

Original Article

GOLM1 upregulates expression of PD-L1 through EGFR/STAT3 pathway in hepatocellular carcinoma

Jiuliang Yan^{1*}, Binghai Zhou^{1,2*}, Lei Guo^{1*}, Zheng Chen^{1*}, Bo Zhang^{1*}, Shuang Liu³, Wentao Zhang¹, Mincheng Yu¹, Yongfeng Xu¹, Yongsheng Xiao¹, Jian Zhou¹, Jia Fan¹, Hui Li¹, Qinghai Ye¹

¹Department of Liver Surgery and Transplantation, Liver Cancer Institute, Zhongshan Hospital, Fudan University, Key Laboratory of Carcinogenesis and Cancer Invasion (Fudan University), Ministry of Education, Shanghai 200032, People's Republic of China; ²Department of Hepatobiliary and Pancreatic Surgery, The Second Affiliated Hospital of Nanchang University, Nanchang 330006, People's Republic of China; ³Department of Neurosurgery, Zhongshan Hospital, Fudan University, Shanghai 200032, People's Republic of China. *Equal contributors.

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Abstract: GOLM1, a type II transmembrane protein, is associated with tumor progression, metastasis and immunosuppression. However, the relationship between GOLM1 and the immunosuppressive molecule PD-L1 in HCC remains largely unclear. Here, we revealed that GOLM1 acts as a novel positive regulator of PD-L1, whose abnormal expression plays a crucial role in cancer immune evasion and progression. We found that GOLM1 is overexpressed and positively correlated with PD-L1 expression in HCC. Mechanistically, we found that GOLM1 promotes the phosphorylation of STAT3 by enhancing the level of EGFR, which in turn upregulates the transcriptional expression of PD-L1. Taken together, we demonstrated that GOLM1 acts as a positive regulator of PD-L1 expression via the EGFR/STAT3 signaling pathway in human HCC cells. This study provides a new insight into the regulatory mechanism of PD-L1 expression in HCC, which may provide a novel therapeutic target for HCC immunotherapy.

Keywords: GOLM1, hepatocellular carcinoma, PD-L1, EGFR, STAT3

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the second leading cause of cancer-related deaths in the world [1]. Although great progress has been made in the diagnosis and treatment of HCC, its overall prognosis is still poor, with a 5-year survival rate of approximately 12% [2]. At present, treatments for early HCC include surgical resection, liver transplantation and local radiofrequency (RF) ablation [3], but their efficacy is still limited. Molecular targeted therapies, such as the small-molecule multikinase inhibitor sorafenib (first-line use) [4], regorafenib (second-line use) [5] and lenvatinib (first-line use) [6] have been used for the treatment of advanced HCC. However, these drugs only extend the median overall survival of patients with advanced HCC by no longer than 4 months [7]. Immune checkpoint blockade (ICB) has brought considerable clinical benefits in the treatment of different

tumors [8-11]. Among these immune checkpoints, the research on programmed cell death protein 1 and its ligand (PD-1/PD-L1) has attracted the most widespread attention [12]. However, the anti-PD-1 therapy approved for HCC treatment only achieved a 20% response rate [13]. Treatment with pembrolizumab or nivolumab failed to reach the primary endpoints of the KEYNOTE-240 and CheckMate-459 HCC clinical trials [14, 15]. Thus, exploring the regulatory mechanism of immune checkpoints and identifying novel therapies to improve HCC patients' long-term outcome is urgently needed.

Golgi membrane protein 1 (GOLM1, also known as Golgi protein 73, GP73 or Golgi phosphoprotein 2, GOLPH2), a type II transmembrane protein, is associated with tumor progression [16, 17], metastasis [18-21] and immunosuppression [22, 23]. It has been reported that the expression level of GOLM1 can be increased

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not only in viral infections [24-27] but also in several types of cancer, such as HCC, lung adenocarcinoma and prostate cancer [28-30]. Our previous study revealed that GOLM1 is one of the leading genes associated with HCC metastasis. We found that GOLM1 expression was closely related to early cancer recurrence, metastasis and poor prognosis in HCC patients. Detailed studies revealed that GOLM1, as a specific mediator (cargo adaptor), selectively interacts with epidermal growth factor receptor (EGFR) to assist EGFR/RTK anchoring on the trans-Golgi network (TGN) and recycling back to the plasma membrane, leading to the continued activation of downstream kinases, and finally mediating HCC metastasis [18]. However, the relationship between GOLM1 and the immunosuppressive molecule PD-L1 in HCC remains largely unclear.

Studies in various tumor types have demonstrated that levels of PD-L1 expression can predict therapeutic responses to monotherapies blocking the PD-1/PD-L1 axis [31-33]. These levels of PD-L1 expression are regulated in highly complicated manners and can be affected by transcriptional control and post-translational regulation [34]. One of the most critical transcription factors in human cancer cells is the signal transducer and activator of transcription 3 (STAT3), which can directly act on the PD-L1 promoter to enhance PD-L1 expression [35]. Some studies have suggested that EGFR is involved in the activation of STAT3 [36-38]. Activated EGFR recruits and phosphorylates STAT3 at Tyr705, then phosphorylated STAT3 enters the nucleus to promote the expression of some cancer-related genes [39-41]. Due to the important regulation of GOLM1 on EGFR, we speculate that there might be a regulatory relationship in the GOLM1/EGFR/STAT3/PD-L1 axis.

Herein, we found that GOLM1 promoted PD-L1 transcriptional expression by enhancing STAT3 Tyr705 phosphorylation. Specifically, we found that GOLM1 enhanced levels of EGFR, hence the levels of phosphorylated EGFR, which phosphorylated STAT3, promoting the expression of PD-L1 in HCC. Our results identified that GOLM1 is a positive regulator of PD-L1 expression, which provides a new insight in the relationship between GOLM1 and immune response.

Materials and methods

Antibodies and reagents

The antibodies listed below were used in Western blotting, immunohistochemical: anti-GOLM1 (ab109628, Abcam; ab92612, Abcam), anti-PD-L1 (#13684T, Cell Signaling Technology; #64988S, Cell Signaling Technology), anti-STAT3 (#9139S, Cell Signaling Technology), anti-p-STAT3 (Tyr705) (#9145S, Cell Signaling Technology), anti-EGFR (ab52894, Abcam), anti-p-EGFR (phospho Y1068) (ab40815, Abcam). BP-1-102 (#T3708) was purchased from Topscience. Gefitinib (ab142052) was purchased from Abcam.

HCC cell lines

The HCC cell lines HCC-LM3, Huh7, Hep3B, Bel-7402 and Hepa1-6 were obtained from Liver Cancer Institute and Zhongshan Hospital, Fudan University, Shanghai, China. These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco). All cell lines were incubated in a humidified incubator containing 5% CO₂ at 37°C.

Tissue microarray and immunohistochemistry

Tissue microarrays containing 239 matched pairs of primary HCC samples and adjacent nontumor liver tissues were constructed as described previously [42, 43]. In brief, tumor specimens were collected from HCC patients who underwent surgical resection from January 2005 to December 2006 in Liver Surgery Department of Zhongshan Hospital, Fudan University, Shanghai, China. The Research Ethics Committee of Zhongshan Hospital approved the research protocol and all patients signed the informed consent forms. Paraffin-embedded implanted tumors were cut into 5- μ m sections. Immunohistochemical (IHC) staining of the HCC samples was performed as described previously [44]. Briefly, each sample was stained with the indicated antibodies and then incubated with an avidin-biotin-peroxidase complex. The chromogen 3-amino-9-ethylcarbazole was used to visualize the target protein. The expression levels of GOLM1 and PD-L1 in

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tissues were evaluated by the H-score method through combining the value of the immunoreaction intensity and the percentage of stained cells [45]. $H\text{-score} = \sum \pi (i + 1)$, where i is the intensity of the stained cells (0 for negative staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining) and π is the value corresponding to the percentage of stained cells with different intensities (0, < 5% positive cells; 1, 6-25% positive cells; 2, 26-50% positive cells; 3, 51-75% positive cells; 4, > 75% positive cells). Tumor tissues with H-scores greater than the median of all scored tumor tissues were classified as high expression.

