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Tumour cell-expressed PD-L1 reprograms lipid metabolism via EGFR/ITGB4/SREBP1c signalling in liver cancer

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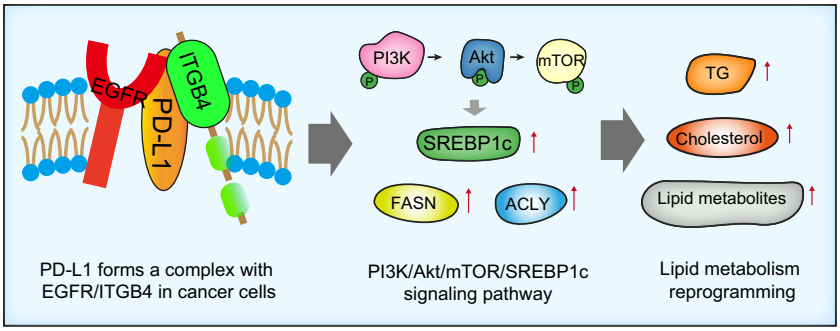
Graphical abstract



Highlights

- Tumour cell-expressed PD-L1 drives reprogramming of lipid metabolism in tumours.
- PD-L1 forms a complex with EGFR/ITGB4 to trigger reprogramming of lipid metabolism in HCC.
- PD-L1 triggers reprogramming of lipid metabolism via EGFR/ITGB4/PI3K/Akt/mTOR/SREBP1c/FASN/ACLY signalling in tumours.
- PD-L1-mediated reprogramming of lipid metabolism supports tumour growth *in vitro* and *in vivo*.

Impact and implications

In this study, we present evidence that PD-L1 drives the reprogramming of lipid metabolism in tumours. PD-L1 forms a complex with epidermal growth factor receptor (EGFR) and ITGB4, activating the PI3K/Akt/mTOR/SREBP1c signalling pathway and thereby contributing to lipid metabolism in cancer progression. Our findings offer novel insights into the mechanisms by which PD-L1 initiates the reprogramming of lipid metabolism in tumours. From a clinical perspective, the anti-PD-L1 antibody may alleviate resistance to the anti-EGFR antibody cetuximab and inhibit the reprogramming of lipid metabolism in tumours.

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Research article

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Tumour cell-expressed PD-L1 reprograms lipid metabolism via EGFR/ITGB4/SREBP1c signalling in liver cancer

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Background & Aims: The programmed death-ligand 1 (PD-L1) is a major co-inhibitory checkpoint factor that controls T-cell activities in tumours. PD-L1 is expressed on immune cells and tumour cells. Whether tumour cell-expressed PD-L1 affects tumour cells in an immune cell-independent fashion remains largely elusive. In this study, we investigated the significance of tumour cell-expressed PD-L1 with a focus on downstream signals and changes in lipid metabolism.

Methods: Immune-independent functions of PD-L1 in tumour growth were investigated *in vitro* and in immuno-deficient mice *in vivo*. The global influence of PD-L1 in targeted/untargeted lipidomic metabolites was studied by comprehensive mass spectrometry-based metabolomic analysis in liver cancer. Effects on lipid metabolism were confirmed by triglyceride and cholesterol assays as well as by Oil Red O staining in liver, pancreatic, breast, and oesophageal squamous cancer. Underlying mechanisms were investigated by real-time quantitative PCR, Western blot analysis, co-immunoprecipitation, pull-down assays, immunofluorescence staining, and RNA sequencing.

Results: PD-L1 enhanced the accumulation of triglycerides, cholesterol, and lipid droplets in tumours. PD-L1 influenced targeted/untargeted lipidomic metabolites in hepatoma, including lipid metabolism, glucose metabolism, amino acid metabolism, nucleotide metabolism, and energy metabolism, suggesting that PD-L1 globally modulates the metabolic reprogramming of tumours. Mechanistically, PD-L1 activated epidermal growth factor receptor (EGFR) and/or integrin β4 (ITGB4) by forming a complex of PD-L1/EGFR/ITGB4 in the cell membrane, prior to activating PI3K/mTOR/SREBP1c signalling, leading to reprogramming of lipid metabolism in tumours. Functionally, PD-L1-mediated lipid metabolism reprogramming supported the tumour growth *in vitro* and *in vivo* through EGFR and/or ITGB4 in an immune cell-independent manner.

Conclusions: Our findings on lipogenesis and EGFR activation by tumour cell-expressed PD-L1 suggest that, in addition to its immunostimulatory effects, anti-PD-L1 may restrict lipid metabolism and EGFR/ITGB4 signalling in liver cancer therapy.

Impact and implications: In this study, we present evidence that PD-L1 drives the reprogramming of lipid metabolism in tumours. PD-L1 forms a complex with epidermal growth factor receptor (EGFR) and ITGB4, activating the PI3K/Akt/mTOR/SREBP1c signalling pathway and thereby contributing to lipid metabolism in cancer progression. Our findings offer novel insights into the mechanisms by which PD-L1 initiates the reprogramming of lipid metabolism in tumours. From a clinical perspective, the anti-PD-L1 antibody may alleviate resistance to the anti-EGFR antibody cetuximab and inhibit the reprogramming of lipid metabolism in tumours.

Keywords: PD-L1; Tumours; Lipid metabolism reprogramming; EGFR; ITGB4; SREBP1c.

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Introduction

The programmed death-ligand 1 (PD-L1), encoded by CD274, serves as the programmed cell death-1 (PD-1) ligand, a key co-inhibitory checkpoint signalling molecule regulating T-cell activities. Various cancers exhibit elevated PD-L1 levels, correlating with a dismal prognosis, and exploit the PD-L1/PD-1 signalling pathway to evade T-cell immunity.[1] Elevated lipid biosynthesis not only enhances the immunosuppressive activity of regulatory T cells (Tregs) but also facilitates glucose metabolism in cervical cancer mediated by PD-L1.[2] However, the relationship between tumour cell-expressed PD-L1 and lipid metabolism reprogramming in cancers is poorly understood.

Reprogramming of lipid metabolism assumes a pivotal role in tumour development.[3,4] Critical lipogenic enzymes, including stearoyl-CoA desaturase (SCD), fatty acid (FA) synthase (FASN), and acetyl-CoA carboxylase,[5] are implicated in hepatocarcinogenesis. In addition, sterol regulatory element-binding protein 1 (SREBP-1), a crucial transcription factor, upregulates ATP-citrate lyase (ACLY), FASN, and SCD, thereby promoting FA synthesis and augmenting cholesterol uptake in hepatocytes.[6] Our previous research revealed that SPIN1 coactivates sterol regulatory element-binding protein-1c (SREBP1c) in the FASN promoter, contributing to the regulation of abnormal lipid metabolism.[7] Furthermore, our group reported that cholesterol upregulates HULC in hepatoma cells.[8] Although metabolomic studies have successfully integrated genomics and transcriptomics, offering deeper insights into disease mechanisms, the precise mechanism governing lipid metabolism reprogramming in tumours remains elusive.

The epidermal growth factor receptor (EGFR) is a 170-kDa transmembrane glycoprotein cell surface receptor belonging to the ErbB/HER family. Activated EGFR influences four major signalling pathways: MAPK, phosphoinositide 3-kinase (PI3K)/Akt/mammalian/mechanistic target of rapamycin (mTOR), PLCγ/PKC, and JAK/STAT.[9] Cetuximab, a human–mouse chimeric IgG1 monoclonal antibody, binds to domain III of the extracellular segment of the tethered inactive state of EGFR, directly blocking ligand binding.[10] PD-L1 binding and its involvement in EGFR activation antagonise TRAIL-induced apoptosis in gastric cancer cells.[11] The EGFR pathway regulates PD-L1 expression via the IL-6/JAK/STAT3 signalling pathway in EGFR-mutated non-small cell lung cancer.[12] However, whether EGFR-mediated lipid metabolism reprogramming is associated with PD-L1 expression in tumours remains unknown. Integrins, which are heterodimeric transmembrane receptors, mediate interactions between cells and extracellular matrix components.[13] Integrin β4 (ITGB4) is aberrantly expressed in several cancers, including breast, colorectal, and lung, and is positively associated with poor prognosis.[14–16] ITGB4 regulates multiple signalling pathways, including ErbB2, PI3K, FAK/Akt, and c-Me, to promote tumour progression.[17–20] However, the role of ITGB4 in lipid metabolism reprogramming in cancer remains unclear. The atypical serine/threonine kinase mTOR serves as the master modulator of cell metabolism and growth by inhibiting catabolic processes such as autophagy and promoting anabolic processes such as lipid, protein, nucleotide, FA synthesis, and ribosome biogenesis.[21] mTOR signalling is typically triggered in cancer cells and promotes lipid metabolism by regulating the expression and/or activity of several key metabolic enzymes.[22] However, the effect of PD-L1 on mTOR signalling in the regulation of lipid metabolism is not well documented.

