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UGGT1 mediates N-glycosylation of LGALS3BP to induce angiogenesis in liver cancer via the NOTCH signaling pathway

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Abstract:

Background: Liver cancer (LC) is one of the leading causes of cancer-related deaths worldwide. Its complex vascular regulatory network and drug resistance severely limit clinical efficacy. Although LGALS3BP is highly expressed in various malignant tumors, its biological function and molecular regulatory mechanisms in LC angiogenesis remain to be elucidated.

Methods: Bioinformatics analysis was used to examine the expression of LGALS3BP in hepatocellular carcinoma (HCC) and its relationship with angiogenesis and the NOTCH signaling pathway. qRT-PCR, Western blot (WB), and ELISA were performed to validate the

mRNA, protein expression, and secretion levels of LGALS3BP. The effects of LGALS3BP on HCC cell viability, proliferation, and invasion were assessed using CCK-8, colony formation, and Transwell assays. A co-culture system of HCC cells and human umbilical vein endothelial cells was established to evaluate pro-angiogenic effects. qRT-PCR and WB were used to detect mRNA and protein expression of the NOTCH pathway. Bioinformatics analysis combined with Co-IP and IF validated the interaction between UGGT1 and LGALS3BP. N-glycosylation sites of LGALS3BP were analyzed, and the role of UGGT1 in LGALS3BP N-glycosylation was investigated through protein stability assays, WB, and ELISA. The impact of UGGT1-mediated LGALS3BP N-glycosylation on the NOTCH pathway and HCC angiogenesis was explored via qRT-PCR, WB, functional cell assays, and co-culture experiments. Finally, *in vivo* validation was conducted using a xenograft tumor model.

Results: LGALS3BP was significantly overexpressed in HCC tissues and cells. Knockdown of LGALS3BP inhibited HCC cell proliferation and invasion, reduced VEGF-A expression, and suppressed tube formation in human umbilical-vein endothelial cells. Mechanistically, UGGT1 maintained LGALS3BP protein stability by mediating its N-glycosylation modification. LGALS3BP promoted VEGF-A expression by activating the NOTCH pathway. Animal experiments demonstrated that UGGT1 knockdown significantly inhibited tumor growth and angiogenesis, an effect that could be partially rescued by LGALS3BP overexpression.

Conclusion: This study reveals that UGGT1 promotes angiogenesis in HCC by mediating N-glycosylation of LGALS3BP and activating the NOTCH/VEGF-A signaling axis, providing a potential target for anti-angiogenic therapy in HCC.

Keywords: liver cancer; UGGT1; LGALS3BP; N-glycosylation modification; angiogenesis

1 Introduction

Liver cancer (LC) ranks as the sixth most common malignancy worldwide, and its steadily increasing burden has become a major global public health challenge¹. Between 2010 and 2021, the annual number of new LC cases rose by 26%, while associated mortality climbed 25%². Its progression and metastasis are highly dependent on pathological angiogenesis³. LC is characterized by disorganized vessel architecture and aberrant vascular networks; this

pathological remodeling not only supplies nutrients but also fosters the highly aggressive behavior of the tumor⁴. Therefore, anti-angiogenic therapy is considered a promising strategy, yet the complexity of vascular-regulatory networks and the emergence of drug resistance have substantially limited its efficacy⁵. Novel, rationally designed anti-angiogenic targets are therefore urgently needed to overcome the limitations of existing therapeutic targets, such as their singularity and susceptibility to drug resistance, thereby improving patient quality of life.

The NOTCH signaling pathway is essential for cell proliferation, differentiation, embryonic development and tissue homeostasis⁶. In LC, NOTCH signaling is frequently upregulated and plays a pivotal oncogenic role⁷. For example, the ADAM17 inhibitor ZLDI-8 sensitizes hepatocellular carcinoma (HCC) cells to sorafenib by suppressing NOTCH activity⁸, indicating that blockade of NOTCH pathway may offer new intervention strategies and enhance current therapies. Moreover, NOTCH signaling pathway is a central regulator of tumor angiogenesis⁹. Ligand-receptor interactions within the pathway govern endothelial-cell function and thereby control neovascularization and vascular remodeling¹⁰. Notably, recent research indicates that in LC, aberrant activation of NOTCH signaling pathway transcriptionally upregulates vascular endothelial growth factor (VEGF), driving robust neovessel formation¹¹. Thus, targeting the NOTCH signaling pathway may exert therapeutic effects by influencing the process of angiogenesis in LC. This finding provides a potential research direction for exploring therapeutic strategies for LC.

LGALS3BP (galectin-3-binding protein) is a secreted glycoprotein that mediates cell-cell communication, immune homeostasis and tumor-microenvironment remodeling¹². LGALS3BP is substantially overexpressed in LC and several other cancers¹². It can activate TGF- β 1 via integrin α V, thereby accelerating liver fibrosis and tumor progression¹³. Importantly, the functional activity of LGALS3BP is modulated by its glycosylation modification. N-glycosylation strengthens LGALS3BP interaction with galectins and subsequently influences intercellular and cell-extracellular matrix interactions, angiogenesis and tumor cell apoptosis¹². We therefore hypothesized that modulation through glycosylation modification of LGALS3BP could critically influence LC angiogenesis.

Here, we uncover a previously unrecognized mechanism in which UGGT1 (UDP-glucose glycoprotein glucosyltransferase 1) mediates N-glycosylation of LGALS3BP to activate

NOTCH signaling and thereby promote LC angiogenesis. Our findings establish the UGGT1-LGALS3BP-NOTCH signaling axis as a key driver of tumor neovascularization and provide new targets for targeting glycosylation events in anti-angiogenic therapies.

2 Methods

2.1 Bioinformatic Analysis

Firstly, the mRNA expression profiles and clinical data of HCC tissues and adjacent non-tumor tissues were downloaded from The Cancer Genome Atlas (TCGA) database. The expression of LGALS3BP and UGGT1 in HCC was analyzed through the GEPIA website (<http://gepia.cancer-pku.cn/>). After grouping based on the median expression of LGALS3BP, GSEA was performed to explore its association with angiogenesis marker genes and the NOTCH pathway. The correlation between LGALS3BP and angiogenesis genes was further verified by Pearson correlation analysis. The protein interaction network was constructed based on the BioGRID database (<https://thebiogrid.org/>) to elucidate the interaction between UGGT1 and LGALS3BP. Finally, the N-glycosylation modification sites of LGALS3BP were predicted using the NetNGlyc website (<http://www.cbs.dtu.dk/services/NetNGlyc/>).

2.2 Cell Cultivation and Reagents

Detailed cell-line information is provided in **Table 1**. MIHA cells were maintained in RPMI-1640 complete medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; BNCC, China) and 1% penicillin/streptomycin (P/S, Solarbio, China). Hep3B cells were cultured in MEM (Procell, China) containing 10% FBS, 1% P/S and 1% non-essential amino acids (Gibco, USA). 293T, MHCC97L and Huh7 cells were grown in DMEM complete medium (Procell, China) with 10% FBS and 1% P/S. Human umbilical-vein endothelial cells (HUVECs) were maintained in a specialized HUVEC complete medium (Procell, China). All cells were incubated in an incubator at 37 °C with 5% CO₂ (Thermo Fisher, USA).

