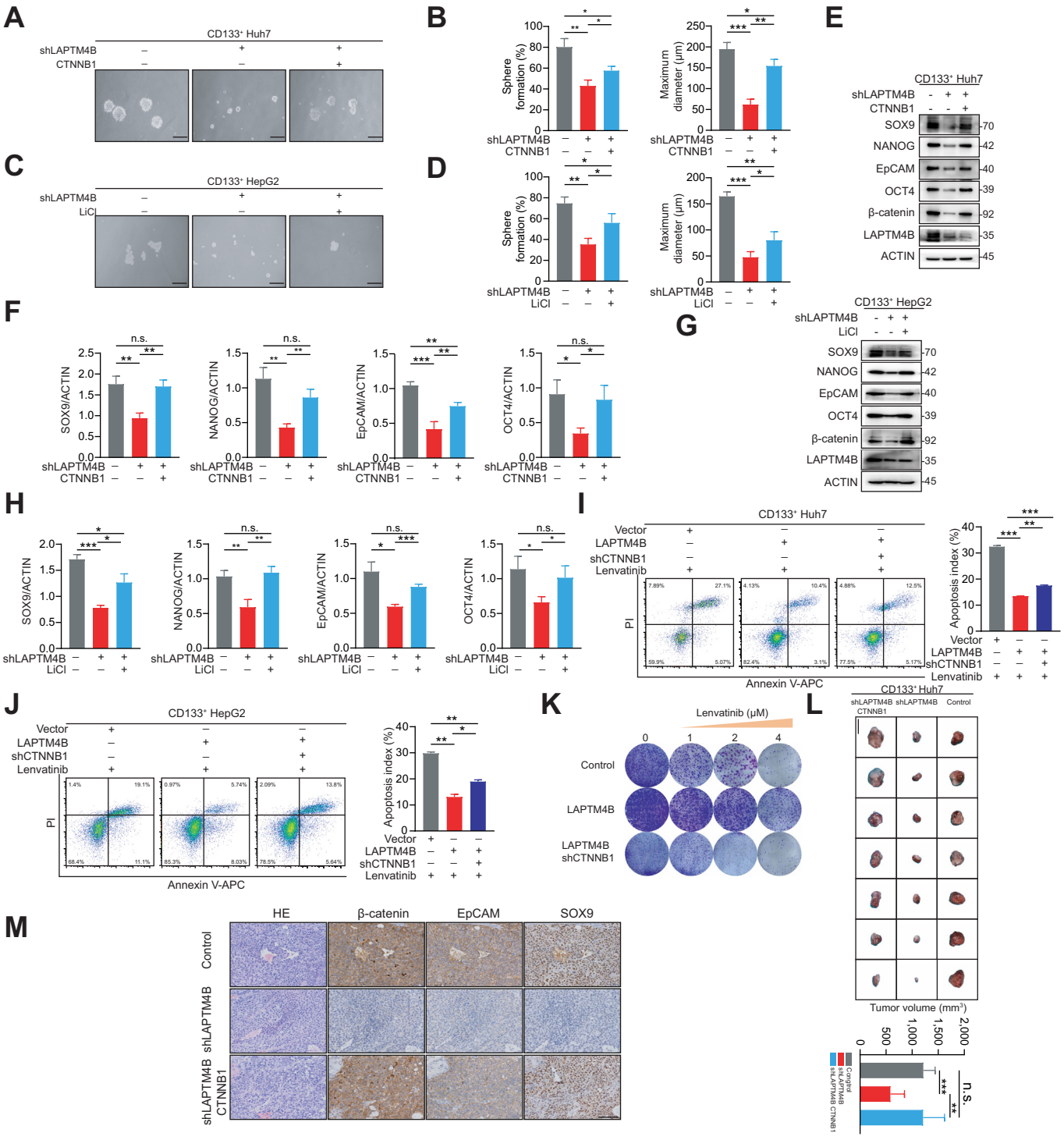

Fig. 5. WNT/β-catenin pathway is responsible for stemness properties of LAPTM4B-mediated CD133⁺ CSLCs. (A,B) Sphere-forming assay of CD133⁺ Huh7 cells with LAPTM4B knockdown and overexpressing *CTNNB1* in stem cell medium. (C,D) Sphere-forming assay of the effect of LiCl (WNT pathway activator) on the sphere formation ability of CD133⁺ HepG2 cells with LAPTM4B knockdown. (E,F) Western blots of *EpCAM*, *SOX9*, *OCT4*, and *NANOG* expression in LAPTM4B-knockdown CD133⁺ Huh7 cells with or without re-introduction of CTNNB1. (G,H) Western blots of *EpCAM*, *SOX9*, *OCT4*, and *NANOG* expression in LAPTM4B-knockdown CD133⁺ HepG2 cells treated with LiCl. (I,J) Effect of overexpressing LAPTM4B and knocking down *CTNNB1* on CD133⁺ Huh7 and CD133⁺ HepG2 cell apoptosis in the presence of lenvatinib (20 μM, 72 h). (K) Colony growth of LAPTM4B-overexpressing CD133⁺ Huh7 cells with knocked-down *CTNNB1* treated with lenvatinib for 7 days. (L) Tumor formation capacity of LAPTM4B-knockdown CD133⁺ Huh7 cells with or without CTNNB1 overexpression (n = 7 per group). (M) Immunohistochemistry of β-catenin, *EpCAM*, and *SOX9* expression in xenografted tumor tissue. Data are means ± SD for at least two independent experiments, analyzed with unpaired Student's *t* tests: **p* <0.05; ***p* <0.01; ****p* <0.001; n.s., no significant difference. Scale bars: 200 μm (A–D). 100 μm (M), 1.5 cm (L). See also Fig. S7. CSLC, cancer stem-like cell; LAPTM4B, lysosome-associated protein transmembrane-4β; WNT, wingless/integrated.