The encyclopedia of RNA interactomes (ENCORI) database

Co-Expression Analysis of GOLM1 and PD-L1 in 374 HCC patients in The Encyclopedia of RNA Interactomes (ENCORI) database is available from the Web site of (<https://starbase.sysu.edu.cn/panGeneCoExp.php#>).

Western blotting

Total proteins extracted from different HCC cell lines with cell lysis buffer supplemented with protease inhibitors were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). After being blocked with 5% nonfat milk in TBST for 1 h at room temperature, the membranes were incubated with the primary antibody at 4°C overnight and then incubated with HRP-conjugated goat anti-mouse or anti-rabbit secondary antibodies for 2 h at room temperature after being washed 3 times with TBST. And then the membranes were washed 3 times and detected with an ECL detection kit (Thermo Fisher Scientific, Waltham, MA). The band intensity was quantified with Image J (National Institutes of Health, Bethesda, MD, USA).

RNA extraction and real-time quantitative reverse-transcription polymerase chain reaction

Total RNA was isolated by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). According to the manufacturer's protocol (Takara), the same amount of RNA was reverse transcribed into cDNA. Real-time quantitative reverse-transcription polymerase chain reaction (RT-qPCR) was

performed using SYBR-Green PCR Master mix (Yeasen Biotechnology Co., Ltd.) according to the manufacturer's instructions. The primers' sequences used were as follows: human GOLM1 forward, 5'-CCGGAGCCTCGAAAAGAG-ATT-3' and reverse, 5'-ATGATCCGTGTCTGGAG-GTC-3'; human PD-L1 forward, 5'-TCACTTG-GTAATTCTGGGAGC-3' and reverse, 5'-CTTTG-AGTTTGTATCTTGGATGCC-3'; human GAPDH forward, 5'-ACAGTCCATGCCATCACTGCC-3' and reverse, 5'-GCCTGCTCACACCTTCTTG-3'.

Lentivirus transfection

Cells were seeded into the 6-well cell culture plates and were cultured for 24 hours to 50% confluence in DMEM containing 10% FBS at 37°C. Then the medium was changed to serum-free medium. According to the pre-experimental infection conditions, the lentiviruses (GeneChem, Shanghai, China) were added to the 6-well cell culture plates. After 12 hours, the cell culture medium was replaced with complete medium. The efficiency of transfection was measured under the fluorescence microscope. After 72 hours, the cells were cultured in medium containing 5 µg/ml puromycin until the fluorescence efficiency reached nearly 100%. Then the concentration of puromycin was reduced to 1 µg/ml to select stably transfected cells. The expression level of GOLM1 was evaluated by Western blotting and RT-qPCR.

Cell proliferation assay

Cell proliferation was assessed with cell counting kit-8 (CCK-8) method (Dojindo, Kumamoto, Japan). Briefly, cells were added into 96-well plates at the initial density of 5×10^3 cells/well. At time points of 24, 48 and 72 hours, 10 µL of CCK-8 reagent was added to the cells in high-glucose DMEM medium for 2 hours. And then the absorbance values were measured at wavelength of 450 nm. Experiments were repeated three times under the same conditions.

Cell migration assay

Cell migration was measured with a Transwell migration apparatus (Becton Dickinson Labware, Franklin Lakes, NJ) of 8-µm pore-size. HCC cells (5×10^4 cells for Hep3B, 2×10^4 cells for Huh7) mixed with 100 µl of serum-free DMEM were added into the upper chamber,

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and 500 μ l of DMEM containing 10% FBS were added into the lower chamber. Then, cells were cultured at 37°C and in a humidified atmosphere with 5% CO₂. After 36 h, cells in the upper chamber without migration were removed with a cotton swab. The membranes were subsequently washed with phosphate buffered saline (PBS), and cells adhered to the membranes were then fixed with 4% paraformaldehyde for 15 min, and stained with Giemsa dye for 10 min. The stained cells were washed with PBS three times and counted under a microscope. The data were presented as mean counts of stained cells in 12 random fields/chamber. Experiments were repeated three times under the same conditions.

In vivo experiment

Hepa1-6 cells with stable knockdown of GOLM1 (Hepa1-6-GOLM1-KD) were constructed through lentiviral transfection. Hepa1-6-NC cells were used as negative control. The C57BL/6 mice (male, 5-6 weeks old, weighing 20-22 g) were randomly divided into 2 groups (Hepa1-6-NC and Hepa1-6-GOLM1-KD, 5 mice per group). Cells were injected (1×10^7 cells, suspended in 0.2 mL PBS, transplanted subcutaneously) to grow into tumors. This study was approved by the Shanghai Medical Experimental Animal Care Committee and performed according to the National Institutes of Health "Guide for the Care and Use of Laboratory Animals". Subcutaneous tumors were measured using a caliper every 3 days. Tumor volumes were calculated using the formula: tumor volume = length \times width²/2. At the end of the experiment, the mice were euthanized by cervical dislocation, and the tumors were weighed and then used for subsequent histological analyses.

Statistical analysis

The data were presented as the means \pm standard deviation (SD). All statistical analyses were carried out with GraphPad Prism 7 (GraphPad Software, CA, USA). Student's t test was performed to analyze the differences between two groups. Survival curves were estimated by the Kaplan-Meier method, and the log-rank test was applied to compare two groups. The association between GOLM1 or PD-L1 expression and clinicopathological variables was analyzed by the χ^2 test or the Fisher's exact test. The correlation between the expres-

sion levels of GOLM1 and PD-L1 was analyzed by Pearson correlation analysis and linear regression analysis. $P < 0.05$ was considered statistically significant.

Results

GOLM1 is overexpressed in human HCC and associated with tumor progression and poor survival

We conducted the IHC analysis on tissue microarrays including tumor samples and corresponding adjacent normal liver tissues from 239 patients. The representative GOLM1 IHC staining images of cancer tissues and corresponding adjacent normal tissues are shown in the **Figure 1A** and **1B**. The expression level of GOLM1 in cancer tissues was significantly higher than that in adjacent normal tissues ($P < 0.0001$) (**Figure 1C**). The correlation between clinicopathological features and GOLM1 expression is shown in **Table 1**. A high expression level of GOLM1 was related to tumor size ($P = 0.0007$), tumor number ($P = 0.0269$), cancer thrombus ($P = 0.0102$) and tumor recurrence ($P = 0.0027$) (**Table 1**), indicating that the high expression of GOLM1 promotes the progression of HCC. We also found that high expression of GOLM1 was closely related to HBV infection ($P = 0.0129$) (**Table 1**). Survival analysis showed that the overall survival (OS) of patients with high GOLM1 expression was poorer than those with low GOLM1 expression ($P = 0.0011$) (**Figure 1F**). These findings suggest that the high expression of GOLM1 is correlated with the progression of HCC and poor survival.