2.3 RNA Interference, Plasmid Construction and Viral Infection

Short hairpin RNAs targeting LGALS3BP (sh-LGALS3BP) or UGGT1 (sh-UGGT1), the corresponding overexpression plasmids (oe-LGALS3BP, oe-UGGT1) and their negative controls were bought from Shanghai Genechem (China). When cells reached 60-80%

confluence, plasmids or shRNAs were transfected with Lipofectamine™ 3000 reagent (Invitrogen, USA); knockdown/overexpression efficiency was verified 48 h later.

To construct a stably transfected Huh7 cell line, 293T cells in the logarithmic growth phase were first seeded into culture dishes. The target gene expression vector, together with the packaging plasmid psPAX2 and the envelope plasmid pMD2.G (Nanjing Jiangyuan, China), was co-transfected using the lipofection method. After 48 h of transfection, the culture supernatant containing viral particles was collected and used to infect Huh7 cells.

2.4 Western Blot (WB)

Cells were lysed on ice for 15 min in RIPA buffer supplemented with protease inhibitors (Beyotime, China). After centrifugation ($12,000 \times g$, 20 min, 4 °C), protein concentrations were determined with a BCA kit (Beyotime, China). Equal amounts of proteins were separated on 10% SDS-PAGE (Epizyme, China) and transferred to PVDF membranes (Baygene, China). Membranes were blocked with 5% non-fat milk (Solarbio, China) for 1 h, incubated with primary and secondary antibodies. Immunoreactive bands were visualized with ECL reagent (Yeasen, China) and imaged using a chemiluminescence imaging system (Bio-Rad, USA). Antibody details are provided in **Table 2**.

2.5 qRT-PCR

Total RNA was isolated with TRIzol reagent (Invitrogen, USA). Concentration and purity (A260/A280) were determined using a NanoDrop One micro-volume spectrophotometer (Thermo Fisher, USA). cDNA was synthesized with the PrimeScript RT Reagent Kit (Takara, Japan). qRT-PCR was conducted with PowerUp SYBR Green Master Mix (Applied Biosystems, USA) on an ABI 7500 PCR System (Thermo Fisher, USA). β -actin served as the internal reference; relative gene expression was calculated by the $2^{-\Delta\Delta C_t}$ method. Primer sequences are displayed in **Table 3**.

2.6 Cell Counting Kit-8 (CCK-8) Assay

Treated cells were seeded at 2×10^3 cells per well in 96-well plates (Corning, USA) with three replicates. CCK-8 solution was added at 0, 24, 48, 72 and 96 h, followed by incubation at 37 °C for 2 h. Absorbance (Optical density (OD) value) at 450 nm was measured on a multimode microplate reader (Tecan Spark, Switzerland) to evaluate cell proliferation.

2.7 Colony Formation Assay

Cells were plated in 6-well plates (Corning, USA) at 1×10^3 per well and cultured for 14 days in complete medium supplemented with 10% FBS (Procell, China) at 37 °C with 5% CO₂. Upon the end of the experiment, colonies were gently rinsed twice with pre-cooled PBS (4 °C) and immobilized with 1 mL of 4% paraformaldehyde (PFA) (Sigma-Aldrich, USA). After removing the fixation solution, cells were stained with 5 mL 0.1% (w/v) crystal violet (Sigma-Aldrich, USA). After thorough washing with deionized water, plates were air-dried and imaged.

2.8 Cell Invasion Assay

Transwell inserts were pre-coated with 50 µL Matrigel (Corning, USA) and allowed to solidify at 37 °C. Cells were digested with 0.25% trypsin (Gibco, USA), resuspended in serum-free medium and seeded at 4×10^4 cells per insert into the upper chamber. Medium containing 10% FBS was placed in the lower chamber as a chemotactic inducer. After 48 h, non-invading cells on the upper surface were gently removed with a cotton swab. Invaded cells were fixed with 4% PFA (Sigma-Aldrich, USA), stained with 0.1% crystal violet (Servicebio, China), rinsed, and photographed under an inverted microscope (Zeiss, Germany).

2.9 ELISA

Expression of VEGF-A in human cells and mouse samples and LGALS3BP secretion in human cells were quantified with commercial ELISA kits. For human cell samples, human-specific VEGF-A ELISA kits (Invitrogen, USA) and human-specific LGALS3BP ELISA kits (FineTest, China) were used; for mouse samples, mouse-specific VEGF-A ELISA kits (Invitrogen, USA) were used. The OD values were measured at 450 nm using a multimode microplate reader (Tecan Spark, Switzerland). The concentrations of the target proteins were calculated through a standard curve.

2.10 Co-culture System

A Transwell co-culture system (0.4 µm pore size; Corning, USA) was used to evaluate interactions between HCC cells and HUVECs. Specifically, HUVECs were seeded in the lower chamber at 2×10^5 cells per insert, and HCC cell lines (Hep3B or Huh7) were plated in the upper chamber at 2×10^5 cells per insert. Cells were co-cultured for 48 h in DMEM containing 5% FBS (Procell, China) before HUVECs were harvested for subsequent tube formation assays.

2.11 Co-Immunoprecipitation (Co-IP)

Huh7 cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (all Beyotime, China). After centrifugation, the supernatant of the lysate was divided into two groups: the experimental group was treated with specific antibodies against LGALS3BP (10281-1-AP, Proteintech, China) or UGGT1 (ab241357, Abcam, UK); the control group was treated with an equivalent amount of rabbit anti-IgG (ab172730, Abcam, UK). The samples were incubated overnight at 4°C. Subsequently, Protein A/G magnetic beads (Invitrogen, USA) were added and the incubation continued for another 4 h. After washing with PBS, the samples were boiled in 5× SDS loading buffer (Beyotime, China) to denature the proteins, and then subjected to WB analysis.

2.12 Protein Stability Assay

Huh7 cells grown to 80% confluence in 6-well plates were treated with 50 µg/ml cycloheximide (CHX, Sigma-Aldrich, USA). Cells were harvested at 0, 2, 4 and 6 h post-CHX, lysed in RIPA buffer (Beyotime, China) and subjected to WB for LGALS3BP.

2.13 Immunofluorescence (IF) Staining

IF staining was performed to detect colocalization of UGGT1 and LGALS3BP in Huh7 cells. Huh7 cells were seeded into confocal culture dishes (NEST, China). After fixation with 75% ethanol and permeabilization with 0.3% Triton X-100 (Solarbio, China), cells were incubated overnight at 4°C with rabbit anti-UGGT1 (14170-1-AP, Proteintech, China) and mouse anti-LGALS3BP (60066-1-Ig, Proteintech, China). Subsequently, the cells were incubated for 1 h at room temperature in the dark with goat anti-rabbit IgG H&L (Alexa Fluor® 488) (ab150077, Abcam, UK) and goat anti-mouse IgG H&L (Alexa Fluor® 647) (ab150115, Abcam, UK). The nuclei were stained with DAPI (Invitrogen, USA). Finally, images were captured by a laser confocal microscope (Zeiss, Germany).