In this study, we investigated immune-independent functions of PD-L1 in tumour cells, focusing on the modulation of lipid metabolism reprogramming. Strikingly, we found that PD-L1 triggered the lipid metabolism reprogramming in liver and gastrointestinal cancers.

Materials and methods

Cell lines and cell culture conditions

The human immortalised normal liver LO2 cell line, Chang Liver cell line, pancreatic cancer cell line ASPC1, breast cancer cell line BT474, and oesophageal squamous cell carcinoma cell lines KYSE180 and KYSE2 were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA). Human hepatoma cell lines Huh7 and HepG2, pancreatic cancer cell line PANC1, and breast cancer cell line MCF7 were maintained in DMEM (Gibco). All cell lines were supplemented with heat-inactivated 10% FBS (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin; grown at 37 °C in a 5% CO_2 atmosphere; and routinely tested for mycoplasma. The cell lines were always mycoplasma negative. The cells were cultured in different flasks or plates for 36 or 48 h and then transfected with plasmids or siRNAs. Transfection was performed using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Statistical analysis

Each experiment was repeated at least thrice. Statistical significance was assessed by comparing mean values (±SD) using Student's *t* test for independent groups, with significance assumed for $^{*}p$ <0.05; $^{**}p$ <0.01; and $^{***}p$ <0.001.

Ethics approval and consent to participate

All experiments involving human participants were performed in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). With informed consent from all participants, paired specimens of HCC and adjacent normal tissues were collected. All experiments involving human participants and animals were approved by the Institute Research Ethics Committee of Tianjin Medical University Cancer Institute and Hospital.

Results

PD-L1 triggers the accumulation of triglycerides, cholesterols, and lipid droplets in tumours

To understand the immune-independent effects of PD-L1, including the effects on lipid metabolites, we investigated lipid alterations in tumour and their correction with PD-L1 expression in hepatocellular carcinoma (HCC). Triglycerides (TGs) and cholesterols displayed remarkable accumulation in HCC tissues relative to their corresponding peritumoral tissues (Fig. S1-1A and B). Intriguingly, a positive correlation existed between PD-L1 and TG and cholesterol levels in the clinical samples (Fig. 1A and B). PD-L1 overexpression increased TG and cholesterol levels in tumour cells in a dose-dependent manner (Fig. 1C and D, and Fig. S1-1C–F). Conversely, PD-L1 knockdown reduced TG and cholesterol levels in these cells in a dose-dependent manner (Fig. 1E and F, Fig. S1-1G–L, and Fig. S1-2A–C). Oil Red O staining revealed that PD-L1 overexpression strongly accelerated lipogenesis in KYSE180 cells (Fig. 1G), whereas treatment with PD-L1 siRNA blocked this effect (Fig. 1G and Fig. S1-2D). In addition, PD-L1 knockout (KO) significantly decreased TG and cholesterol levels, whereas reconstitution of PD-L1 reversed this phenotype (Fig. 1H and I, and Fig. S1-3A).

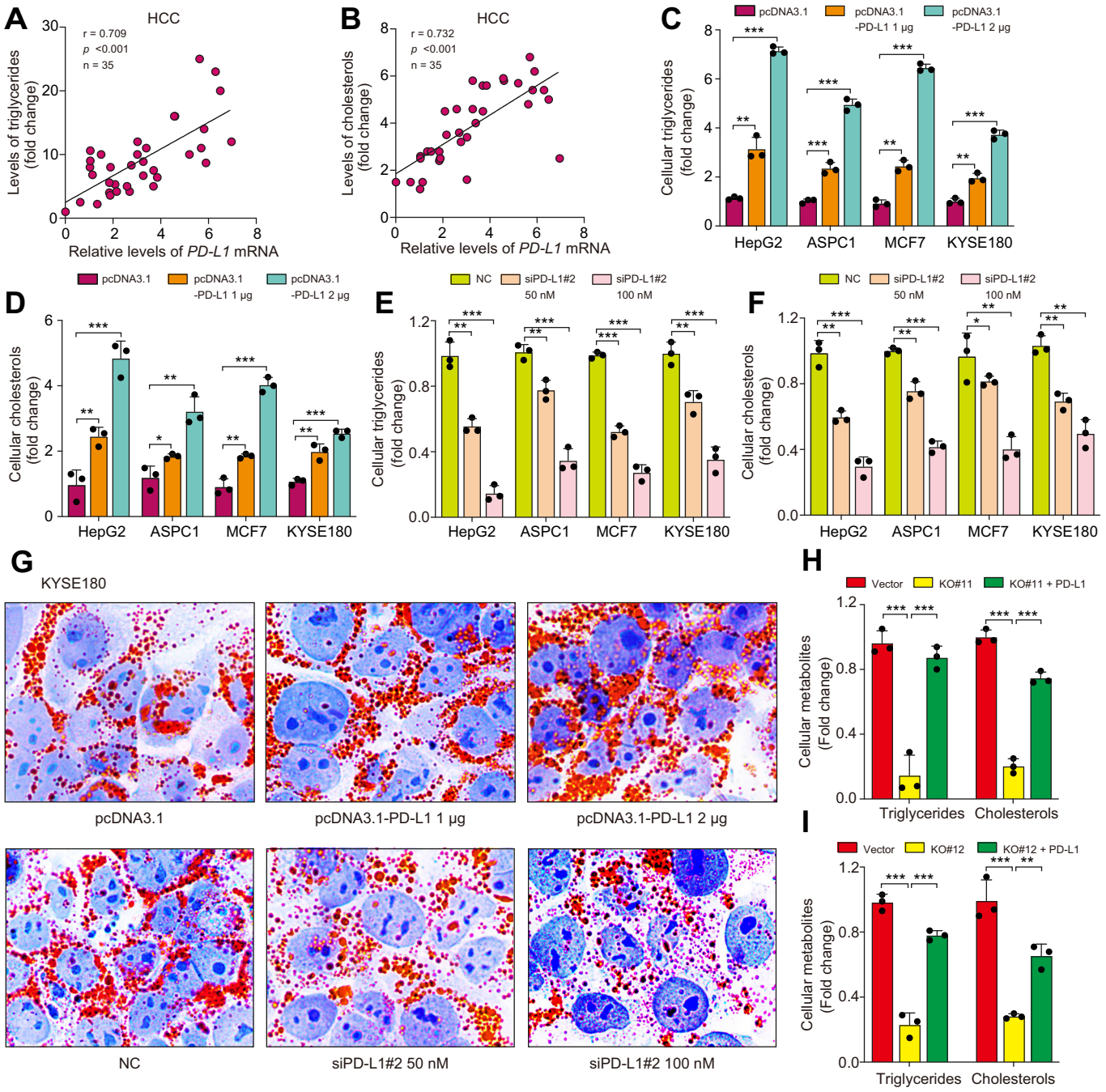

Fig. 1. PD-L1 triggers the accumulation of triglycerides, cholesterols, and lipid droplets in tumours. (A and B) Investigation of the relationship between PD-L1 and triglycerides/cholesterols in 35 HCC tissues using RT-qPCR and tissue total triglyceride and cholesterol assay kits. (C and D) Assessment of the impact of PD-L1 overexpression on intracellular levels of triglycerides and cholesterols in tumour cells using tissue total triglyceride and cholesterol assay kits. (E and F) Determination of the effect of PD-L1 siRNA on intracellular triglycerides and cholesterols in tumour cells using tissue total triglyceride and cholesterol assay kits. (G) Evaluation of the influence of PD-L1 on lipid droplets in KYSE180 cells using Oil Red O staining. (H and I) Measurement of triglyceride and cholesterol levels after the overexpression of PD-L1 plasmid in PD-L1 KO HepG2 cells. HCC, hepatocellular carcinoma; KO, knockout; PD-L1, programmed death-ligand 1; RT-qPCR, real-time quantitative PCR.

We screened for the effect of PD-L1 on HCC gene expression using RNA-sequencing (RNA-Seq) analysis. The profiling data revealed that the expressions of 332 genes were upregulated, and those of 1,113 genes were downregulated by 1.5-fold when PD-L1 was knocked down in HepG2 cells (Fig. S1-3B). Interestingly, KEGG pathway analysis demonstrated that PD-L1 is involved in the biosynthesis of unsaturated FAs, the peroxisome proliferator-activated receptor (PPAR) signalling pathway, and cholesterol metabolism (Fig. S1-3C). We validated the expression levels of PPAR signalling pathway-responsive genes using real-time quantitative PCR (RT-qPCR) in HepG2 cells (Fig. S1-3D).