GOLM1 is positively correlated with PD-L1 expression in human HCC tissue

GOLM1 was reported to play an important role in tumor progression [16, 17], metastasis [18-21] and immunosuppression [22, 23], and its overexpression has been associated with cancer recurrence, metastasis and poor prognosis in different tumors [16, 18-21, 46], but the effect of GOLM1 on the immunosuppressive molecule PD-L1 remains largely unknown. To determine the relation between GOLM1 and PD-L1 expression, we measured their expression in HCC and corresponding adjacent normal tissues by tissue microarray. The representative PD-L1 IHC staining images of cancer tissues and corresponding adjacent normal tis-

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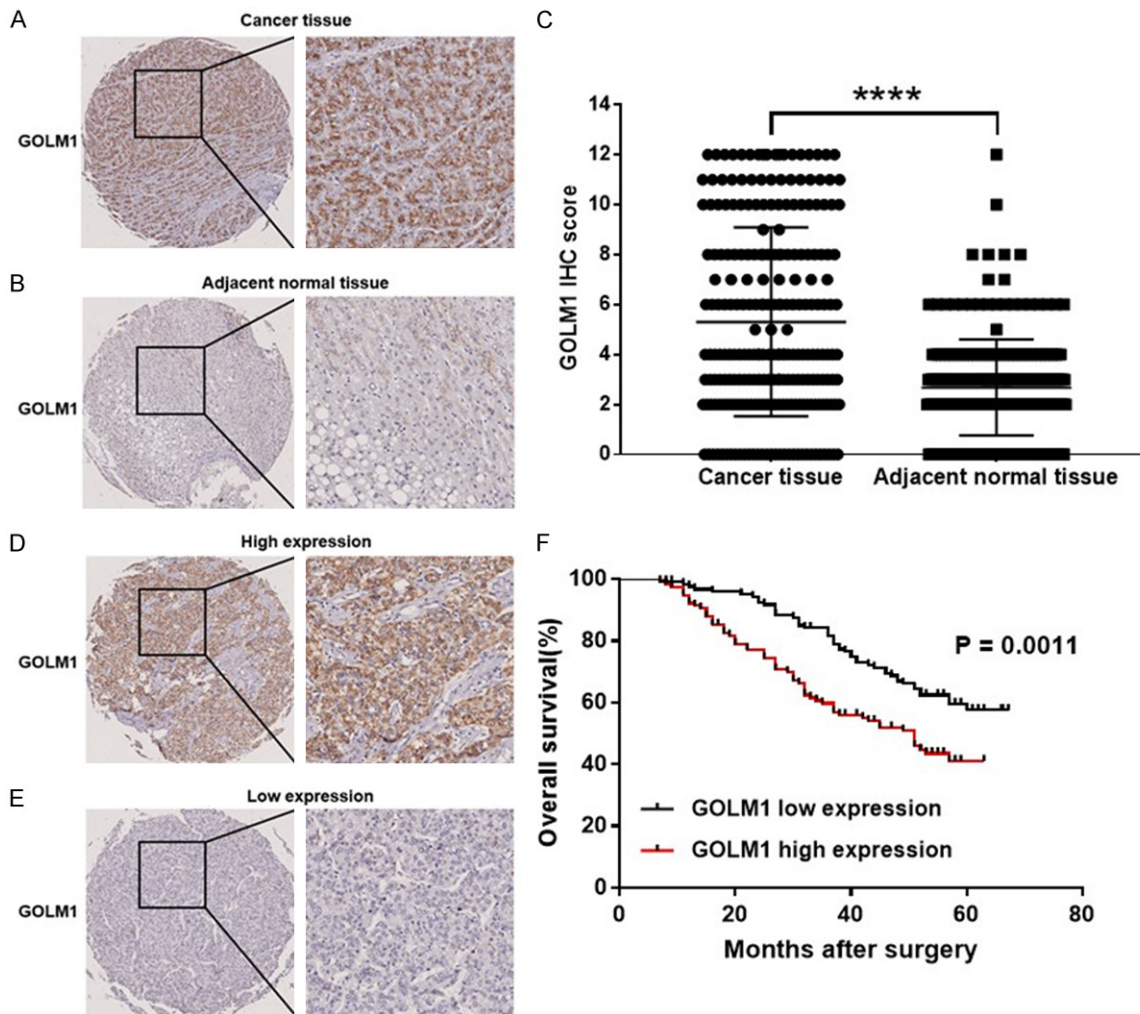


Figure 1. Expression of GOLM1 in HCC. GOLM1 IHC staining of (A) cancer tissues and (B) corresponding adjacent normal tissues (Scale bar, 100 μ m). (C) H-score of GOLM1 expression of cancer tissues and adjacent normal tissues. (D) High GOLM1 expression in human HCC tissues (Scale bar, 100 μ m). (E) Low GOLM1 expression in human HCC tissues (Scale bar, 100 μ m). (F) Survival curves of HCC patients with GOLM1 high expression and GOLM1 low expression. Significance was evaluated using the Log-rank test ($P = 0.0011$). Tumor tissues with H-scores greater than the median of all scored tumor tissues were classified as high GOLM1 expression. **** $P < 0.0001$.

sues are shown in the **Figure 2A** and **2B**. We found that the expression level of PD-L1 in cancer tissues was significantly higher than that in adjacent nontumor tissues (**Figure 2C**). The correlation between PD-L1 and the clinicopathological factors was summarized in **Table 1**. A high expression level of PD-L1 was associated with alpha fetoprotein (AFP) level ($P = 0.0163$) and tumor recurrence ($P = 0.0182$) (**Table 1**). Survival analysis showed the OS of patients with high PD-L1 expression was poorer than those with low PD-L1 expression ($P = 0.0326$) (**Figure 2F**). Representative pictures of IHC staining of HCC for GOLM1 and PD-L1 are shown in the **Figure 3A**. We then investigated the correlation between GOLM1 and PD-L1

using Pearson correlation and linear regression analysis and found that PD-L1 expression was positively correlated with GOLM1 expression ($r = 0.3919$, $P < 0.0001$) (**Figure 3B**). This finding was further confirmed using the ENCORI database, which showed a positive correlation between GOLM1 and PD-L1 mRNA expression ($r = 0.224$, $P = 0.0000128$, **Figure 3C**), implying that the regulation between GOLM1 and PD-L1 may occur at the transcriptional level.

GOLM1 upregulates PD-L1 mRNA expression in HCC cell lines

Our previous study found that compared with HCC cell lines with lower metastatic potentials,

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Table 1. Correlation of GOLM1 or PD-L1 expression with clinicopathological factors

Parameters	n	GOLM1		P value	PD-L1		P value
		Low	High		Low	High	
Age (yr)							
≤ 50	89	49	40	0.7203	45	44	0.5387
> 50	150	79	71		82	68	
Gender							
Male	197	103	94	0.3931	104	93	0.8163
Female	42	25	17		23	19	
HBsAg							
Positive	225	116	109	0.0129*	117	108	0.1575
Negative	14	12	2		10	4	
AFP (ng/mL)							
≤ 20	94	55	39	0.2163	59	35	0.0163*
> 20	145	73	72		68	77	
Tumor size (cm)							
≤ 5	156	96	60	0.0007***	87	69	0.2638
> 5	83	32	51		40	43	
Tumor number							
Solitary	197	112	85	0.0269*	108	89	0.2584
Multiple	42	16	26		19	23	
Cancer thrombus							
Yes	81	34	47	0.0102*	37	44	0.0980
No	158	94	64		90	68	
Tumor capsule							
Yes	132	72	60	0.7335	72	60	0.6282
No	107	56	51		55	52	
Tumor recurrence							
Yes	90	37	53	0.0027**	39	51	0.0182*
No	149	91	58		88	61	
Clinical stage							
I+II	236	127	109	0.4797	125	111	0.6365
III	3	1	2		2	1	

*P < 0.05, **P < 0.01, ***P < 0.001.