IF staining was performed to detect CD31 expression. Frozen sections of tumor tissue were fixed and permeabilized, then blocked with 5% bovine serum albumin (BSA, Sigma-Aldrich, USA). The sections were incubated overnight at 4°C with rabbit anti-CD31 primary antibody (ab222783, Abcam, UK). The following day, the sections were incubated for 1 h at room temperature in the dark with goat anti-rabbit IgG H&L (Alexa Fluor® 488) secondary antibody (ab150077, Abcam, UK). After nuclear staining with DAPI (Invitrogen, USA), images were captured using a laser confocal microscope (Zeiss, Germany).

2.14 Xenograft Tumor Model

BALB/c nude mice from Beijing Vital River Laboratory Animal Technology Co., Ltd. (China) were used to construct a xenograft tumor model. All procedures were approved by the Animal Ethics Committee of Zhejiang Luoxi Medical Technology Co., Ltd., Hangzhou, China (Approval No. LX4825021202). Eighteen 4-6 week mice were housed under specific pathogen free conditions (22 ± 1 °C, 50% humidity, 12-h light/dark cycle), and were randomly assigned to three groups ($n = 6$ each): sh-NC+oe-NC, sh-UGGT1+oe-NC, and sh-UGGT1+oe-LGALS3BP.

Stably transfected Huh7 cells (1×10^6 cells per mouse) were mixed 1:1 with Matrigel (Corning, USA) and injected subcutaneously into the right flank. Tumor volumes were measured every 7 days. On day 28, mice were euthanized. Tumor tissues and mouse serum were harvested for further assays.

2.15 Immunohistochemistry (IHC)

Paraffin-embedded tissue sections were dewaxed in xylene (Merck, Germany) and rehydrated through graded ethanol (Servicebio, China). Antigen retrieval was performed by microwave heating in EDTA buffer (Beyotime, China). Endogenous peroxidase was quenched with 3% H_2O_2 (Sigma-Aldrich, USA) at room temperature, followed by blocking with 5% BSA (Beyotime, China) for 30 min. Sections were incubated overnight at 4 °C with primary antibodies (all rabbit): anti-UGGT1 (14170-1-AP, Proteintech, USA), anti-LGALS3BP (MA5-50412, Invitrogen, USA), anti-NOTCH1 (ab52627, Abcam, UK) or anti-Ki67 (ab16667, Abcam, UK). The following day, HRP-conjugated goat anti-rabbit secondary antibody (ab205718, Abcam, UK) was applied for 1 h at room temperature. Immunoreactivity was visualized with DAB (Beyotime, China) and nuclei were counterstained with hematoxylin (Solarbio, China). Images were captured using an inverted microscope (Zeiss, Germany) and analyzed with ImageJ (NIH, USA).

2.16 Statistical Analysis

All data were analyzed with GraphPad Prism 8.0 (GraphPad Software Inc., USA). Continuous variables are presented as $\bar{x}\pm s$ ($n\geq 3$). Comparisons between two groups were performed with t-tests; multiple groups were compared by one-way ANOVA. $P < 0.05$ was considered statistically significant.

238 **3 Results**

239 **3.1 LGALS3BP is Highly Expressed in HCC and Induces Tumor Angiogenesis**

240 To elucidate the role of LGALS3BP in HCC, we first queried the GEPIA database and
241 found that LGALS3BP mRNA is upregulated in HCC tissues (**Fig. 1A**). qRT-PCR and WB
242 analyses further demonstrated that both mRNA and protein levels of LGALS3BP were
243 significantly upregulated in HCC cell lines (Hep3B, MHCC97H, Huh7) than in the normal
244 hepatocytes MIHA (**Fig. 1B-C**). Consistently, ELISA revealed that the secretion levels of
245 LGALS3BP were also significantly higher in Hep3B, MHCC97L and Huh7 cells than in MIHA
246 cells (**Fig. 1D**). Collectively, these data indicate that LGALS3BP is highly expressed in HCC
247 and may serve as a potential diagnostic biomarker and therapeutic target. To explore the
248 underlying mechanisms, GSEA was performed to reveal that LGALS3BP was significantly
249 enriched in angiogenesis-related pathways and positively correlated with angiogenesis
250 signatures in HCC (**Fig. 1E-F**). We next selected Hep3B cells, which highly express
251 LGALS3BP, and stably knocked down LGALS3BP using shRNA; knockdown efficiency was
252 confirmed by qRT-PCR and WB (**Fig. 1G**). Functional assays showed that LGALS3BP
253 knockdown significantly reduced Hep3B cell viability, proliferation and invasion (**Fig. 1H-J**).

254 VEGF-A, as a key member of the vascular endothelial growth factors, plays a crucial role
255 in tumor angiogenesis. WB and ELISA demonstrated that LGALS3BP knockdown
256 significantly decreased both protein expression and secretion of VEGF-A (**Fig. 1K-L**). We
257 co-cultured LGALS3BP-knockdown Hep3B cells with HUVECs for a tube formation assay.
258 Tube formation abilities of HUVECs were significantly reduced in the
259 LGALS3BP-knockdown group compared to the sh-NC group (**Fig. 1M**). Taken together, these
260 findings demonstrate that LGALS3BP is overexpressed in HCC and promotes tumor
261 angiogenesis by upregulating VEGF-A.

262 **3.2 LGALS3BP Induces Angiogenesis in HCC via the NOTCH Signaling Pathway**

263 To dissect the molecular mechanism by which LGALS3BP promotes angiogenesis, we
264 performed pathway-enrichment analyses. Results revealed that LGALS3BP was significantly
265 enriched in the NOTCH signaling pathway (**Fig. 2A**). qRT-PCR and WB analyses in

LGALS3BP-knockdown Hep3B cells showed that the mRNA and protein levels of NOTCH signaling pathway-related proteins, NOTCH1, HES1 and HEY1, were significantly downregulated. This indicates that LGALS3BP can possibly influence the expression of downstream effector molecules by modulating NOTCH signaling pathway. Notably, the protein expression of N1ICD, the active form of the NOTCH1 receptor, was also significantly downregulated (**Fig. 2B-C**). Subsequently, we overexpressed LGALS3BP in Huh7 cells; transfection efficiency was verified by qRT-PCR (**Fig. 2D**). Rescue experiments were then performed with the NOTCH signaling pathway inhibitor DAPT (HY-13027, MCE, USA). Functional assays revealed that LGALS3BP overexpression significantly enhanced Huh7 cell viability, proliferation and invasion, whereas co-treatment with DAPT reversed these pro-tumorigenic effects (**Fig. 2E-G**). These data confirm the oncogenic role of LGALS3BP and the key mediating role of NOTCH signaling pathway. WB and ELISA showed that LGALS3BP overexpression increased both VEGF-A protein expression and its secretion, and this increase was inhibited by DAPT (**Fig. 2H-I**). In co-culture assays, compared with the control group, the angiogenic capacity of HUVECs was enhanced in the co-culture system overexpressing LGALS3BP, while this effect was reversed by DAPT treatment (**Fig. 2J**). These results suggest that LGALS3BP promotes HCC angiogenesis by upregulating VEGF-A expression through activation of the NOTCH signaling pathway.