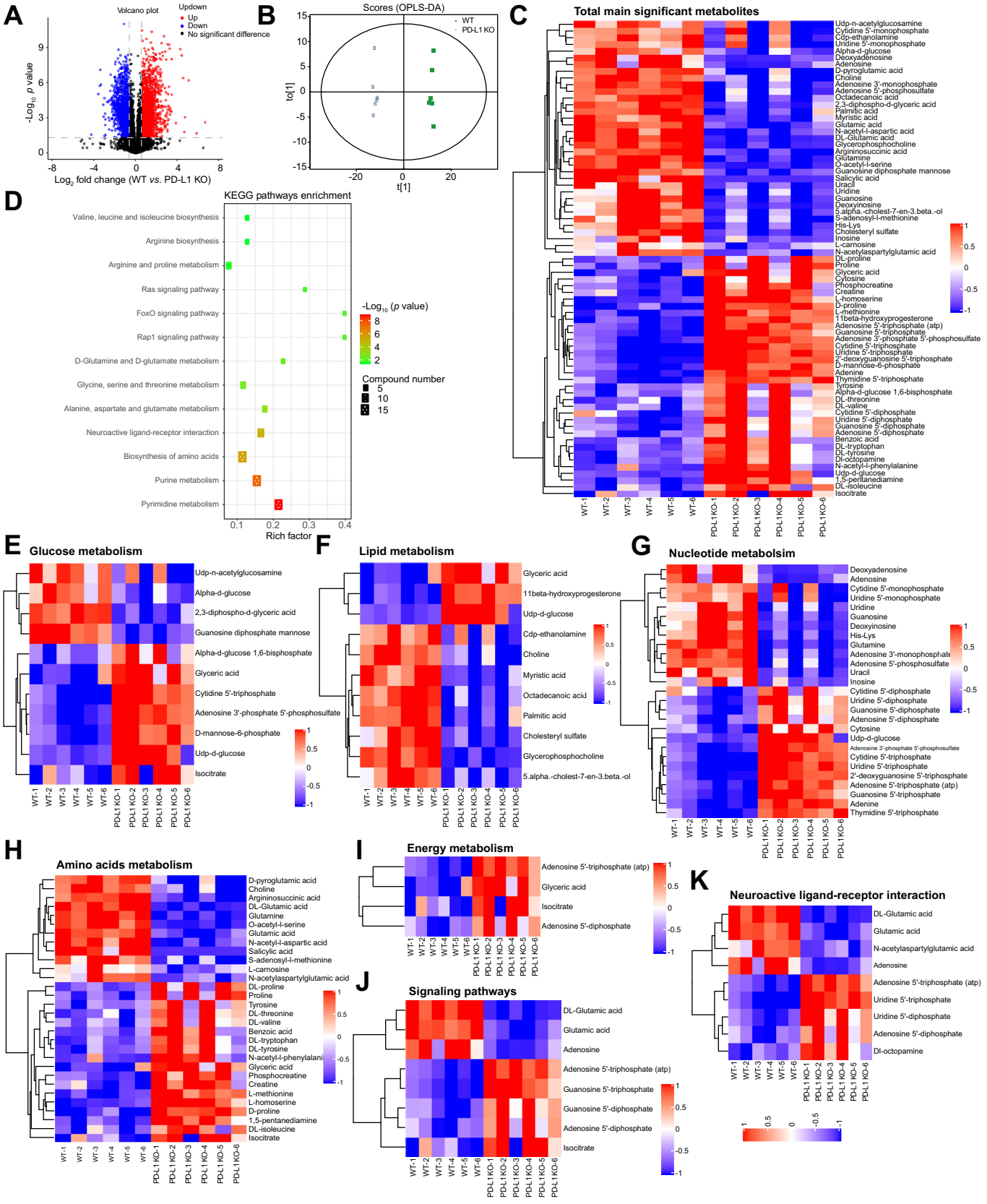

Fig. 2. PD-L1 induces lipid metabolism reprogramming in liver cancer. (A) Differential analysis of all detected metabolites (including unidentified ones) in positive ion modes based on univariate analysis. Volcano graph visualisation of differential metabolites with FC >1.5 or FC <0.67 and p <0.05. (B) OPLS-DA score chart in positive ion mode. (C) Hierarchical clustering heat map of significant difference metabolites. (D) KEGG pathways enrichment. (E–K) Hierarchical clustering heat map showing significant differences in glucose metabolites, lipid metabolites, nucleotide metabolites, amino acids metabolites, energy metabolites, signalling pathways, and neuroactive ligand–receptor interaction. Each column represents the mean ± SD of three independent experiments. PD-L1 KO denotes PD-L1 knockout cells. *Statistically significant difference, p <0.05, Student's t test. FC, fold change; KEGG, Kyoto Encyclopedia of Genes and Genomes; KO, knockout; OPLS-DA, Orthogonal Partial Least Squares Discriminant Analysis; PD-L1, programmed death-ligand 1; WT, wild type.