HCC cells with higher invasion and metastasis capabilities have significantly increased GOLM1 levels [18]. To determinate whether GOLM1 regulates PD-L1 expression, we first detected the expression of GOLM1 and PD-L1 in HCC cell lines: HCC-LM3, Huh7, Hep3B and Bel-7402 cells (HCC-LM3 and Huh7 cells with higher invasion and metastasis capabilities; Hep3B and Bel-7402 cells with lower invasion and metastasis potentials). We found that the expression levels of GOLM1 and PD-L1 were relatively high in HCC-LM3 and Huh7 cell lines and relatively low in Hep3B and Bel-7402 cell lines at both protein (**Figure 4A, 4C**) and mRNA levels (**Figure**

4B, 4D). Among these four cell lines, the expression of GOLM1 was highest in Huh7 cells and lowest in Hep3B cells. We then 1) knocked down GOLM1 expression in Huh7 cells and 2) overexpressed GOLM1 in Hep3B cells by the lentivirus transfection (**Supplementary Figure 1A, 1B**). We found that PD-L1 expression was significantly decreased when knocking down GOLM1 expression in Huh7 cells (**Figure 4E, 4F**), while its expression was significantly up-regulated when GOLM1 expression was overexpressed in Hep3B cells (**Figure 4G, 4H**). These results indicate that GOLM1 may positively regulate PD-L1 mRNA expression.

GOLM1 upregulates PD-L1 expression via the EGFR/STAT3 pathway

Given STAT3 can directly act on the PD-L1 promoter to increase PD-L1 expression in human cancer cells [35], EGFR is suggested to be involved in STAT3 activation [36-38], and GOLM1 can modulate EGFR levels [18], we

hypothesized that GOLM1 regulates PD-L1 mRNA expression by activating EGFR/STAT3 pathway.

First, we found that STAT3 Tyr705 phosphorylation was inhibited after knocking down GOLM1 in Huh7 cells, while it was enhanced after overexpressing GOLM1 in Hep3B cells (**Figure 5A**).

To investigate whether the GOLM1-mediated upregulation of PD-L1 expression is dependent on STAT3 Tyr705 phosphorylation in HCC, we further inhibited STAT3 with selective small-molecule inhibitors (BP-1-102) in GOLM1-over-

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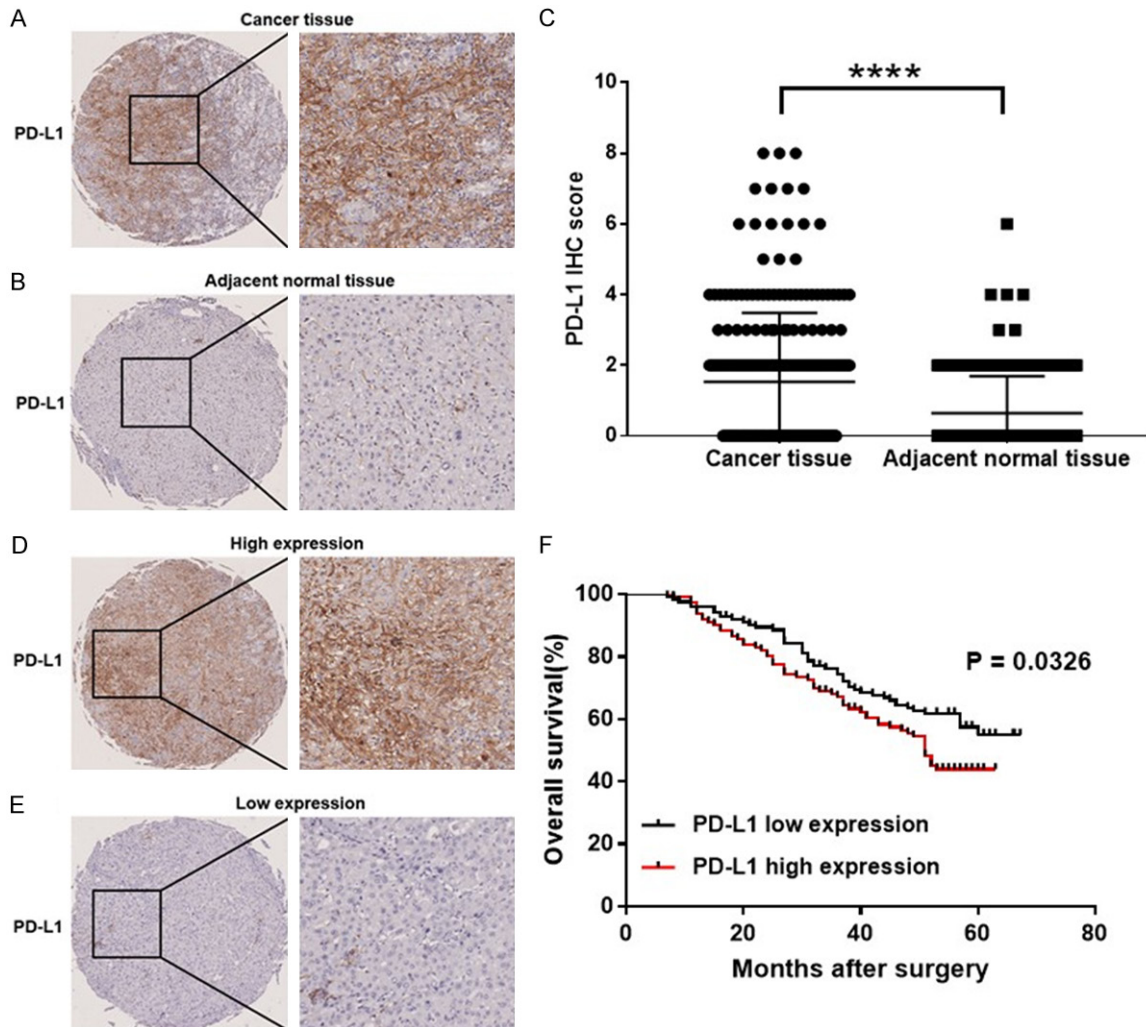


Figure 2. Expression of PD-L1 in HCC. PD-L1 IHC staining of (A) cancer tissues and corresponding (B) adjacent normal tissues (Scale bar, 100 μ m). (C) H-score of PD-L1 expression of cancer tissues and adjacent normal tissues. (D) High PD-L1 expression in human HCC tissues (Scale bar, 100 μ m). (E) Low PD-L1 expression in human HCC tissues (Scale bar, 100 μ m). (F) Survival curves of HCC patients with PD-L1 high expression and PD-L1 low expression. Significance was evaluated using the Log-rank test ($P = 0.0326$). Tumor tissues with H-scores greater than the median of all scored tumor tissues were classified as high PD-L1 expression. **** $P < 0.0001$.

expressed Hep3B cells. PD-L1 expression was decreased when STAT3 Tyr705 phosphorylation was inhibited (Figure 5B, 5C), suggesting that the upregulation of PD-L1 expression induced by GOLM1 is dependent on STAT3 Tyr705 phosphorylation.

To determine whether the GOLM1-mediated upregulation of STAT3 Tyr705 phosphorylation is dependent on EGFR phosphorylation in HCC, we investigated the effect of GOLM1 on the phosphorylation of EGFR. We found that the phosphorylated EGFR were significantly increased after overexpressing GOLM1 in Hep3B

cells. We then blocked the phosphorylation of EGFR with the inhibitor Gefitinib and found that the phosphorylation of STAT3 was significantly inhibited in Huh7 cells (Figure 5D, 5E). Furthermore, we found that expression of PD-L1 and the phosphorylation of STAT3 were simultaneously suppressed after Gefitinib treatment in GOLM1 overexpressed Hep3B cells (Figure 5F, 5G), indicating that GOLM1 regulates phosphorylation of STAT3 and expression of PD-L1 by promoting phosphorylation of EGFR. Taken together, the above data suggests that GOLM1 upregulates the expression of PD-L1 via the EGFR/STAT3 pathway.