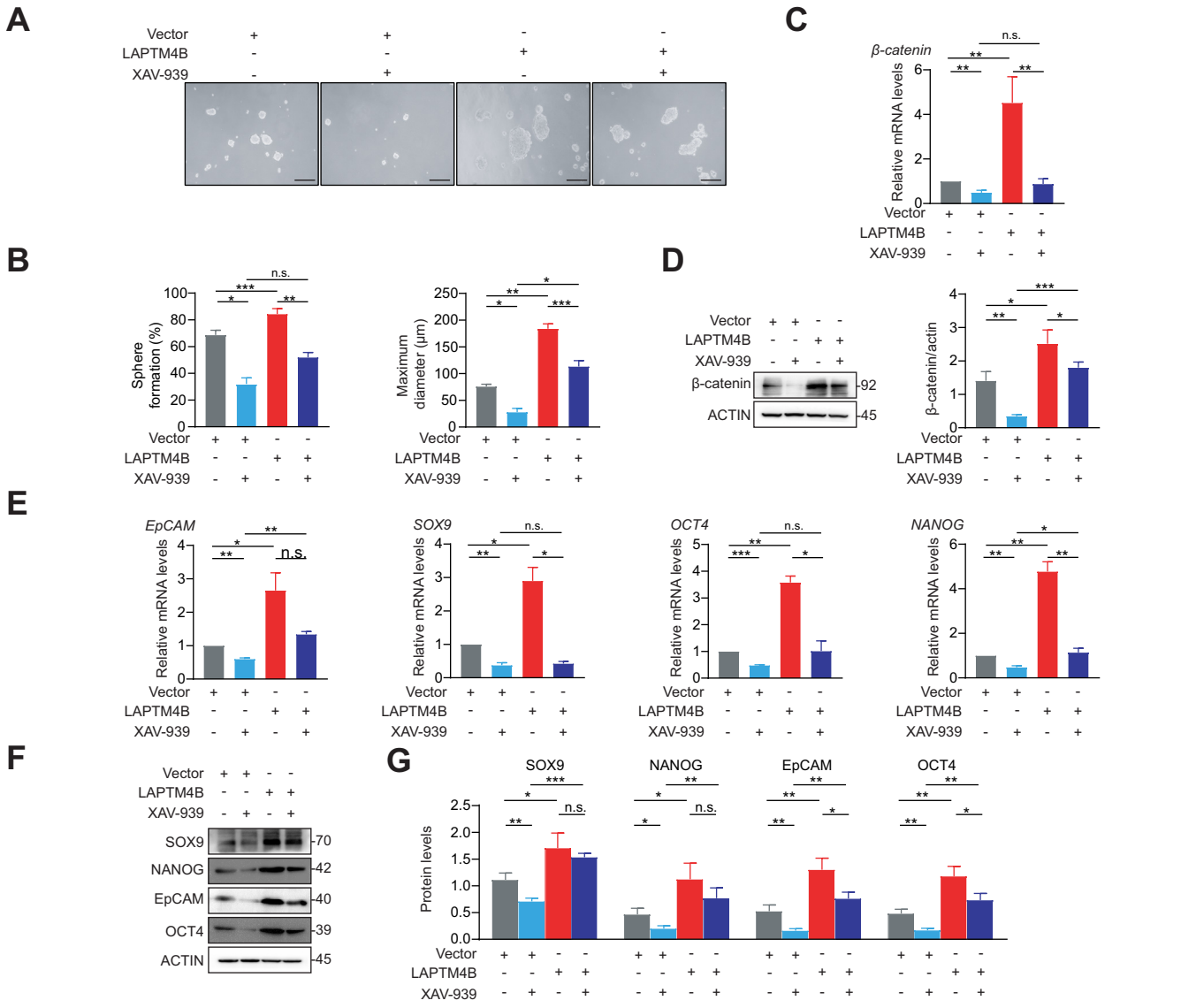

Fig. 6. LAPTM4B-induced stemness activation of CD133⁺ CSLCs is inhibited by WNT inhibition. (A,B) Sphere formation assays in LAPTM4B-overexpressing CD133⁺ Huh7 cells treated with or without XAV-939 (WNT pathway inhibitor). (C–G) qPCR and western blots of (C,D) β-catenin and (E–G) *EpCAM*, *SOX9*, *OCT4*, and *NANOG* expression in LAPTM4B-overexpressing CD133⁺ Huh7 cells treated with or without XAV-939. Data are means ± SD for at least two independent experiments, analyzed with unpaired Student's *t* tests: **p* <0.05; ***p* <0.01; ****p* <0.001; n.s., no significant difference. Scale bars: 200 μm. CSLC, cancer stem-like cell; LAPTM4B, lysosome-associated protein transmembrane-4β; WNT, wingless/integrated.

levels of these proteins demonstrated prolonged survival (p = 0.0043) (Fig. 7G). The 3-year overall survival rate was significantly decreased in patients with HCC with high LAPTM4B and β-catenin expression compared with those with low expression (42.9% *vs.* 74.4%, HR 5.174, 95% CI 2.280–11.741, p <0.001, Table S3). In summary, these results emphasize that overactivation of LAPTM4B and β-catenin indicates a negative prognosis in patients with HCC. They also reveal a significant pathological connection between LAPTM4B and β-catenin expression and the development of HCC.

Discussion

Cancer stem cells significantly contribute to tumor initiation, progression, metastasis, recurrence, and drug resistance.[3,24,33] Given the emergence of targeted therapies, the concept of cancer stem cells offers novel perspectives for diagnosing and predicting the prognosis of tumors. However, the molecular mechanisms by which cancer stem cells regulate and maintain their stemness characteristics remain unclear, and the clinical application of targeted therapy is subject to various limitations. Therefore, exploring the regulatory mechanism of stemness is particularly important. In this study, we identified that LAPTM4B enhances CD133⁺ CSLCs stemness through the WNT/β-catenin pathway.