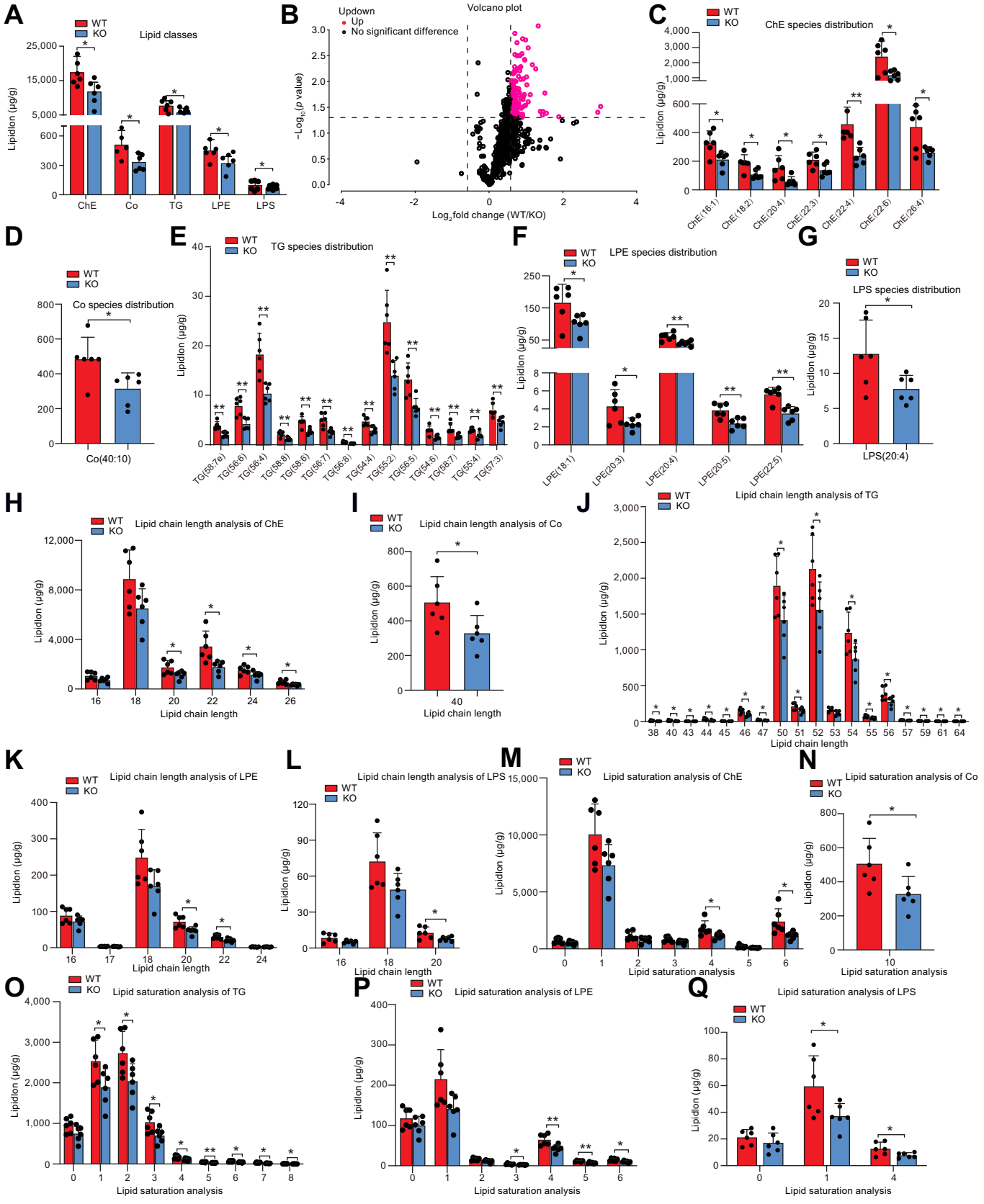

Fig. 3. PD-L1-mediated lipid metabolism reprogramming in liver cancer. (A) Lipid subclass content of major lipid species in HepG2 WT and KO cell lines analysed by LC-MS/MS. (B) Volcano plot displaying differential analysis of all detected lipid molecules in the targeted metabolomics of WT *vs.* PD-L1 KO cells. Rose red dots represent significantly different lipids (FC >1.5 or FC <0.67). (C–G) Content of major ChE, Co, TG, LPE, and LPS distributions in HepG2 WT and PD-L1 KO cells. (H–L) Contents of lipid molecules with different carbon chain lengths in each subcategory in HepG2 WT and PD-L1 KO cells. (M–Q) Contents of lipid molecules with different numbers of unsaturated bonds in each subcategory in HepG2 WT and PD-L1 KO cells. Each column represents the mean ± SD of three independent experiments. Statistically significant difference, $^{*}p$ <0.05, Student's *t* test. ChE, cholesterol ester; Co, coenzyme; FC, fold change; KO, knockout; LC-MS/MS, liquid chromatography with tandem mass spectrometry; LPE, lysophosphatidylethanolamine; LPS, lysophosphatidylserine; PD-L1, programmed death-ligand 1; TG, triglyceride; WT, wild type.

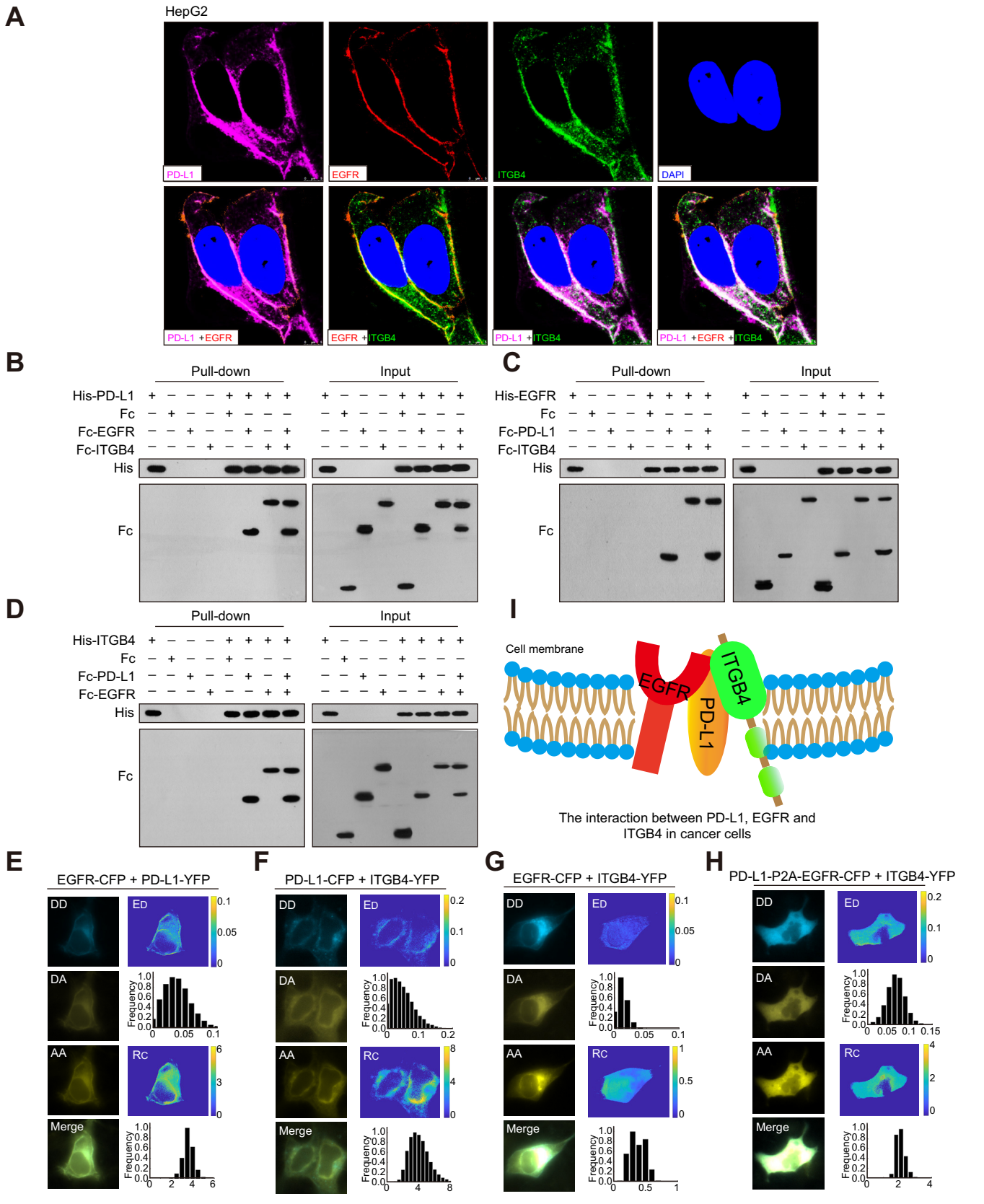

Fig. 4. PD-L1 forms a complex by interacting with EGFR and/or ITGB4. (A) Confocal immunofluorescence microscopy in HepG2 cells documented the co-localisation of PD-L1, EGFR, and ITGB4 proteins. Scale bar = 5/2.5 μm. (B) Pull-down assays using Fc antibody in Western blot analysis demonstrated PD-L1 binding to purified Fc, Fc-EGFR fusion protein, or Fc-ITGB4 fusion protein. (C) Western blot analysis revealed EGFR binding to purified Fc, Fc-PD-L1 fusion protein, or Fc-ITGB4 fusion protein in pull-down assays. (D) Pull-down assays and Western blot analysis showed ITGB4 binding to purified Fc, Fc-PD-L1 fusion

The relapse-free survival analysis of patients with HCC revealed that those with high PD-L1 expression had lower survival rates in The Cancer Genome Atlas (TCGA) database (Fig. S1-4A). Immunohistochemical (IHC) analysis showed that the PD-L1 positivity rate was 80% (64/80) in the HCC tissue microarray (Fig. S1-4B). Moreover, PD-L1 levels significantly increased in 35 clinical HCC tissues relative to their corresponding peritumoral tissues (Fig. S1-4C and D). Consistently, high PD-L1 levels were observed in hepatoma cell lines compared with normal liver cell lines (Fig. S1-4E). Thus, we conclude that PD-L1 accumulates TGs, cholesterols, and lipid droplets in tumours.

PD-L1 confers lipid metabolism reprogramming in liver cancer

To further substantiate the influence of PD-L1 on lipid metabolism reprogramming, we conducted untargeted/targeted metabolite analysis using comprehensive mass spectrometry metabolomics. In the untargeted metabolite analysis, diverse metabolites were visually presented in the form of a volcano plot (Fig. 2A). The Orthogonal Partial Least Squares Discriminant Analysis model score chart effectively distinguished between the two sample sets (Fig. 2B). Hierarchical cluster analysis revealed significant differences in metabolites (Fig. 2C). KEGG pathway enrichment analysis indicated PD-L1 involvement in metabolism-related pathways, with lipid metabolism reprogramming being one of them (Fig. 2D–K).

Using the shotgun lipidomic analysis of targeted metabolites, we extensively identified and quantified the lipid composition of HepG2 cells. The metabolomics experiments throughout the study identified 1,019 unique lipid species, including cholesterol ester (chE), coenzyme (Co), TG, phosphatidylethanolamine, sphingomyelin, and other sterol lipids (Fig. 3A). A volcano plot illustrated changes in the expression of different lipid molecules (Fig. 3B), and these lipid molecules were mapped and displayed according to their subclasses (Fig. 3C–G). PD-L1 KO reduced the number of lipid molecules with varying carbon chain lengths and diminished lipid saturation in HepG2 cells (Fig. 3H–Q). In conclusion, our findings support the assertion that PD-L1 contributes to lipid metabolism reprogramming in liver cancer.