3.3 UGGT1 Mediates N-Glycosylation of LGALS3BP

To further dissect the regulatory mechanism of LGALS3BP in HCC, we performed protein-protein interaction analysis and identified UGGT1 as a potential interacting partner of LGALS3BP (**Supplementary Fig. 1**). TCGA data confirmed that UGGT1 mRNA was significantly upregulated in HCC tissues compared with adjacent normal tissues (**Fig. 3A**). Co-IP assays demonstrated a specific binding between UGGT1 and LGALS3BP (**Fig. 3B**), and IF co-localization showed that both proteins were predominantly localized in the endoplasmic reticulum (ER) (**Fig. 3C**). These results confirm that UGGT1 interacts with LGALS3BP.

LGALS3BP is a secreted glycoprotein whose function may be controlled by N-glycosylation¹². *In silico* prediction revealed seven putative N-glycosylation sites within LGALS3BP (**Fig. 3D**). Subsequently, to test whether UGGT1 mediates N-glycosylation of LGALS3BP, we established UGGT1-overexpressing Huh7 cells; transfection efficiency was

verified by qRT-PCR (**Fig. 3E**). CHX chase assays showed that UGGT1 overexpression significantly prolonged the half-life of LGALS3BP protein (**Fig. 3F**). WB analysis revealed that UGGT1 overexpression significantly increased total LGALS3BP protein expression, compared to controls; however, upon treatment with the N-glycosylation inhibitor tunicamycin (HY-A0098, MCE, USA), the molecular weight of LGALS3BP decreased from approximately 97 kDa to approximately 65 kDa, and its protein expression level significantly decreased compared to the UGGT1-overexpressing group (**Fig. 3G**). Consistently, ELISA demonstrated that UGGT1 overexpression enhanced LGALS3BP secretion, an effect that was inhibited by tunicamycin (**Fig. 3H**). Together, these data establish that UGGT1 stabilizes LGALS3BP protein and facilitates its secretion by mediating the N-glycosylation of LGALS3BP.

3.4 UGGT1 Activates the NOTCH Signaling Pathway by Mediating the N-Glycosylation of LGALS3BP to Facilitate Angiogenesis in HCC

To determine whether UGGT1-mediated N-glycosylation of LGALS3BP enhances angiogenesis in HCC via the NOTCH signaling pathway, we established three stable Huh7 groups: control (sh-NC+oe-NC), UGGT1 knockdown (sh-UGGT1+oe-NC), and UGGT1 knockdown and LGALS3BP overexpression (sh-UGGT1+oe-LGALS3BP). These models were used for rescue experiments; qRT-PCR confirmed efficient transfection (**Fig. 4A**). WB and ELISA showed that UGGT1 knockdown significantly reduced protein expression and secretion levels of LGALS3BP, whereas these effects were partially reversed by LGALS3BP overexpression (**Fig. 4B-C**). Consistently, NOTCH signaling pathway-related molecules, NOTCH1, HES1, HEY1 and NICD, were significantly downregulated at mRNA and protein levels after UGGT1 knockdown, as demonstrated by qRT-PCR and WB; these decreases were largely rescued by LGALS3BP overexpression (**Fig. 4D-E**). Functional assays revealed that UGGT1 knockdown significantly impaired Huh7 viability, proliferation and invasion, and each defect was partially reversed when LGALS3BP was re-introduced (**Fig. 4F-H**). VEGF-A expression and secretion were likewise diminished by UGGT1 knockdown and were partially reversed by LGALS3BP overexpression, demonstrated by WB and ELISA (**Fig. 4I-J**). Moreover, in tube formation assays, the angiogenic capacity of HUVECs co-cultured with the sh-UGGT1 group was significantly reduced compared to the control group, while the sh-UGGT1+oe-LGALS3BP group partially restored the angiogenic ability (**Fig. 4K**).

Collectively, these data demonstrate that UGGT1 activates the NOTCH/VEGF-A signaling pathway by modulating the N-glycosylation of LGALS3BP to promote angiogenesis in HCC.

3.5 *In vivo* Validation That UGGT1/LGALS3BP Induces HCC Angiogenesis via the NOTCH Signaling Pathway

To validate the pro-angiogenic UGGT1/LGALS3BP/NOTCH axis *in vivo*, we established a xenograft tumor model by subcutaneously introducing Huh7 cells stably transfected with sh-NC+oe-NC, sh-UGGT1+oe-NC, or sh-UGGT1+oe-LGALS3BP into BALB/c nude mice. Results showed that compared with the control group, the tumor volume and weight in the UGGT1 knockdown group of nude mice were significantly reduced, and tumor growth was significantly slowed down. However, overexpression of LGALS3BP on the basis of UGGT1 knockdown partially restored tumor volume, weight, and growth rate (**Fig. 5A-C**). IHC of resected tumors revealed that UGGT1 knockdown significantly reduced the protein expression of UGGT1, LGALS3BP, NOTCH1 and the proliferation marker Ki67; each reduction was partially reversed by LGALS3BP overexpression in the rescue group (**Fig. 5D**). ELISA showed that VEGF-A secretion was significantly diminished upon UGGT1 silencing and was partially restored when LGALS3BP was overexpressed (**Fig. 5E**). IF staining for the endothelial marker CD31 demonstrated that microvessel density was substantially decreased in sh-UGGT1 tumors but was significantly increased in the sh-UGGT1+oe-LGALS3BP group (**Fig. 5F**). Collectively, these data confirm that UGGT1-mediated N-glycosylation of LGALS3BP activates the NOTCH/VEGF-A signaling axis *in vivo*, thereby fueling angiogenesis and tumor growth in HCC.

4 Discussion

This study delineates a previously unrecognized axis in which UGGT1-mediated N-glycosylation of LGALS3BP activates the NOTCH signaling pathway, thereby driving angiogenesis in LC. First, by integrating bioinformatic analyses and experiments, we documented that LGALS3BP is highly expressed in HCC and acts as a potent pro-angiogenic factor. Mechanistically, LGALS3BP activates NOTCH signaling, which in turn transcriptionally upregulates VEGF-A. Moreover, we demonstrated that UGGT1 stabilizes

LGALS3BP and facilitates its secretion through N-glycosylation of LGALS3BP, thereby activating the NOTCH signaling pathway and the resulting angiogenic response.

LGALS3BP, a secreted glycoprotein, upregulates in multiple tumors, including LC, neuroblastoma and clear-cell renal cell carcinoma¹⁴⁻¹⁶. Our bioinformatic and experimental data corroborate LGALS3BP's high expression in LC tissues and cell lines. Since LC is a highly vascularized tumor whose progression is closely linked to neovascularization¹⁷, we hypothesized that LGALS3BP drives tumor aggressiveness by inducing angiogenesis. Indeed, through GSEA and correlation analyses, we found a positive association between LGALS3BP and angiogenesis. Functional assays confirmed that LGALS3BP upregulates VEGF-A expression and facilitates tube formation. These findings establish the LGALS3BP-VEGF axis as a critical driver of LC angiogenesis and identify LGALS3BP as an attractive target for anti-angiogenic therapy.