Overexpression of LAPTM4B results in uncontrolled cell proliferation and malignant transformation, which could contribute to HCC progression as an oncogene.[34,35] Previous studies demonstrated that LAPTM4B can enhance the proliferation and invasion of HCC cells, while maintaining their stemness through a positive feedback loop with Yes-related

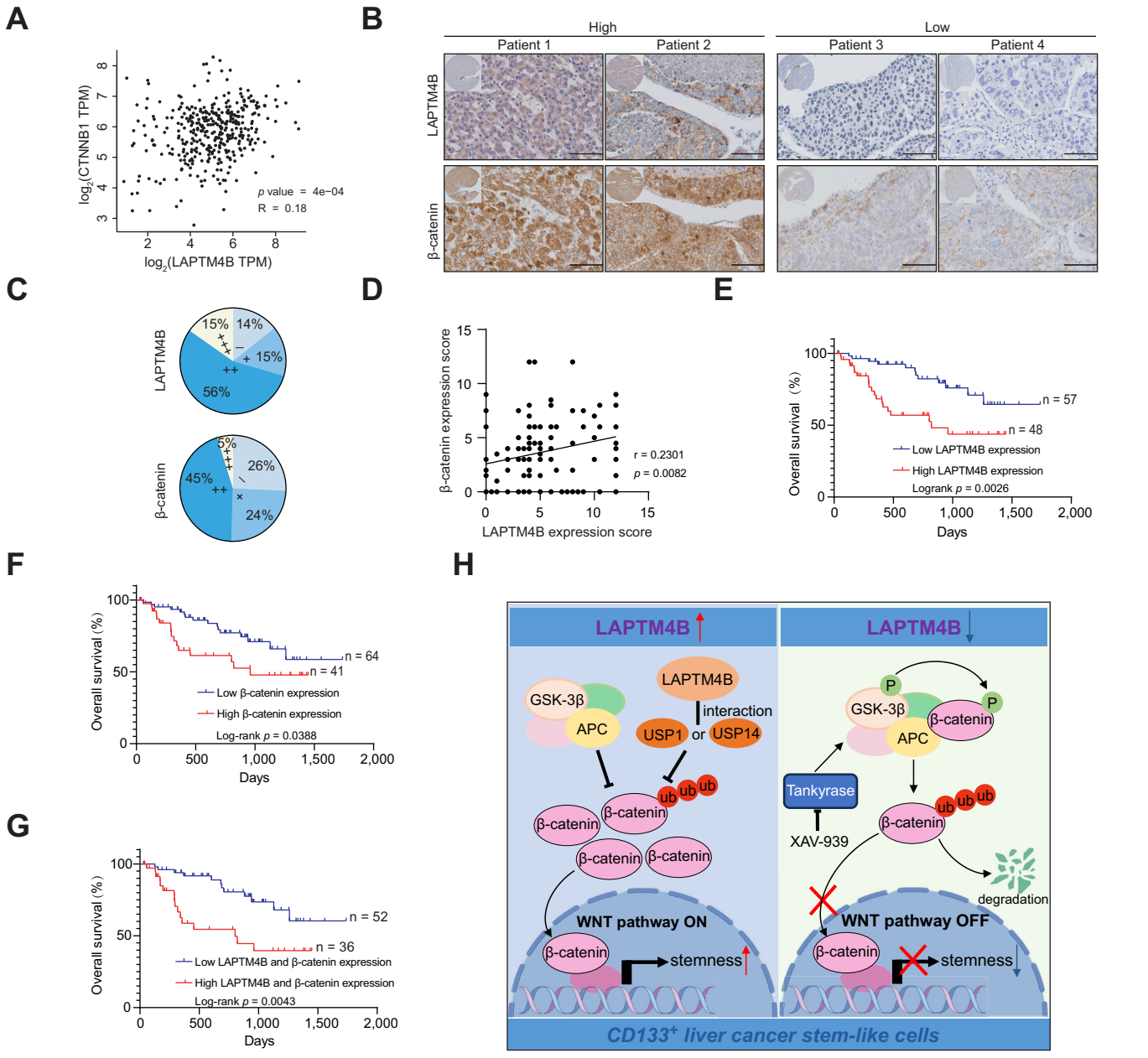

Fig. 7. LAPTM4B expression is correlated with β-catenin in HCC tissues. (A) Correlation between LAPTM4B and β-catenin expression in TCGA database confirmed by Pearson's correlation analysis. (B) Representative images of LAPTM4B and β-catenin expression based on immunohistochemistry of tissue microarrays. (C) Expression level of LAPTM4B and β-catenin in HCC tissues indicated as negative (–), weak (+), moderate (++), and strong (+++) expression. (D) Pearson's correlation analysis of LAPTM4B and β-catenin expression in patients with HCC. (E,F) Kaplan-Meier curves for overall survival of patients with HCC with high or low LAPTM4B/β-catenin expression. (G) Kaplan-Meier curves for overall survival of patients with high or low levels of LAPTM4B and β-catenin. (H) Working model. Survival rate was estimated by the Kaplan-Meier method and groups were compared by Log rank test. Scale bars: 100 μm. HCC, hepatocellular carcinoma; LAPTM4B, lysosome-associated protein transmembrane-4β; TCGA, the Cancer Genome Atlas.

protein (YAP), thereby promoting HCC progression.[8,20] Here, we further investigated WNT signaling, a regulatory pathway by which LAPTM4B promotes stemness in HCC.

WNT/β-catenin signaling is commonly activated in HCC and is crucial for regulating stem cell renewal.[36] In this study, we revealed additional evidence supporting the role of LAPTM4B in enhancing the functional capacity of the WNT/β-catenin pathway by inhibiting β-catenin phosphorylation and ubiquitination degradation, as well as its involvement in regulating stemness in HCC. In addition, our study provides evidence confirming that the combined high expression of LAPTM4B and β-catenin is associated with a poor prognosis for patients with HCC. Thus, targeting the LAPTM4B–WNT/β-catenin axis could improve the antitumor efficacy of drugs by attenuating tumor cell proliferation, invasion, and stemness in HCC. Encouragingly, various small-molecule inhibitors of the WNT/β-catenin pathway are now commercially available. E7386 is in phase I clinical trials as the first-in-class orally

active β-catenin-CBP antagonist inhibiting the WNT/β-catenin pathway in tumor xenograft models derived from patients with HCC.[37] Nonetheless, more comprehensive clinical trials are needed to further evaluate potential targets and explore personalized treatment options.