PD-L1 forms a complex by interacting with EGFR and/or ITGB4

Next, we endeavoured to elucidate the underlying mechanism by which PD-L1 regulates lipid metabolism reprogramming. Given that PD-L1 binds to EGFR and influences the sensitivity of gastric cancer cells,[11] we confirmed the interaction between PD-L1 and EGFR in HepG2 and Huh7 cells (Fig. S2-1A–F). As cetuximab is a monoclonal antibody that binds to EGFR,[23] we assessed the impact of cetuximab on the PD-L1–EGFR interaction. Interestingly, cetuximab significantly inhibited the interaction between PD-L1 and EGFR in HepG2 and Huh7 cells (Fig. S2-1A, B, D, and E). The working concentration of cetuximab was determined in HepG2 and Huh7 cells (Fig. S2-2A and B). However, cetuximab failed to affect EGFR mRNA and protein levels (Fig. S2-2C and D). EGFR knockdown or cetuximab treatment mitigated the influence of PD-L1 on intracellular TGs and cholesterols, whereas EGFR overexpression exhibited the opposite effect (Fig. S2-2E–N), suggesting that PD-L1 induces lipid metabolism reprogramming by interacting with EGFR in the cells (Fig. S2-2O).

Given that PD-L1 functions in glucose metabolism reprogramming by directly binding to ITGB4,[24] we investigated whether the interaction between PD-L1 and ITGB4 contributes to lipid metabolism reprogramming. As expected, we confirmed the interaction between PD-L1 and ITGB4 (Fig. S2-3A–D) and observed that siITGB4 blocked PD-L1-mediated intracellular TG and cholesterol accumulation in HepG2 and Huh7 cells (Fig. S2-3E–J). Conversely, ITGB4 overexpression rescued siPD-L1-mediated events (Fig. S2-3K–N). PD-L1 modulates lipid metabolism reprogramming by binding to ITGB4 in liver cancer cells (Fig. S2-3O).

Based on positive correlations between PD-L1 and EGFR/ITGB4 in clinical samples from 35 HCC donors (Fig. S2-4A), we hypothesised that PD-L1, EGFR, and ITGB4 form a complex that modulates the reprogramming of lipid metabolism. Immunofluorescence staining demonstrated co-localisation of PD-L1/EGFR, PD-L1/ITGB4, EGFR/ITGB4, and PD-L1/EGFR/ITGB4 in HepG2 and Huh7 cells (Fig. 4A and Fig. S2-4B). Moreover, pull-down assays showed that the purified His-tagged PD-L1 extracellular domain protein could be pulled down by Fc-EGFR and Fc-ITGB4 extracellular domains (as bait proteins) (Fig. 4B). Similar results were observed for the EGFR extracellular domain binding to the PD-L1 and ITGB4 extracellular domains (Fig. 4C) and for the ITGB4 extracellular domain binding to the PD-L1 and EGFR extracellular domains (Fig. 4D). The co-localisation of PD-L1, EGFR, and ITGB4 proteins was also validated by fluorescence resonance energy transfer (FRET) assays in HepG2 cells (Fig. 4E–G). PD-L1 enhanced low-affinity interactions between EGFR and ITGB4 (Fig. 4H). Pembrolizumab, a PD-1 antibody, failed to bind PD-L1 to EGFR/ITGB4 (Fig. S2-4C and D). We analysed the precise interaction sites for the complex through bioinformatics using Discovery Studio software. Interestingly, we observed that several amino acid residues in the PD-L1 extracellular domain were responsible for the interaction between the EGFR and ITGB4 extracellular domains (PD-L1, Protein Data Bank ID: 3BIS; EGFR, Protein Data Bank ID: 1IVO; ITGB4, predicted) (Fig. S2-5A–F). Accordingly, our results showed that the PD-L1 mutants (R125A and D215A) attenuated the binding of EGFR to ITGB4 (Fig. S2-5G). Taken together, we concluded that PD-L1 forms a complex by interacting with EGFR and/or ITGB4 (Fig. 4I).

PD-L1 triggers lipid metabolism reprogramming via EGFR/ITGB4/PI3K/Akt/mTOR/SREBP1c/FASN/ACLY signalling

PD-L1 promotes cell growth via mTOR signalling in head and neck squamous cell carcinomas.[25] The mTOR signalling pathway contributes to lipid metabolism by upregulating the expression of lipogenic enzymes such as FASN and ACLY.[26,27] Therefore, we hypothesised that the PD-L1/EGFR/ITGB4 complex promotes the reprogramming of lipid metabolism via PI3K/Akt/mTOR/SREBP1c signalling. We validated the effects of PD-L1, PD-L1/siEGFR, PD-L1/cetuximab, PD-L1/siITGB4, PD-L1/siEGFR/siITGB4, and PD-L1/cetuximab/siITGB4 on p-PI3K, p-Akt, and p-mTOR in HepG2 and Huh7 cells (Fig. 5A, B). Conversely, treatment with siPD-L1, siPD-L1/EGFR, siPD-L1/ITGB4, and siPD-L1/EGFR/ITGB4 showed contrasting results (Fig. 5C and D). PD-L1 KO also decreased the levels

protein, or Fc-EGFR fusion protein. (E–H) FRET assays in HepG2 cells validated the co-localisation of PD-L1, EGFR, and ITGB4 proteins with indicated treatments. Quantitative FRET measurements (left), and corresponding pseudo-colour ED and RC images and their histograms (right) are presented. (I) A model depicting the interaction among PD-L1, EGFR, and ITGB4 in cancer cells. AA, acceptor-acceptor FRET; CFP, cyan fluorescent protein; DA, donor-acceptor FRET; DD, donor-donor FRET; E_D, Donor-centric FRET efficiency; EGFR, epidermal growth factor receptor; FRET, fluorescence resonance energy transfer; ITGB4, integrin β4; PD-L1, programmed death-ligand 1; Rc, acceptor-to-donor concentration ratio; YFP, yellow fluorescent protein.