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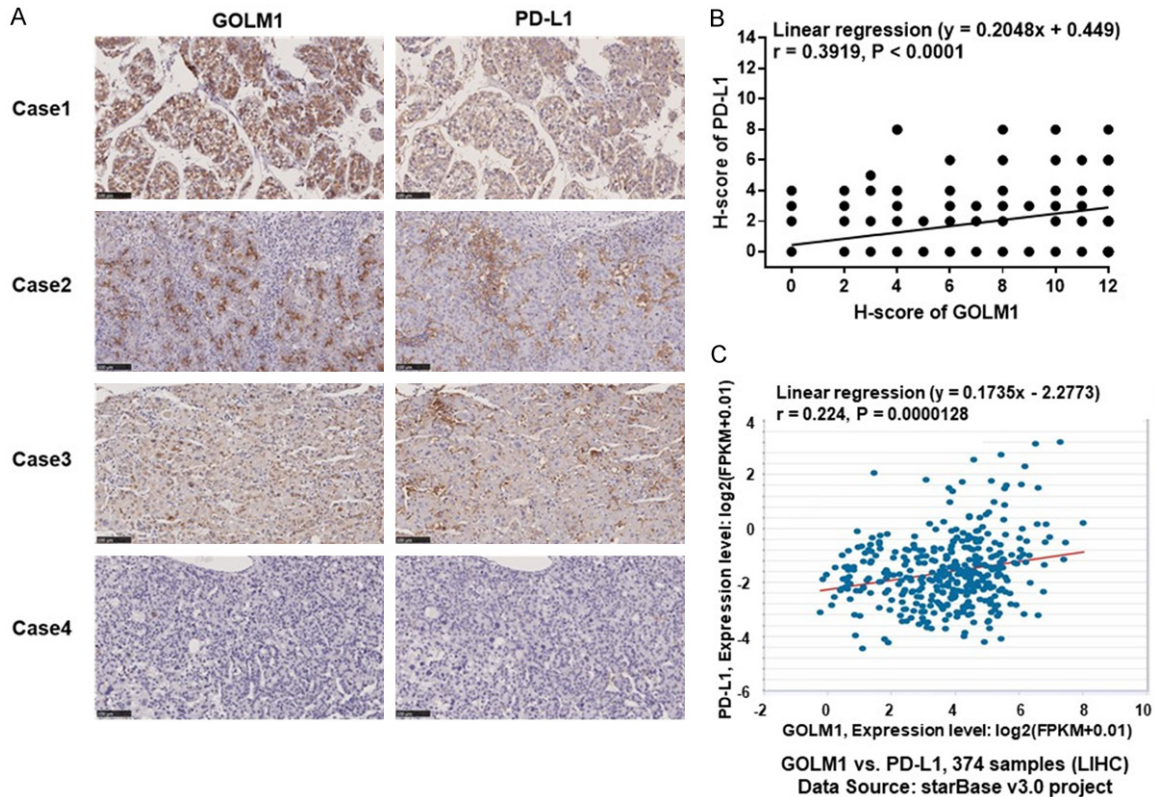


Figure 3. Correlation of GOLM1 and PD-L1. (A) Patient tissue samples were stained for GOLM1 and PD-L1. Representative pictures of IHC staining of HCC tumors for GOLM1 and PD-L1. (B) Correlation analysis of GOLM1 and PD-L1 expression in human HCC tissue microarray (n = 239, Linear regression (y = 0.2048x+0.449), r = 0.3919, P < 0.0001). (C) Correlation of GOLM1 and PD-L1 gene expression in the ENCORI database (C) from the ENCORI database.

GOLM1 promotes the proliferation and migration abilities of HCC cells in vitro

We conducted the CCK-8 assay to evaluate the effects of GOLM1 on cell proliferation in vitro. We observed that GOLM1 knockdown decreased the proliferation of Hep3B cells and GOLM1 overexpression promoted the proliferation of Huh7 cells significantly in vitro compared to their respective negative controls at the time point of 72 h (Supplementary Figure 2A, 2B). Transwell migration assay was conducted to investigate the modulation of GOLM1 on cell migration in vitro. As shown in Supplementary Figure 2C-F, GOLM1 overexpression promoted the migration of Hep3B cells compared with the negative control group. In contrast, the knockdown of GOLM1 decreased the migration ability of Huh7 cells.

GOLM1 knockdown inhibits tumor growth and downregulates the expression of PD-L1 in Hepa1-6 cells in vivo

The subcutaneous tumor model was used to evaluate the effect of GOLM1 on HCC cell prolif-

eration in vivo. Mice were randomly divided into 2 groups (Hepa1-6-NC and Hepa1-6-GOLM1-KD, 5 mice per group). Comparison of the two groups of mice showed that the tumors in GOLM1-KD group were significantly smaller than those in NC group (Figure 6A-C). IHC staining of PD-L1 showed that GOLM1 knockdown downregulates PD-L1 expression. PD-L1 expression in the GOLM1-KD group was significantly lower than that in the NC group (Figure 6D, 6E). Thus, GOLM1 plays an important role in promoting tumor growth and regulating PD-L1 expression.

Discussion

GOLM1 was reported to play an important role in tumor progression [16, 17], metastasis [18-21] and immunosuppression [22, 23], and its overexpression was associated with cancer recurrence, metastasis, and poor prognosis in different tumors [16, 18-21, 46]. Especially, in our previous study, we found that GOLM1 could lead to the continued activation of downstream kinases by stabilizing EGFR expression, and

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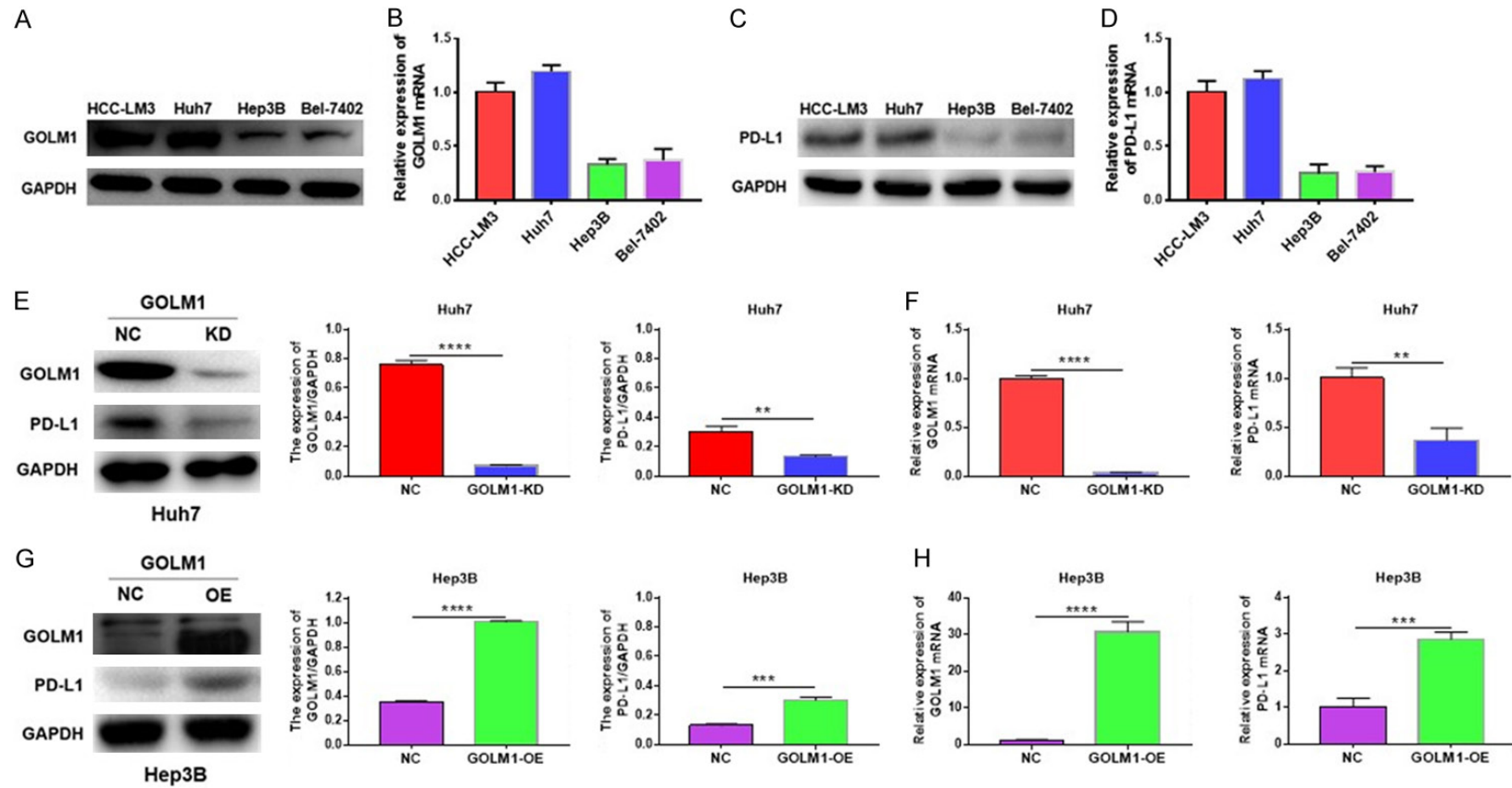