To uncover the specific mechanism underlying the modulation of angiogenesis by LGALS3BP, we performed pathway enrichment analyses. Results revealed the over-representation of the NOTCH signaling cascade. NOTCH signaling is a conserved pathway that governs cell proliferation, differentiation and vascular development under multiple physiological and pathological conditions¹⁸. In LC, NOTCH enhances epithelial-mesenchymal transition and metastatic dissemination¹⁹. Moreover, NOTCH is hyper-activated in drug-resistant LC cells and promotes tumor vascularization by upregulating VEGF; pharmacologic blockade with the NOTCH pathway inhibitor DAPT effectively inhibits tumor growth and angiogenesis²⁰. Consistently, we observed that after LGALS3BP overexpression, LC cell viability, proliferation and invasion were increased, whereas DAPT treatment reversed these pro-tumorigenic effects. Collectively, these data underscore NOTCH inhibition as a promising therapeutic strategy for LC.

N-glycosylation promotes the invasiveness and angiogenesis of tumors by affecting key signaling pathways and protein functions, thus playing an important role in the progression and angiogenesis of LC^{21, 22}. For instance, N-glycosylation of Wnt3 modulates its binding to FZD receptors to regulate Wnt/ β -catenin signaling and consequent proliferation, migration and invasion of LC cells²³. The biological activity of the secreted glycoprotein LGALS3BP is N-glycosylation-dependent, with its protein stability essentially relying on it¹². The regulation

of N-glycosylation relies on the activity of the ER-localized glycosyltransferase UGGT1²⁴. UGGT1 is responsible for recognizing and repairing the N-glycan structures of misfolded glycoproteins. By mediating the reglycosylation of N-glycans, it competes with the ER-associated degradation pathways, thereby regulating the metabolism of unstable or misfolded glycoproteins²⁵. UGGT1 can enhance the stability of PD-L1 protein by mediating its N-glycosylation, thereby promoting tumor immune evasion²⁶. Additionally, research has indicated that the knockdown of UGGT1-AS1 leads to malignant proliferation of breast cancer cells, suggesting that UGGT1 may function as a tumor suppressor gene²⁷. We have confirmed through bioinformatic analyses and cellular experiments that UGGT1 maintained protein stability and promoted the secretion of LGALS3BP by mediating its N-glycosylation, and activated the NOTCH signaling pathway, thereby promoting angiogenesis in HCC. Our *in vivo* experiments have demonstrated that UGGT1 knockdown significantly inhibited HCC angiogenesis and tumor growth, while overexpression of LGALS3BP could partially reverse this effect. These results establish the UGGT1-LGALS3BP-NOTCH/VEGF axis as a key target for regulating LC angiogenesis.

This study elucidates the critical regulatory role of UGGT1 in LC angiogenesis and its underlying molecular mechanisms. UGGT1 promotes tumor angiogenesis by regulating the N-glycosylation level of LGALS3BP and subsequently activating the downstream NOTCH signaling pathway. However, there are several limitations. First, the findings have not yet been validated in tissue samples from patients. Second, whether the UGGT1-LGALS3BP axis is involved in disease progression through other signaling pathways in addition to the NOTCH pathway remains to be explored. Additionally, the upstream regulatory mechanisms underlying the overexpression of UGGT1 in LC are still unclear. Future research will further expand the mechanistic studies of this molecular regulatory network and explore therapeutic strategies targeting the UGGT1-LGALS3BP interaction, providing a new theoretical basis and potential target for anti-angiogenic therapy in LC.

Author contribution

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Conflict of Interests

The authors have no conflicts of interest to declare.

Data Availability Statement

The data and materials in the current study are available from the corresponding author on reasonable request.

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Ethics approval and consent to participate

The animal experiments in this study were approved by the Animal Ethics Committee of Zhejiang Luoxi Medical Technology Co., Ltd., Hangzhou, China (Approval No. LX4825021202).

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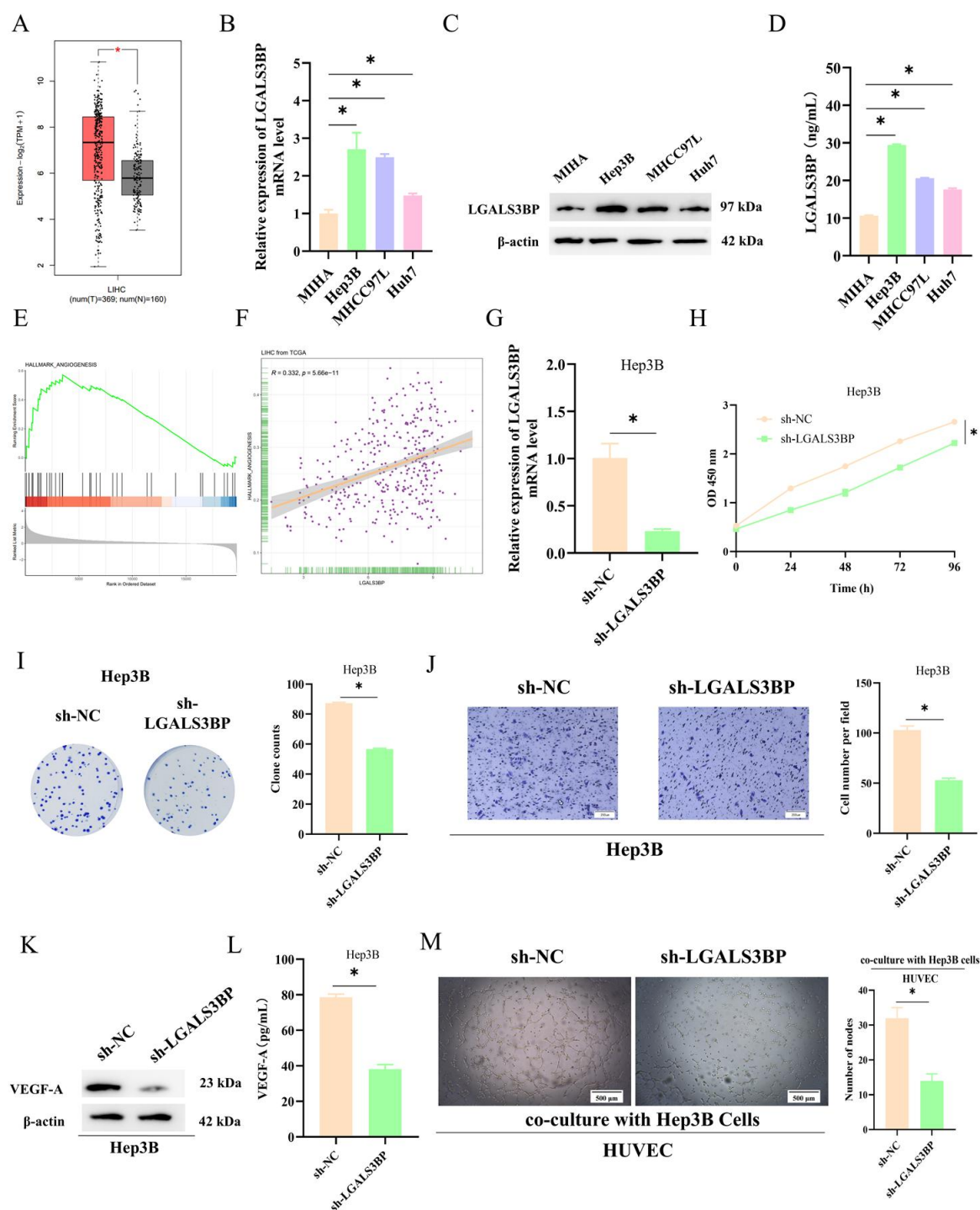
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528