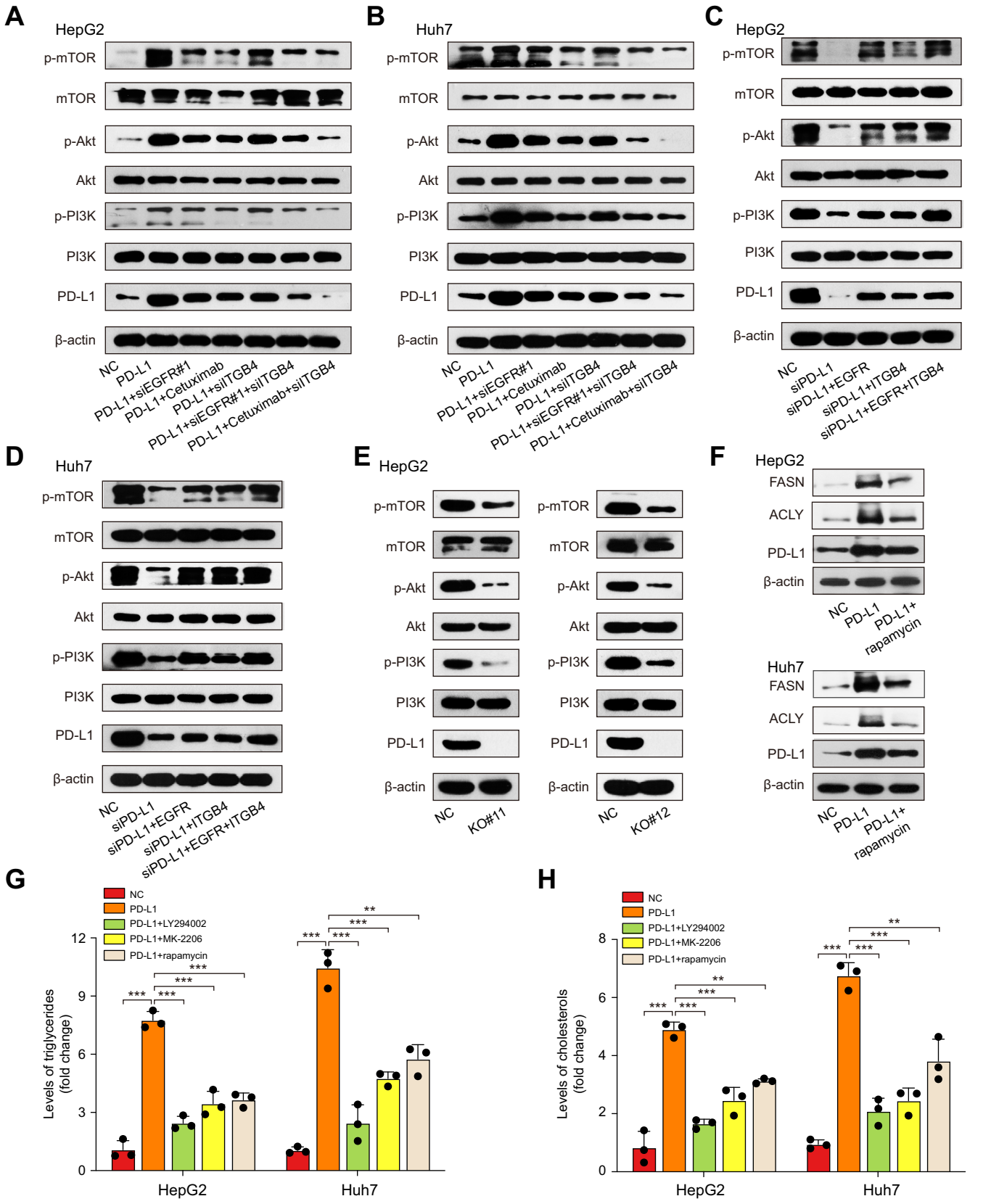

Fig. 5. PD-L1 triggers lipid metabolism reprogramming via EGFR/ITGB4/PI3K/Akt/mTOR/SREBP1c/FASN/ACLY signalling. (A–E) Western blot analysis evaluated the protein levels of total PI3K/p-PI3K, total Akt/p-Akt, and total mTOR/p-mTOR in cells with indicated treatments. (F) Western blot analysis determined the expression levels of FASN and ACLY in HepG2 and Huh7 cells following the indicated treatments. (G and H) Tissue total triglyceride assay kit or tissue total cholesterol kit measured intracellular triglyceride and cholesterol levels in HepG2 and Huh7 cells after treatment. Statistically significant differences are

of p-PI3K, p-Akt, and p-mTOR compared with the negative control (NC) group in HepG2 cells (Fig. 5E). We used the PI3K inhibitor LY294002, Akt inhibitor MK-2206, and mTOR inhibitor rapamycin to validate this hypothesis. As expected, PD-L1 overexpression increased FASN and ACLY expression, and rapamycin treatment significantly blocked these effects (Fig. 5F). Functionally, PD-L1 contributes to lipid metabolism reprogramming by activating PI3K/Akt/mTOR/ACLY signalling in liver cancer (Fig. 5G and H). We investigated the effects of EGFR and ITGB4 on the PI3K/Akt/mTOR pathway (Fig. S3-1A–D). The orthotopic liver mouse model showed that treatment with an anti-PD-L1 antibody, but not an anti-PD-1 antibody, inhibited tumour growth and reduced the levels of tissue TGs and cholesterols, and the Akt inhibitor MK-2206 enhanced this effect (Fig. S3-2A–C). PD-L1 knockdown reduced the expression of SREBP1c but not PPARs and CCAAT/enhancer-binding protein alpha (CEBP-α) in HepG2 cells (Fig. S3-2D–F). Remarkably, PD-L1 modulated SREBP1c and its downstream lipogenic enzymes FASN and ACLY in HepG2 cells via EGFR, ITGB4, and mTOR signalling (Fig. S3-3A). siSREBP1c attenuated the PD-L1/EGFR/ITGB4-mediated increase in both TG and cholesterol levels (Fig. S3-3B–E). Thus, we conclude that PD-L1 triggers lipid metabolism reprogramming by driving EGFR/ITGB4/PI3K/Akt/mTOR/SREBP1c/FASN/ACLY signalling in liver cancer.

PD-L1-mediated lipid metabolism reprogramming supports tumour cell proliferation by EGFR/ITGB4/SREBP1c signalling *in vitro*

We investigated the impact of PD-L1-mediated lipid metabolism on cell proliferation. PD-L1 overexpression significantly enhanced the proliferation of HepG2 and Huh7 cells, as demonstrated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, EdU incorporation assays, colony formation assays, and flow cytometry assays (Fig. 6, Fig. S4-1). The use of siEGFR and cetuximab effectively nullified PD-L1-mediated events (Fig. 6A–I). The overexpression of EGFR robustly rescued siPD-L1-mediated suppression of cell proliferation (Fig. 6B–J). We also assessed the role of ITGB4 in PD-L1-mediated lipid metabolism reprogramming for modulating cell proliferation *in vitro* (Fig. S4-1A–J), suggesting that PD-L1 promotes cell proliferation via EGFR and/or ITGB4. It has been reported that Hippo signalling, regulated by the mevalonate pathway, can modulate PD-L1,[28,29] and the mevalonate pathway is implicated in cholesterol genesis.[30,31] Our data showed that PD-L1 knockdown reduced the proliferation of HepG2 cells, and treatment with cholesterol rescued this effect (Fig. S4-2A–C). PD-L1 overexpression promoted the proliferation of HepG2 cells, as determined by MTT, EdU incorporation, and colony formation assays (Fig. S4-3A–C). SREBP1c knockdown effectively nullified PD-L1-mediated events (Fig. S4-3A–C). Conversely, PD-L1 KO decreased the proliferation of HepG2 cells, and SREBP1c overexpression rescued PD-L1 KO-mediated events (Fig. S4-4A–C). In conclusion, we assert that PD-L1-mediated lipid metabolism reprogramming supports cell proliferation via EGFR/ITGB4/SREBP1c signalling *in vitro*.

PD-L1-mediated lipid metabolism reprogramming confers tumour growth via EGFR/ITGB4/SREBP1c *in vivo*

To extend these observations *in vivo*, we treated male nude mice carrying allografts of hepatoma cell lines with subcutaneous xenografts. As expected, we found that the overexpression of PD-L1 remarkably increased the volume and weight of tumours; however, siEGFR/siITGB4 and cetuximab treatment blocked PD-L1-enhanced tumour growth in mice (Fig. 7A–C and Fig. S5-1A–C). PD-L1 increased the expression levels of Ki-67, and both EGFR and ITGB4 were implicated in the PD-L1-mediated growth of HCC cells (Fig. 7D and Fig. S5-1D). We confirmed that the mRNA expression levels of PD-L1 increased in the PD-L1 overexpressed group (Fig. 7E and Fig. S5-1E). TG and cholesterol levels were markedly reduced in tumour tissues from mice treated with PD-L1 overexpression/cetuximab/siITGB4 (Fig. 7F and G, and Fig. S5-1F and G). Interestingly, a similar trend was observed with Oil Red O staining (Fig. 7D and Fig. S5-1D).

Moreover, we repeated the above experiments in the C57BL/6 orthotopic tumour mouse model using Hepa1-6 wild-type (WT) and Hepa1-6 pd-l1 KO cells. The obtained results were consistent with the findings in immunodeficient mice (Fig. S5-2A–D). PD-L1 overexpression increased tumour growth, whereas SREBP1c knockdown attenuated this effect in mice (Fig. S5-3A–C). The levels of Ki-67 expression, lipid droplets, TGs, and cholesterols were markedly increased in tumour tissues from mice overexpressing PD-L1, whereas SREBP1c knockdown blocked these effects (Fig. S5-3D–G). However, PD-L1 KO resulted in the opposite effect, and SREBP1c overexpression rescued PD-L1 KO-mediated events (Fig. S5-4). Collectively, we conclude that PD-L1-mediated lipid metabolism reprogramming promotes tumour growth via EGFR/ITGB4/SREBP1c signalling *in vivo*.

Discussion

The expression of PD-L1 by cancer cells serves as a mechanism to evade T-cell-mediated immunosurveillance.[32,33] In addition, cancer cells, characterised by hyperactive growth, use an intricate network of lipid metabolic pathways to sustain their burgeoning biomass. However, the immune-independent roles of PD-L1, such as modulation of lipid metabolism in tumours, are poorly understood. Given the limited comprehension of PD-L1, this study aimed to reveal the novel roles of PD-L1 in tumorigenesis.

Analysis of TCGA data revealed that patients with liver cancer and displaying elevated PD-L1 levels exhibited diminished survival rates. Furthermore, we observed a PD-L1 positivity rate of 80% (64/80) in HCC tissues, with significantly heightened PD-L1 mRNA and protein expression in liver cancer tissues compared with peritumoral tissues. Our investigation also revealed pronounced PD-L1 expression in various tumour cell lines. RNA-Seq analysis illustrated that PD-L1 plays a role in the biosynthesis of unsaturated FAs, the PPAR signalling pathway, and cholesterol metabolism. This finding suggests that PD-L1 actively participates in the modulation of lipid metabolic reprogramming in HCC.