Figure 4. GOLM1 upregulates PD-L1 expression in HCC cell lines. A and B. Expression levels of GOLM1 in different HCC cell lines: HCC-LM3, Huh7, Hep3B and Bel-7402. C and D. Expression levels of PD-L1 in different HCC cell lines: HCC-LM3, Huh7, Hep3B and Bel-7402. Protein expression was detected by Western blotting. mRNA expression was detected by RT-qPCR. E and F. Western blot and RT-qPCR analysis of PD-L1 levels in GOLM1-knockdown Huh7 cells. Quantitative analysis of GOLM1 and PD-L1 expression after GOLM1 was knockdown in Huh7 cells through Image J intensity measurement. G and H. Western blot and RT-qPCR analysis of PD-L1 levels in GOLM1-overexpressed Hep3B cells. Quantitative analysis of GOLM1 and PD-L1 expression after GOLM1 was overexpressed in Hep3B cells through Image J intensity measurement. **P < 0.01, ***P < 0.001, ****P < 0.0001.

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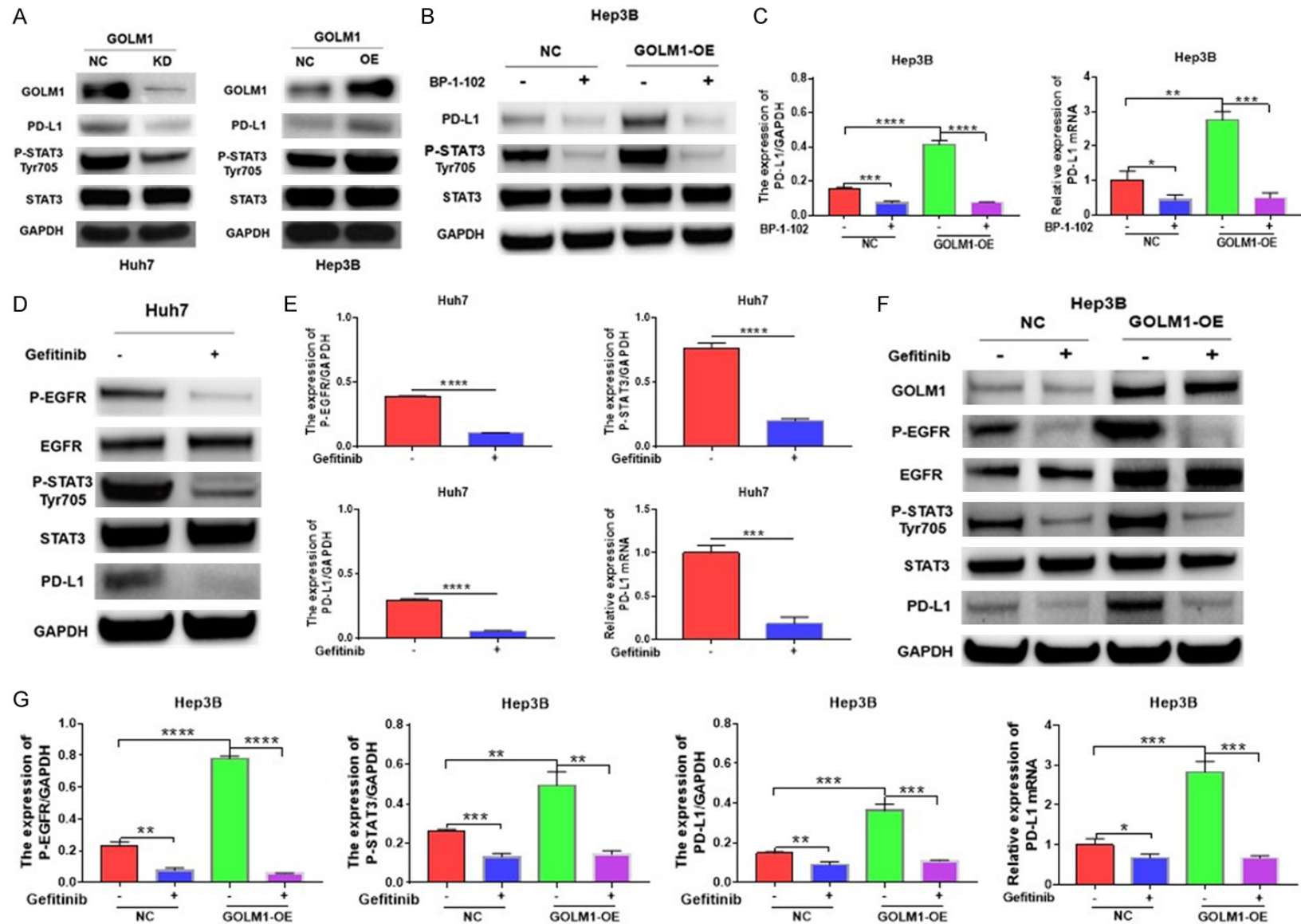


Figure 5. GOLM1 promotes PD-L1 expression via the EGFR/STAT3 pathway. **A.** Western blot analysis of PD-L1 and phosphorylation of STAT3 Tyr705 in GOLM1-knockdown Huh7 cells and in GOLM1-overexpressed Hep3B cells. **B.** GOLM1 upregulates the expression of PD-L1 through promoting phosphorylation of STAT3 Tyr705 (P-STAT3). The expression of PD-L1, P-STAT3 and STAT3 was detected by Western blot after incubation with BP-1-102 (5 μ M) for 12 hours in Hep3B cells. **C.**

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Quantitative analysis of PD-L1 expression after treatment with BP-1-102 through Image J intensity measurement. The relative expression of PD-L1 mRNA in Hep3B cells and GOLM1-overexpressed Hep3B cells was detected by RT-qPCR after incubation with BP-1-102 (5 μ M) for 12 hours. D. The expression of EGFR, the phosphorylation of EGFR (P-EGFR), STAT3, P-STAT3 and PD-L1 was detected by Western blot after treatment with Gefitinib (4 μ M) for 6 hours in Huh7 cells. E. Quantitative analysis of P-EGFR, P-STAT3 and PD-L1 expression after treatment with Gefitinib through Image J intensity measurement. The relative expression of PD-L1 mRNA was detected by RT-qPCR after treatment with Gefitinib in Huh7 cells. F. GOLM1 enhances the phosphorylation of STAT3 Tyr705 through the regulation of P-EGFR and EGFR. The expression of GOLM1, EGFR, P-EGFR, STAT3, and P-STAT3 was detected by Western blot in Hep3B cells and GOLM1-overexpressed Hep3B cells after incubation with Gefitinib. G. Quantitative analysis of P-EGFR, P-STAT3 and PD-L1 expression after treatment with Gefitinib through Image J intensity measurement. The relative expression of PD-L1 mRNA in Hep3B cells and GOLM1-overexpressed Hep3B cells was detected by RT-qPCR after treatment with Gefitinib. Cells were incubated with EGF (50 ng/ml).