529 **Figure 1 LGALS3BP is overexpressed in HCC and induces tumor angiogenesis.**

530 **A:** Analysis of LGALS3BP mRNA expression levels in HCC tissues and normal tissues from

531 the GEPIA database. **B:** qRT-PCR detection of LGALS3BP mRNA expression in normal

532 hepatocytes (MIHA) and HCC cells (Hep3B, MHCC97L, Huh7). **C:** WB detection of

533 LGALS3BP protein expression in normal hepatocytes and HCC cells. **D:** ELISA detection of
534 LGALS3BP secretion levels in normal hepatocytes and HCC cells. **E:** GSEA of the association
535 between LGALS3BP and angiogenesis-related gene sets. **F:** Pearson correlation analysis of
536 LGALS3BP and angiogenesis pathway enrichment scores. Cell experimental groups: sh-NC,
537 sh-LGALS3BP. **G:** qRT-PCR detection of transfection efficiency. **H:** CCK-8 assay for cell
538 viability. **I:** Colony formation assay for cell proliferation. **J:** Transwell assay for cell invasion.
539 **K:** WB detection of VEGF-A protein expression. **L:** ELISA detection of VEGF-A secretion
540 levels. Hep3B cells from sh-NC and sh-LGALS3BP groups were co-cultured with HUVECs.
541 **M:** Tube formation assay to assess angiogenesis capability. *, $P<0.05$.

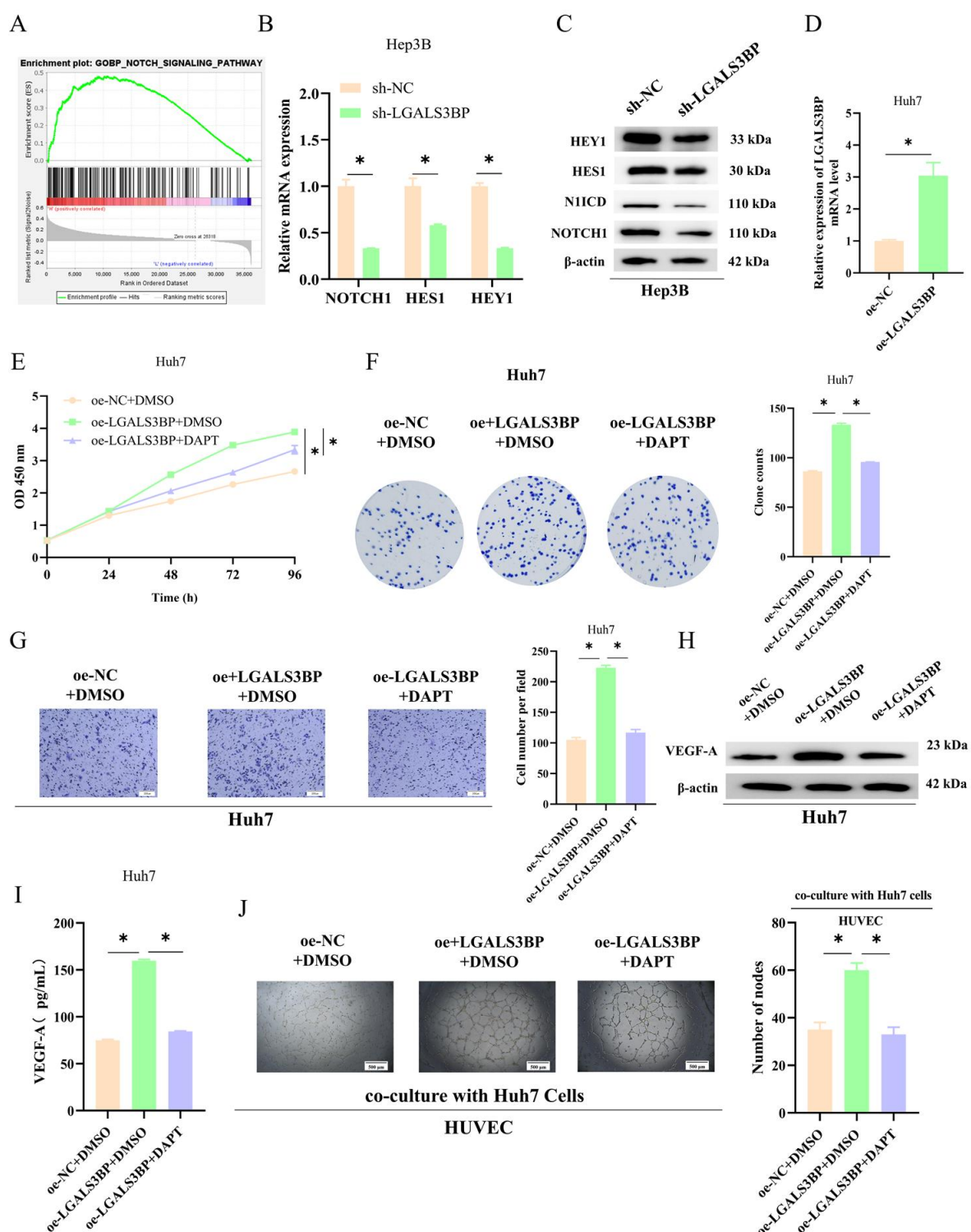


Figure 2 LGALS3BP induces angiogenesis in HCC via the NOTCH signaling pathway. A: GSEA. Cell experimental groups: sh-NC, sh-LGALS3BP. **B:** qRT-PCR detection of NOTCH1, HES1, and HEY1 mRNA levels. **C:** WB detection of NOTCH1, N1ICD, HES1, and HEY1 protein expression. Cell experimental groups: oe-NC, oe-LGALS3BP. **D:** qRT-PCR detection of transfection efficiency. The NOTCH pathway inhibitor DAPT was added to the

oe-LGALS3BP group for the following groups: oe-NC+DMSO, oe-LGALS3BP+DMSO, oe-LGALS3BP+DAPT. **E:** CCK-8 assay for cell viability. **F:** Colony formation assay for cell proliferation. **G:** Transwell assay for cell invasion. **H:** WB detection of VEGF-A protein expression. **I:** ELISA detection of VEGF-A secretion levels. Huh7 cells transfected with oe-NC+DMSO, oe-LGALS3BP+DMSO, and oe-LGALS3BP+DAPT were co-cultured with HUVECs. **J:** Tube formation assay to assess angiogenesis capability. *, $P < 0.05$.