In this study, we illustrated that PD-L1 contributes to the accumulation of TGs, cholesterols, and lipid droplets in tumours. Lipogenic enzymes critical for tumour growth and alterations, such as metabolite abundance and the accumulation of lipid metabolic products, play a role in tumour development.[6] Notably, PD-L1 strongly modulates the levels of lipid metabolism transcription factors, synthases, oxidases, and intracellular lipid metabolism products. Comprehensive mass

indicated: $^{**}p$ <0.01; $^{***}p$ <0.001; ns, no significance; Student's *t* test. The experiment was repeated at least three times. ACLY, ATP citrate lyase; CEBP-α, CCAAT/enhancer-binding protein alpha; EGFR, epidermal growth factor receptor; FASN, fatty acid synthase; ITGB4, integrin β4; KO, knockout; mTOR, mammalian/mechanistic target of rapamycin; PD-L1, programmed death-ligand 1; PI3K, phosphoinositide 3-kinase; SREBP1c, sterol regulatory element-binding protein-1c.

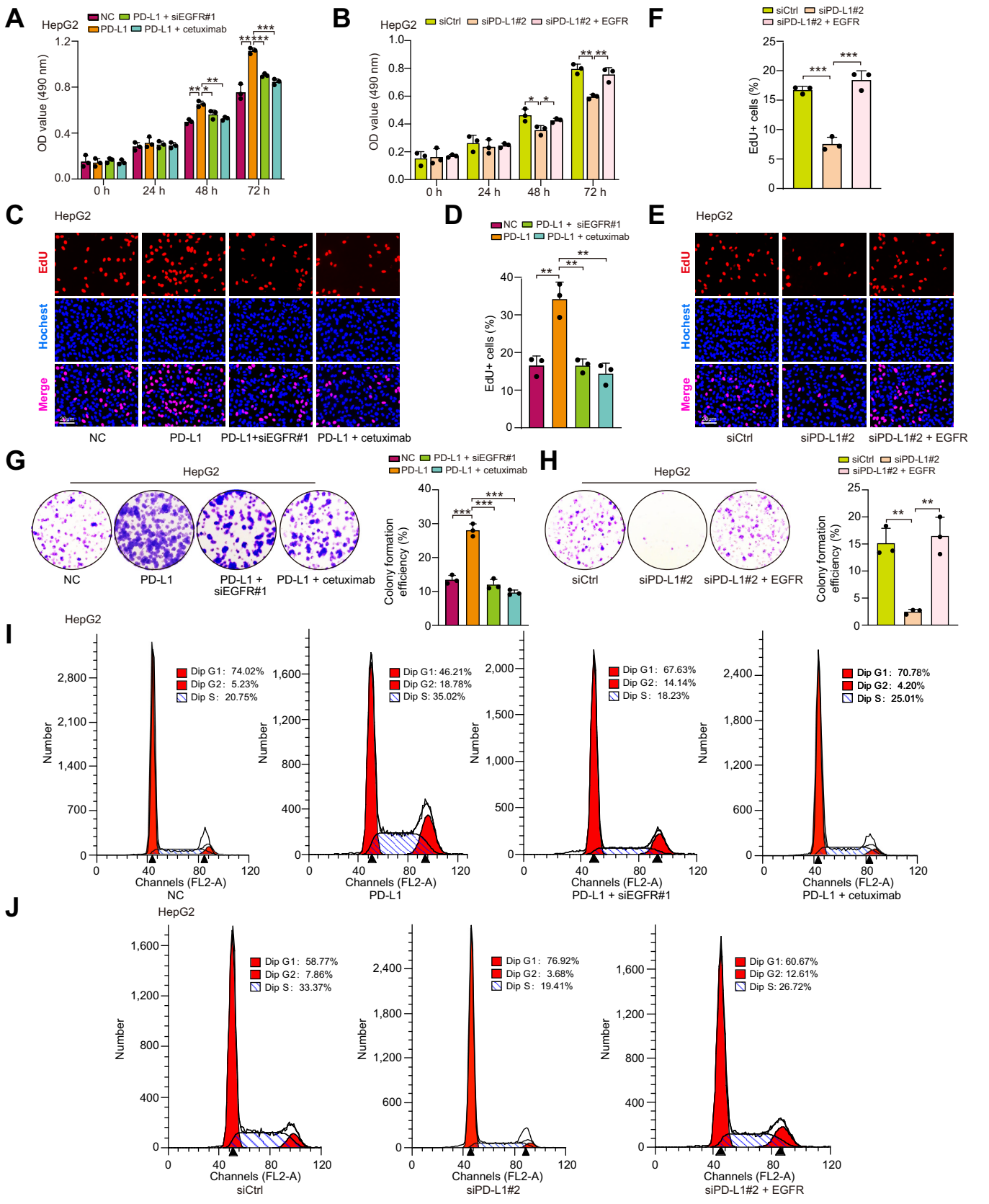

Fig. 6. PD-L1-mediated lipid metabolism reprogramming supports tumour cell proliferation by EGFR/ITGB4/SREBP1c signalling *in vitro*. (A, B) MTT assays were employed to detect cell proliferation in HepG2 cells subjected to the indicated treatments. (C–F) Cell proliferation was assessed using EdU incorporation assays in HepG2 cells following the indicated treatments. Scale bar = 20 μm. (G and H) Colony formation assays were conducted to examine HepG2 cell proliferation under the indicated treatments. (I and J) Flow cytometry assays were used to detect cell proliferation in HepG2 cells with the indicated treatment. Statistically significant differences are denoted: $^{*}p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001; Student's *t* test. The experiment was repeated at least three times. EGFR, epidermal growth factor receptor; ITGB4, integrin β4; PD-L1, programmed death-ligand 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD, optical density; SREBP1c, sterol regulatory element-binding protein-1c.