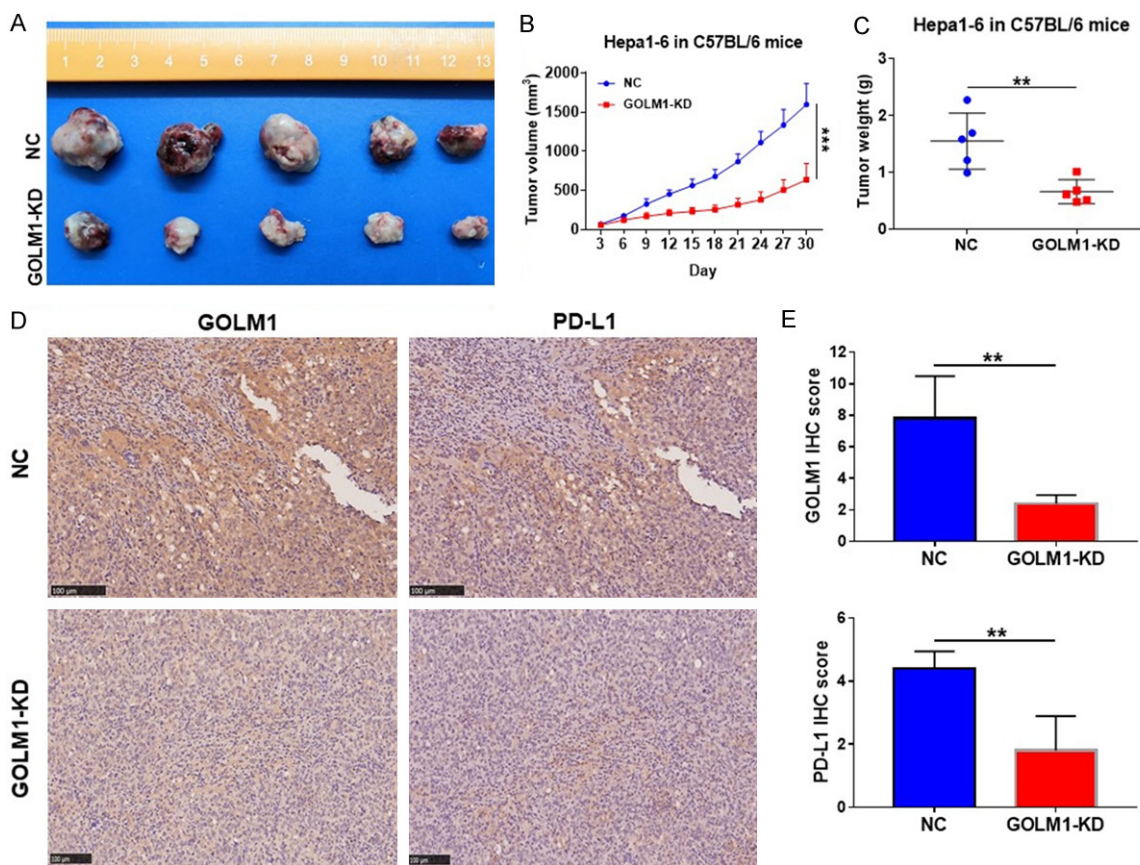


Figure 6. GOLM1 knockdown inhibits tumor growth and downregulates the expression of PD-L1 in Hepa1-6 cells in vivo. A. Images of Hepa1-6 subcutaneous HCC tumors from each group ($n = 5$). B. Tumor growth curves in C57BL/6 mice from the Hepa1-6-NC and -GOLM1-KD groups. C. Tumor weight from the Hepa1-6-NC and -GOLM1-KD groups. D. Representative IHC images of GOLM1 and PD-L1 expression in Hepa1-6 subcutaneous HCC tumors. Scale bar = 100 μ m. E. Graphic representation of IHC score of GOLM1 and PD-L1 expression in Hepa1-6 subcutaneous HCC tumors from NC and GOLM1-KD groups. ** $P < 0.01$, *** $P < 0.001$.

finally mediating HCC metastasis [18]. In addition, GOLM1 was reported inducing immunosuppression, but the effect of GOLM1 on the immunosuppressive molecule PD-L1 remains largely unknown. In this study, we unexpectedly found that GOLM1 upregulated PD-L1 transcriptional expression by promoting STAT3

Tyr705 phosphorylation. Furthermore, we found that GOLM1 enhanced the level of EGFR, further leading to upregulation of phosphorylation of STAT3, and finally promoted the expression of PD-L1 in HCC. In conclusion, our results suggest a novel molecular regulatory mechanism - GOLM1/EGFR/STAT3/PD-L1 signaling pathway

GOLM1 upregulates expression of PD-L1 in HCC

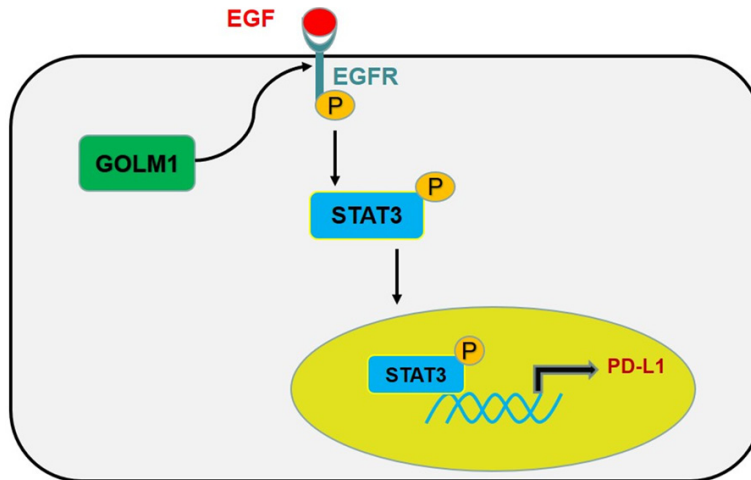


Figure 7. A brief illustration of the proposed working model. GOLM1 can upregulate the level of EGFR. After binding to its ligand EGF, EGFR is phosphorylated, which then promotes phosphorylation of STAT3. Phosphorylated STAT3 plays an important role in promoting the expression of PD-L1.

(**Figure 7**), which may serve as a novel target axis in searching of a new and effective HCC immunotherapy.