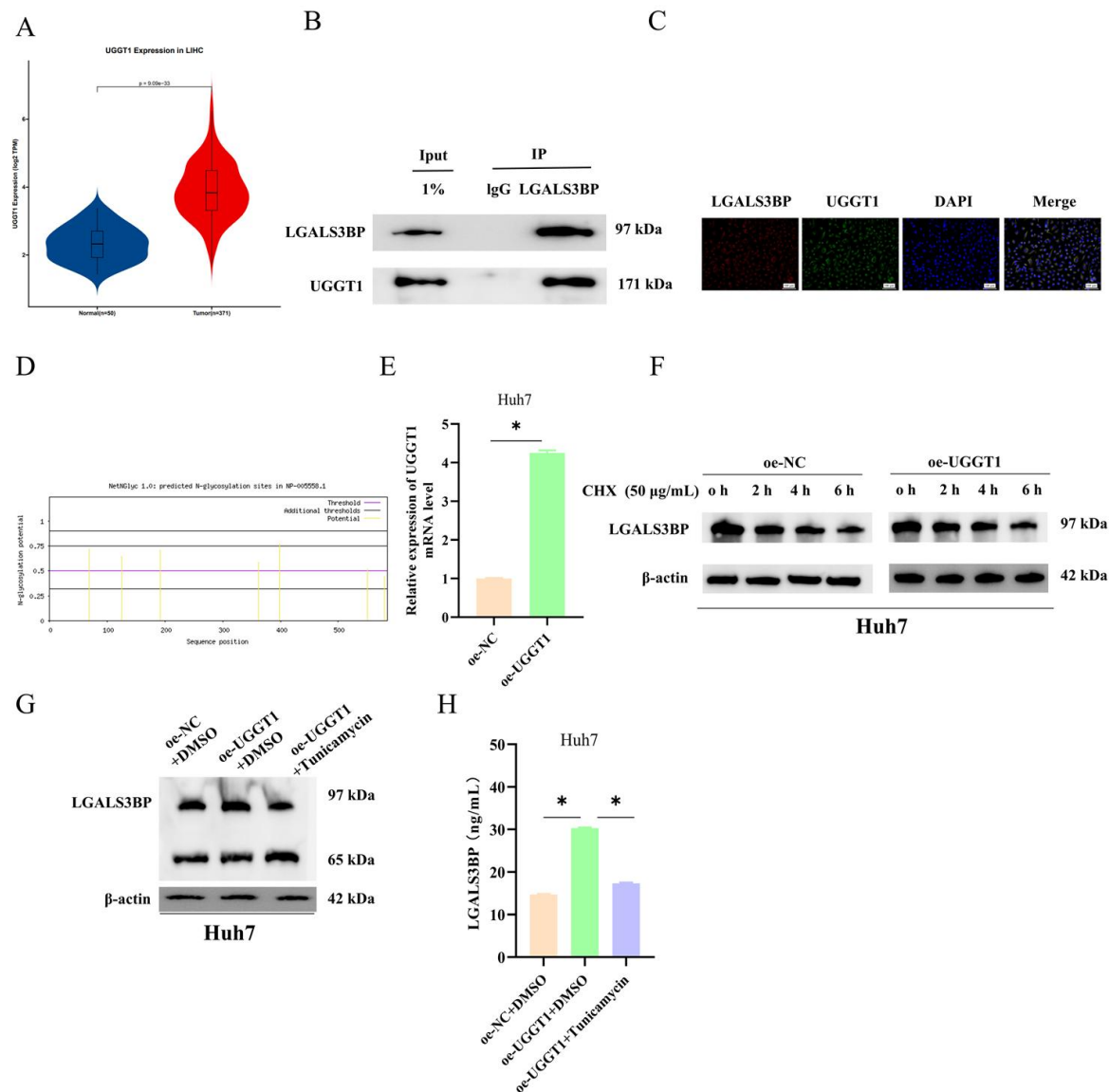


Figure 3 UGGT1 mediates N-glycosylation of LGALS3BP. **A:** Analysis of UGGT1 expression in HCC tissues and normal tissues from TCGA-LIHC. **B:** Co-IP detection of the interaction between UGGT1 and LGALS3BP proteins. **C:** IF detection of co-localization of UGGT1 and LGALS3BP in cells. **D:** Analysis of LGALS3BP N-glycosylation sites using the

559 NetNGlyc website. Cell groups: oe-NC, oe-UGGT1. **E:** qRT-PCR detection of transfection
560 efficiency. **F:** Cells treated with CHX to assess LGALS3BP stability. Cell groups:
561 oe-NC+DMSO, oe-UGGT1+DMSO, oe-UGGT1+Tunicamycin. **G:** WB detection of
562 LGALS3BP expression. **H:** ELISA detection of LGALS3BP secretion levels. *, $P < 0.05$.