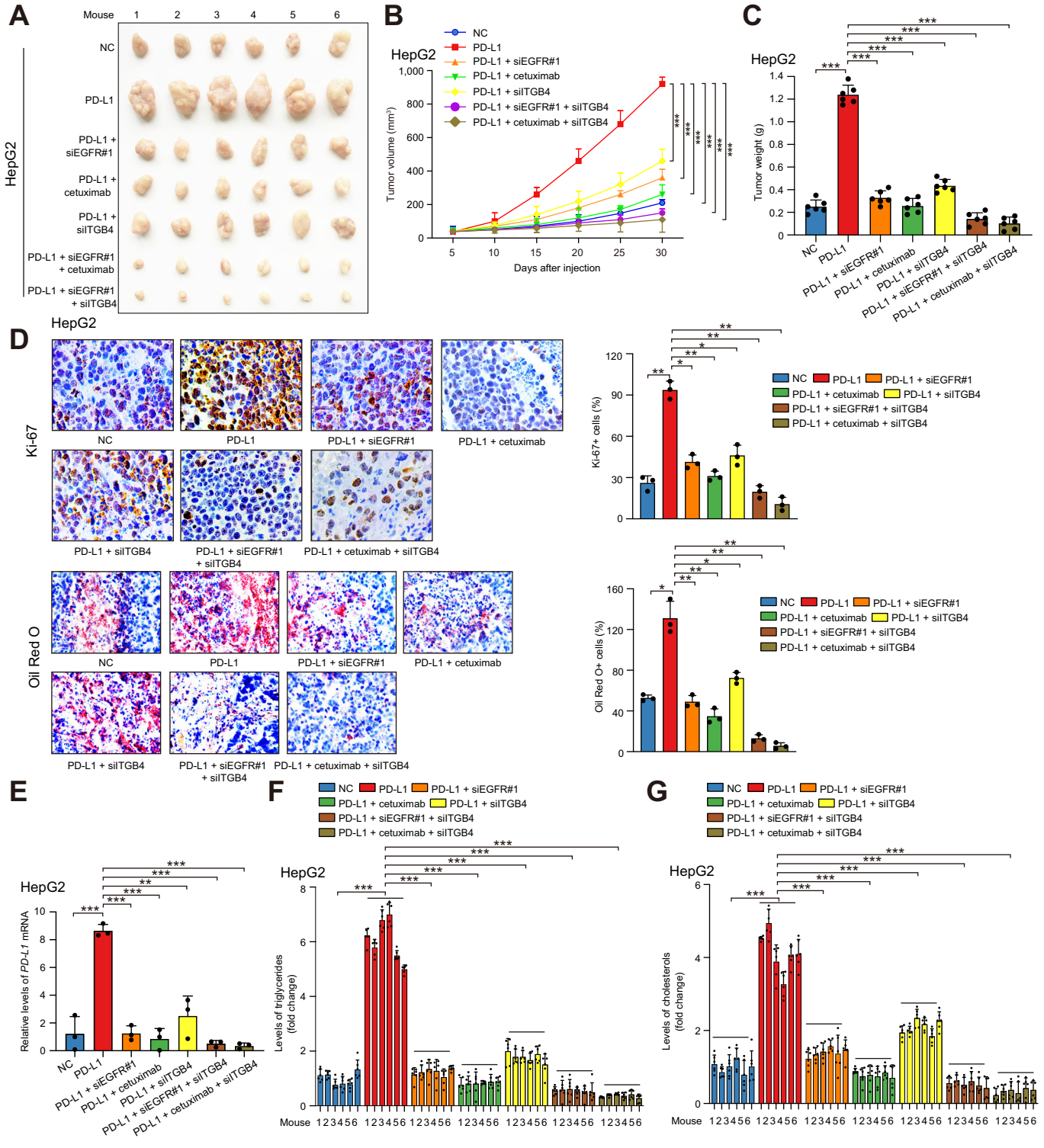

Fig. 7. PD-L1-mediated lipid metabolism reprogramming confers tumour growth via EGFR/ITGB4/SREBP1c *in vivo*. (A) Photographs depict dissected tumours from nude mice injected with HepG2 cells following the indicated treatment (n = 6 mice/group). (B) Growth curves illustrate tumour progression in nude mice. (C) Average tumour weight is reported for each group. (D) Ki-67 expression in tumour tissues from nude mice was assessed by IHC assays. Lipogenesis in the tumour tissues from mice transplanted with HepG2 cells was determined by Oil Red O staining using frozen sections. (E) PD-L1 mRNA expression levels in tumour tissues from mice were determined by RT-qPCR analysis. (F and G) Triglyceride or cholesterol levels were measured using the tissue triglyceride assay kit or tissue total cholesterol kit in the tumour tissues from mice. Statistically significant differences are denoted: $^{**}p$ <0.01; $^{***}p$ <0.001; Student's *t* test. The experiment was repeated at least three times. EGFR, epidermal growth factor receptor; IHC, immunohistochemical; ITGB4, integrin β4; OD, optical density; PD-L1, programmed death-ligand 1; RT-qPCR, real-time quantitative PCR; SREBP1c, sterol regulatory element-binding protein-1c.

spectrometry-based targeted/untargeted metabolomic analysis demonstrated that PD-L1 influences metabolites, including lipids, glucose, amino acids, nucleotides, and energy metabolism, participating in crucial signalling pathways in HCC cells. This observation aligns with the RNA-Seq transcriptome sequence analysis of PD-L1 in tumours. Thus, PD-L1 appears to influence lipid metabolism reprogramming and modulate multiple metabolic reprogramming processes in tumours globally.

Next, we excavated PD-L1 binding membrane proteins to determine the mechanism by which PD-L1 regulates lipid metabolism reprogramming in tumours. Interestingly, we found that PD-L1 interacts with EGFR to trigger the reprogramming of lipid metabolism in HCC cells. Moreover, the EGFR-specific monoclonal antibody cetuximab inhibits lipid metabolism reprogramming by blocking the interaction of EGFR with PD-L1. Considering that PD-L1 functions in glucose metabolism reprogramming by directly binding to ITGB4 in cervical cancer,[24] we hypothesised that PD-L1 drives lipid metabolism reprogramming by interacting with ITGB4 in HCC. These events were observed in these cells. We further validated that PD-L1 directly interacts with EGFR and ITGB4 to form complexes in cells. This suggests that PD-L1 triggers the reprogramming of lipid metabolism in tumours, depending on the complex. It is possible that the roles of EGFR and ITGB4 inhibition are independent of PD-L1, which is consistent with a previous study that EGFR regulates lipid metabolism in liver cancer.[34] It has been reported that cytoplasmic PD-L1 plays a critical role in cancer development.[35] However, the involvement of cytoplasmic PD-L1 in the regulation of lipogenesis in cancer cells remains unclear. Patients who respond to the anti-EGFR antibody cetuximab often develop resistance to this therapy.[36] Our findings suggest that in the event of cetuximab resistance, PD-L1-mediated lipid metabolism reprogramming might be involved, providing a new key to understanding cetuximab resistance in the clinic. Thus, the anti-PD-L1 antibody might release resistance to the anti-EGFR antibody, cetuximab, in cancer therapy. Clinically, the application of an anti-PD-L1 antibody might inhibit lipid metabolism reprogramming in tumours.

The mTOR signalling is implicated in the reprogramming of lipid metabolism in tumours.[37] We demonstrated that the PD-L1/EGFR/ITGB4 complex contributes to lipid metabolism reprogramming by activating PI3K/Akt/mTOR/SREBP1c/FASN/ACLY signalling in liver cancer. TGs and other fat metabolites influence the activity of PPARs, a class of transcription factors that regulate lipid oxidative enzymes.[38] Our data suggest that PD-L1-modulated lipid metabolite accumulation positively influences lipid metabolism-related enzymes in a feedback loop. Given the role of PD-L1 in inhibiting antitumour immunity,[39] we hypothesised that PD-L1-mediated lipid metabolism reprogramming might be involved in these events. Functionally, we validated that PD-L1 promotes the reprogramming of lipid metabolism by interacting with EGFR/ITGB4 *in vivo*. Currently, antibodies targeting the PD-L1/PD-1 axis are under evaluation in many clinical trials and have been approved for cancer treatment.[39] Our study redefines the role of PD-L1 as an immunotherapy target for tumours in the clinic, enhancing the understanding of resistance to anti-PD-L1 antibodies in cancer therapy. Abnormal lipogenesis is a hallmark of cancer cells.[40] In this study, we found that PD-L1-mediated lipid metabolism contributes to cancer progression *in vitro* and *in vivo*. A study in nude mice excluded the major effects of PD-L1 via the immune system. Our findings provide new insights into the mechanisms by which PD-L1 modulates lipid metabolism reprogramming in tumours. Therapeutically, anti-PD-L1 antibodies are available for the treatment of lipid metabolism reprogramming in tumours.

Conclusion

Our findings indicate that tumour cell-expressed PD-L1 initiates the reprogramming of lipid metabolism by activating the EGFR/ITGB4/PI3K/Akt/mTOR/SREBP1c/FASN/ACLY axis in tumours. In this model, PD-L1 induces lipid metabolism reprogramming via the activation of EGFR and/or ITGB4, forming a complex with EGFR and ITGB4. Subsequently, this complex activation leads to the initiation of PI3K/Akt/mTOR/SREBP1c/FASN/ACLY signalling, ultimately resulting in the accumulation of lipid metabolites within tumours. PD-L1-mediated lipid metabolism reprogramming actively supports tumour progression.

Abbreviations

ACLY, ATP citrate lyase; chE, cholesterol ester; Co, coenzyme; EGFR, epidermal growth factor receptor; FA, fatty acid; FASN, fatty acid synthase; FRET, fluorescence resonance energy transfer; HCC, hepatocellular carcinoma; IHC, immunohistochemical; ITGB4, integrin β4; KEGG, Kyoto Encyclopedia of Genes and Genomes; KO, knockout; mTOR, mammalian/mechanistic target of rapamycin; PD-1, programmed cell death-1; PD-L1, programmed death-ligand 1; PI3K, phosphoinositide 3-kinase; PPAR, peroxisome proliferator-activated receptor; RNA-Seq, RNA sequencing; RT-qPCR, real-time quantitative PCR; SCD, stearoyl-CoA desaturase; SREBP-1, sterol regulatory element-binding protein 1; SREBP1c, sterol regulatory element-binding protein-1c; TCGA, The Cancer Genome Atlas; TG, triglyceride; Treg, regulatory T cell; WT, wild type.

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Conflicts of interest

The authors declare no potential Conflicts of interest.

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

Authors' contributions

Designed the experiments: MZ, HFY, XDZ, WL, NNZ. Performed the experiments: MZ, HFY, GY. Analysed the data: MZ, HFY, GY. Interpreted the results: MZ, HFY, GY. Performed the cell experiments and *in vivo* studies: YFW, YNB, HHZ. Provided the methodology: LNZ, PL, HLY, YG. Contributed to the interpretation and conceptual advancement: SW, XDZ, WL, NNZ. Wrote the manuscript: MZ, HFY, XDZ. Revised the manuscript: GY, XDZ.

Data availability statement

All data are available upon request from the corresponding author.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jhepr.2024.101009.

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Author names in bold designate shared co-first authorship

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