STAT3 mediates a variety of cellular biological processes, including tumor cell proliferation, metastasis and immune response [47-49]. In malignant tumors, excessive activation of STAT3 leads to inflammation-driven repair, thereby promoting drug resistance or tumor progression [50-53]. STAT3 was reported to induce immunosuppression in cancer by upregulating PD-L1 and overexpression of PD-L1 significantly associates with the level of phosphorylated STAT3 [54, 55]. PD-L1 is an important immunomodulatory molecule that can inhibit the immune response by binding to its receptor, PD-1 [56]. In several human cancers, including melanoma, non-small cell lung cancer, hepatocellular carcinoma, gastric cancer, esophageal cancer, and urothelial carcinoma, high PD-L1 expression is associated with tumor immunosuppression and poor prognosis [57-59]. In the current study, we found that GOLM1 upregulated PD-L1 transcriptional expression by promoting STAT3 Tyr705 phosphorylation. This positive regulation effect is attenuated by STAT3 inhibitor due to decreased Tyr705 phosphorylation of STAT3. Our findings reveal GOLM1 as an upstream positive regulator of STAT3 and uncover a new mechanism that regulates the expression of PD-L1 in HCC.

Regarding the mechanism, we found that upon GOLM1 overexpression in HCC cells, EGFR

phosphorylation levels were significantly increased, thereby enhancing STAT3 phosphorylation at Tyr705, which finally led to upregulation of PD-L1 in HCC cells. Together this suggests that the phosphorylation level of EGFR regulated by GOLM1 not only affects tumor metastasis but also participates in immune escape in HCC. EGFR is a member of the EGFR/ErbB subfamily of receptor tyrosine kinases (RTKs), which plays an important role in the development of various cancers, cell proliferation, survival and metastasis [60, 61]. Furthermore, we previously showed that EGFR recycle regulated by

GOLM1 promotes HCC metastasis [18]. Our present study reveals another mechanism by which GOLM1 affects the prognosis of HCC patients.

In summary, we demonstrated that GOLM1 promoted the expression of PD-L1 by regulating the EGFR/STAT3 signaling pathway. Our data reveals a novel regulatory mechanism of PD-L1 expression in HCC, which may provide a novel therapeutic target for HCC immunotherapy.

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Disclosure of conflict of interest

None.

Address correspondence to: Drs. Hui Li and Qinghai Ye, Department of Liver Surgery and Transplantation, Liver Cancer Institute, Zhongshan Hospital, Fudan University, 180 Feng Lin Road, Shanghai 200032, People's Republic of China. Tel: +86-21-64041990; Fax: +86-21-64037181; E-mail: li.hui1@zs-hospital.sh.cn (HL); ye.qinghai@zs-hospital.sh.cn (QHY)

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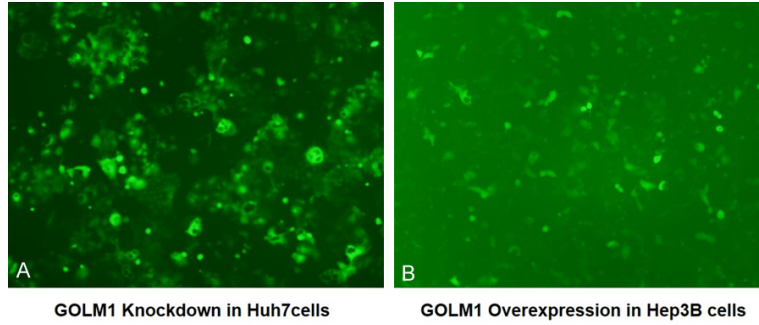
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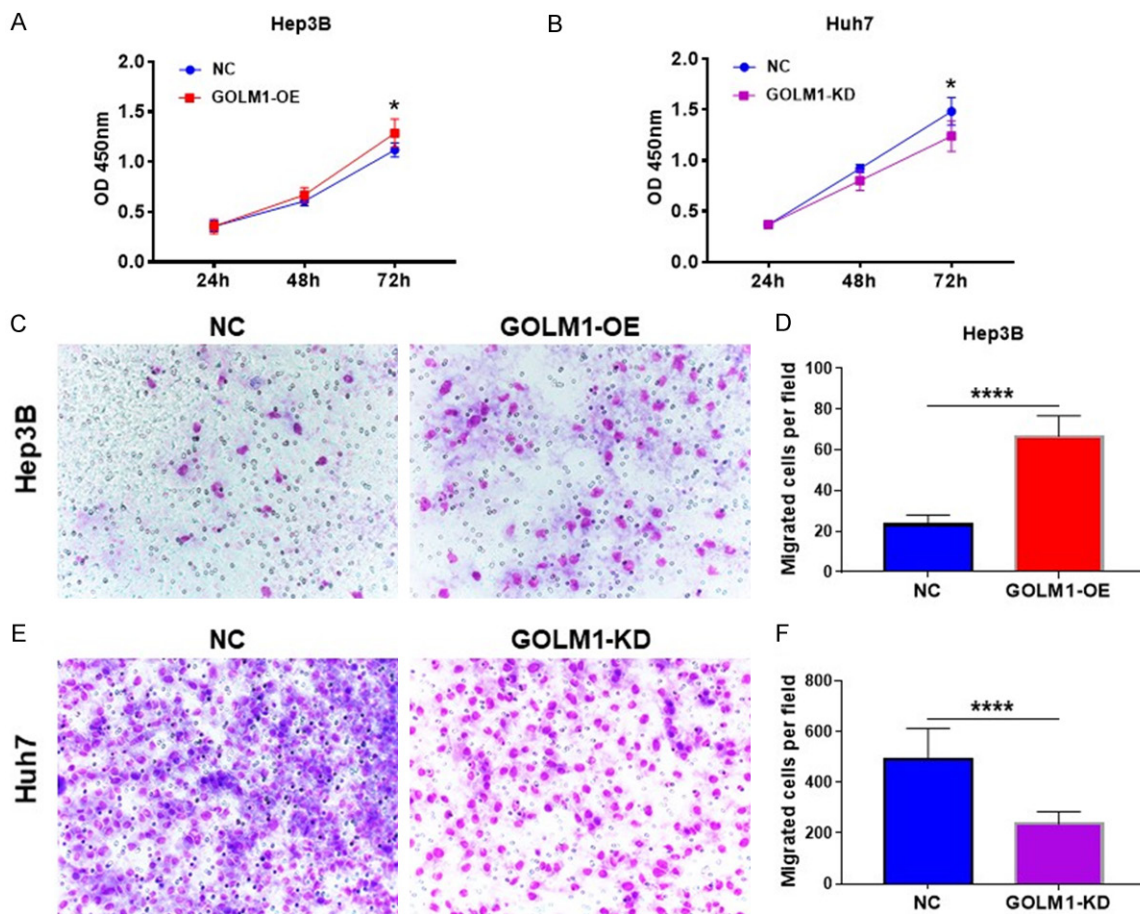
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Supplementary Figure 1. Lentivirus transduction. GOLM1 was knocked down in Huh7 cells (A) and overexpressed in Hep3B cells (B). The efficiency of transduction was measured under the fluorescence microscope. Puromycin was used to select stably transduced cells.



Supplementary Figure 2. Roles of GOLM1 in promoting growth and metastasis of HCC cells. A and B. CCK-8 assays were performed to evaluate the proliferation status of Hep3B cells (overexpression (OE) of GOLM1 vs. negative control (NC)) and Huh7 cells (knockdown (KD) of GOLM1 vs. NC). C. Images of Transwell assays for Hep3B cells (OE of GOLM1 vs. NC). Scale bar = 50 μ m. D. Quantification of migrated Hep3B cells in Transwell assays after incubation for 36 h. E. Images of Transwell assays for Huh7 cells (KD of GOLM1 vs. NC). Scale bar = 50 μ m. F. Quantification of migrated Huh7 cells in Transwell assays after incubation for 36 h. Data were presented as mean \pm SD. * $P < 0.05$, **** $P < 0.0001$.