In conclusion, this study revealed that LAPTM4B activates the WNT/β-catenin pathway, which is involved in regulating HCC stemness. Our study confirms that LAPTM4B inhibits β-catenin phosphorylation. Furthermore, LAPTM4B interacts with USP1 and USP14 to inhibit the ubiquitinated degradation of β-catenin. This contributes to increased β-catenin stability and promotes β-catenin nuclear localization. Accumulation of β-catenin in the nucleus promotes activation of the WNT/β-catenin pathway and enhances tumor stemness. Abnormal upregulation of LAPTM4B and β-catenin enhances stemness and correlates with a poorer HCC prognosis (Fig. 7H). The findings of our study provide insights into stemness regulation in HCC and potential therapeutic targets for individuals with this condition.

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Abbreviations

APC, adenomatous polyposis coli; c-MYC, cellular myelocytomatosis; CD133, cluster of differentiation 133; CHX, cycloheximide; Co-IP, co-immunoprecipitation; CSLC, cancer stem-like cell; EMT, epithelial-to-mesenchymal transition; GEPIA, gene expression profiling interactive analysis; GSK-3β, glycogen synthase kinase 3β; HCC, hepatocellular carcinoma; HR, hazard ratio; IF, immunouorescent staining; IHC, immunohistochemistry; LAPTM4B, lysosome-associated protein transmembrane-4β; MMP7, matrix metalloproteinase 7; p-GSK-3β, phosphorylated glycogen synthase kinase 3β; qPCR, quantitative real-time PCR; RNA-seq, RNA sequencing; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; TCGA, The Cancer Genome Atlas; TMAs, tissue microarrays; USP, ubiquitin carboxyl-terminal hydrolase; WNT, wingless/integrated; YAP, Yes-related protein.

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Conflicts of interest

The authors declare no conflicts of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Concept and design: AMH, JPL, and JHW. Performed experiments and analyzed data: JHW, JPL, YC, and MRC. Collected human tissues and clinical data: JHW and YC. Animal experiments: JHW and JPL. Wrote the manuscript: JHW, JPL, and AMH. Reviewed the manuscript: all authors.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and/or its supplementary materials and methods. Any additional data are available after approval from the corresponding author.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2024.101306.

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Author names in bold designate shared co-first authorship

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