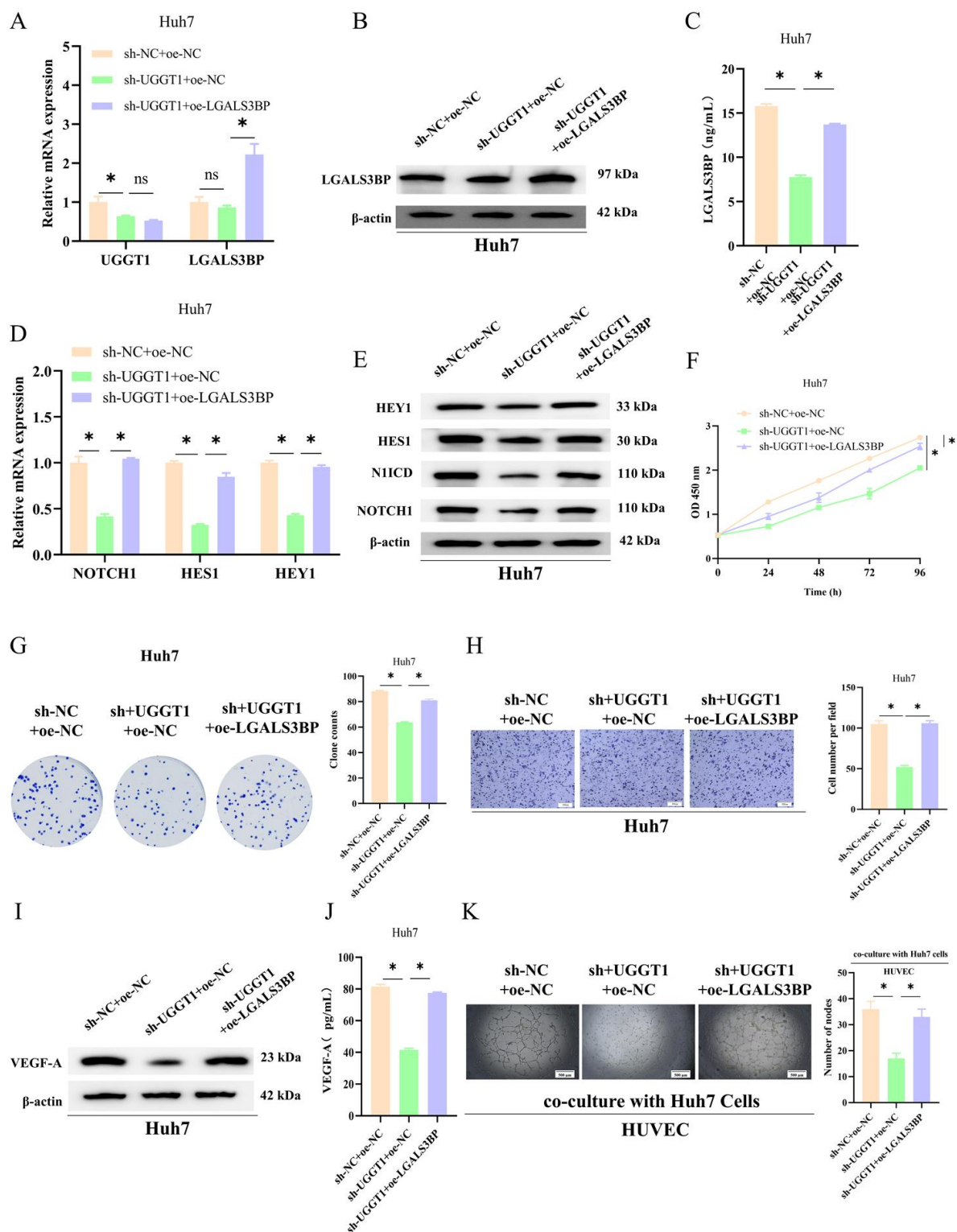


Figure 4 UGGT1 activates the NOTCH signaling pathway by mediating the N-glycosylation of LGALS3BP to facilitate angiogenesis in HCC. Cell experimental groups: sh-NC+oe-NC, sh-UGGT1+oe-NC, sh-UGGT1+oe-LGALS3BP. **A:** qRT-PCR detection of transfection efficiency. **B:** WB detection of LGALS3BP protein expression levels in cells. **C:** ELISA detection of LGALS3BP secretion levels in cells. **D:** qRT-PCR detection of NOTCH1, HES1, and HEY1 mRNA expression levels in cells. **E:** WB detection of NOTCH1, N1ICD, HES1, and HEY1 protein expression levels in cells. **F:** CCK-8 assay for cell viability. **G:** Colony formation assay for cell proliferation. **H:** Transwell assay for cell invasion. **I:** WB detection of VEGF-A protein expression. **J:** ELISA detection of VEGF-A secretion levels. Huh7 cells transfected with sh-NC, sh-UGGT1, and sh-UGGT1+oe-LGALS3BP were co-cultured with HUVECs. **K:** Tube formation assay to assess angiogenesis capability. *, $P<0.05$; ns, not statistically significant.

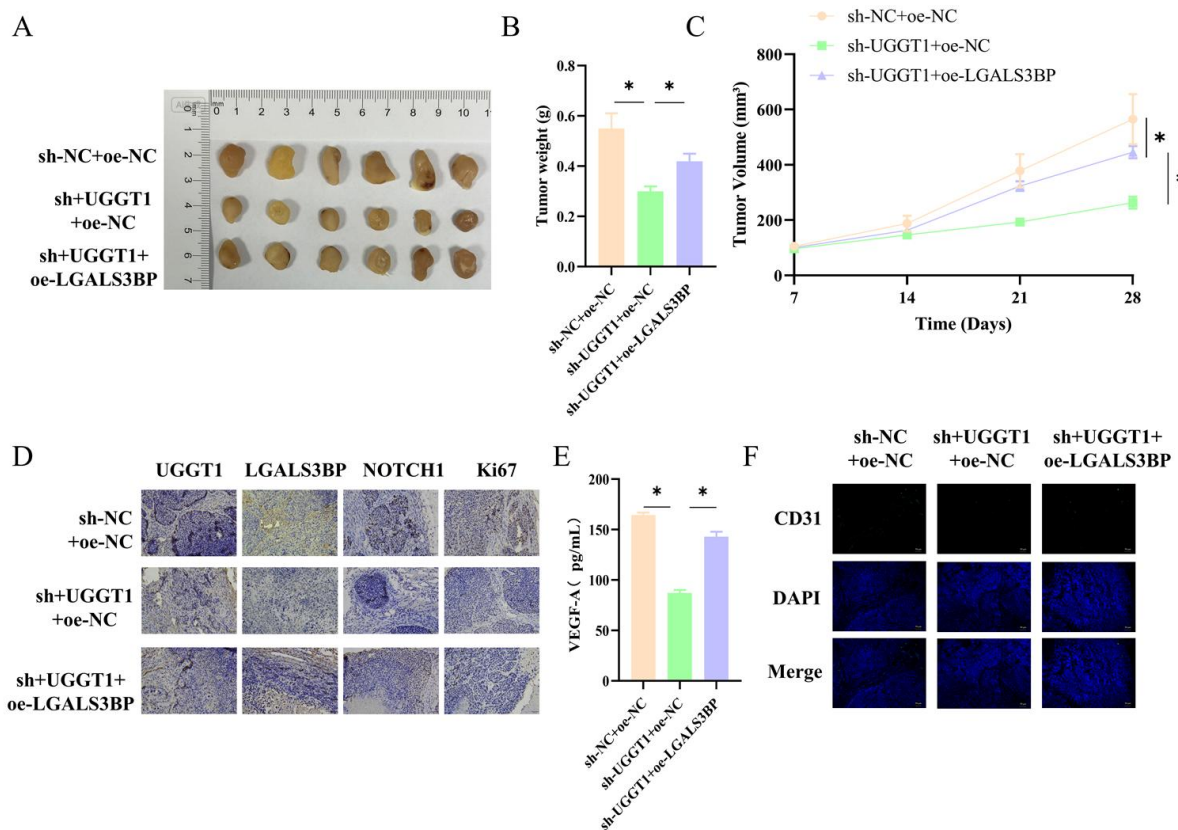


Figure 5 *In vivo* validation that UGGT1/LGALS3BP induces angiogenesis in HCC via the NOTCH signaling pathway. Huh7 cells transfected with sh-NC+oe-NC, sh-UGGT1+oe-NC, and sh-UGGT1+oe-LGALS3BP were subcutaneously injected into BALB/c nude mice to establish a human HCC xenograft model. After 4 weeks, the nude mice

were euthanized. **A:** Representative images of tumors in nude mice. **B-C:** Measurement of tumor weight (**B**) and volume (**C**). **D:** IHC detection of UGGT1, LGALS3BP, NOTCH1, and the proliferation marker Ki67 protein expression. **E:** ELISA detection of VEGF-A secretion levels. **F:** IF observation of CD31 expression. *, $P < 0.05$.

Supplementary Figure

Supplementary Figure 1. Analysis of protein interaction between UGGT1 and LGALS3BP using the BioGRID website.

Supplementary Files

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