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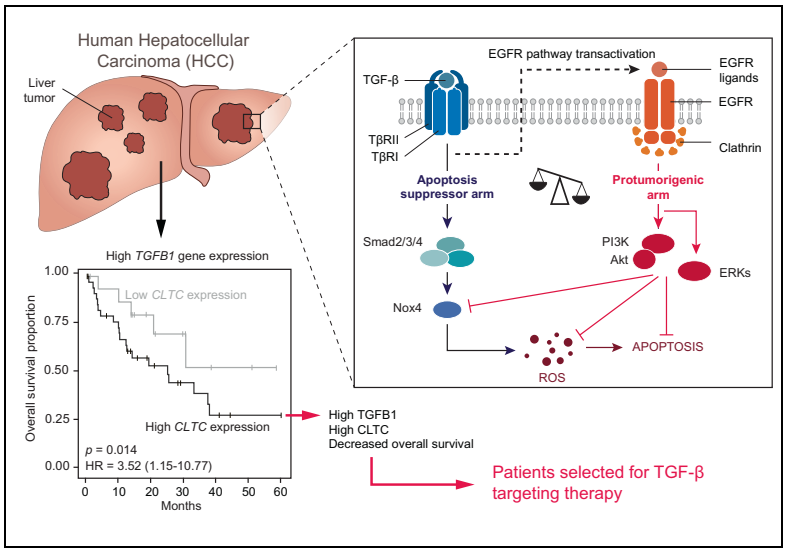
Clathrin switches transforming growth factor-β role to pro-tumorigenic in liver cancer

Graphical abstract



Highlights

- *CLTC* expression increases during liver tumorigenesis in humans and mice.
- *CLTC* expression is required for TGF-β-induced anti-apoptotic signals in liver cells.
- Autocrine TGF-β signalling in invasive HCC cells upregulates *CLTC* expression.
- High levels of *TGFB* and *CLTC* correlate with lower overall survival in patients with HCC.
- *CLTC* expression may help to select patients that will benefit from anti-TGF-β therapy.

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Lay summary

Clathrin heavy-chain expression increases during liver tumorigenesis in humans (*CLTC*) and mice (*Cltc*), altering the cellular response to TGF-β in favour of anti-apoptotic/pro-tumorigenic signals. A positive correlation between *TGFB1* and *CLTC* was found in HCC cells and patients. Patients expressing high levels of *TGFB1* and *CLTC* had a worse prognosis and lower overall survival. *CLTC* expression in HCC human samples could help select patients that would benefit from therapies targeting TGF-β.

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Clathrin switches transforming growth factor-β role to pro-tumorigenic in liver cancer

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Background & Aims: Upon ligand binding, tyrosine kinase receptors, such as epidermal growth factor receptor (EGFR), are recruited into clathrin-coated pits for internalization by endocytosis, which is relevant for signalling and/or receptor degradation. In liver cells, transforming growth factor-β (TGF-β) induces both pro- and anti-apoptotic signals; the latter are mediated by the EGFR pathway. Since EGFR mainly traffics via clathrin-coated vesicles, we aimed to analyse the potential role of clathrin in TGF-β-induced signalling in liver cells and its relevance in liver cancer.

Methods: Real-Time PCR and immunohistochemistry were used to analyse clathrin heavy-chain expression in human (CLTC) and mice (*Cltc*) liver tumours. Transient knockdown (siRNA) or overexpression of *CLTC* were used to analyse its role on TGF-β and EGFR signalling *in vitro*. Bioinformatic analysis was used to determine the effect of *CLTC* and *TGFB1* expression on prognosis and overall survival in patients with hepatocellular carcinoma (HCC).

Results: Clathrin expression increased during liver tumorigenesis in humans and mice. *CLTC* knockdown cells responded to TGF-β phosphorylating SMADs (canonical signalling) but showed impairment in the anti-apoptotic signals (EGFR transactivation). Experiments of loss or gain of function in HCC cells reveal an essential role for clathrin in inhibiting TGF-β-induced apoptosis and upregulation of its pro-apoptotic target *NOX4*. Autocrine TGF-β signalling in invasive HCC cells upregulates *CLTC* expression, switching its role to pro-tumorigenic. A positive correlation between *TGFB1* and *CLTC* was found in HCC cells and patients. Patients expressing high levels of *TGFB1* and *CLTC* had a worse prognosis and lower overall survival.

Conclusions: This work describes a novel role for clathrin in liver tumorigenesis, favouring non-canonical pro-tumorigenic TGF-β pathways. *CLTC* expression in human HCC samples could help select patients that would benefit from TGF-β-targeted therapy.

Lay summary: Clathrin heavy-chain expression increases during liver tumorigenesis in humans (*CLTC*) and mice (*Cltc*), altering the cellular response to TGF-β in favour of anti-apoptotic/pro-tumorigenic signals. A positive correlation between *TGFB1* and *CLTC* was found in HCC cells and patients. Patients expressing high levels of *TGFB1* and *CLTC* had a worse prognosis and lower overall survival. *CLTC* expression in HCC human samples could help select patients that would benefit from therapies targeting TGF-β.

Introduction

Primary hepatic endocytic functions are important in several physiological and pathological processes. Despite this, their molecular mechanisms remain poorly defined and remarkably understudied.[1] Ligand-induced internalization and degradation of receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR) or hepatocyte growth factor receptor (c-MET), are relevant for maintenance and inhibition of their signalling pathways. Upon binding their respective ligands, each of these receptors are recruited into clathrin-coated pits eventually leading to endocytosis. However, clathrin might play additional roles, since Akt signalling following EGFR or MET activation requires clathrin, but could not require receptor endocytosis.[2] EGFR mediates differential signalling depending on its localization in the cell.[3,4] At the plasma membrane, clathrin is present in microdomains,[5,6] where EGFR clustering occurs.[7] In these microdomains, clathrin may act as a scaffold protein, recruiting signalling adaptors. In human hepatocellular carcinoma (HCC),

Keywords: Clathrin; HCC; TGF-β; EGFR; Liver; Hepatocyte; Intracellular traffic; Cancer biology; Anti-TGF-beta therapy.
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levels of clathrin heavy-chain protein help to distinguish early HCC from benign tumours and its expression is stronger in poorly differentiated HCC than in well differentiated HCC.[8–10] However, little is known about the molecular mechanisms behind these results.

In hepatocytes, transforming growth factor-β (TGF-β) induces both pro- and anti-apoptotic signals.[11] The anti-apoptotic signals are mediated by the EGFR pathway, which is transactivated by TGF-β through a mechanism that involves EGFR ligands' upregulation and activation of the metalloprotease TACE/ADAM17, responsible for their shedding.[12–14] In HCC cells, TGF-β also upregulates the expression of EGFR ligands, which transactivates the EGFR pathway, counteracting its pro-apoptotic response.[15] EGFR targeting knockdown, or pharmacological inhibition, significantly enhances TGF-β-induced cell death, correlating with higher levels of the NADPH oxidase NOX4 and changes in the expression of BCL-2 and IAP families. Once cells overcome apoptosis, they respond to TGF-β by undergoing epithelial-mesenchymal transition (EMT), which confers migratory/invasive capacities and stem cell properties.[16]

We have recently reported that caveolin-1, a protein involved in intracellular traffic for which a role in HCC has been proposed,[17] is necessary for the TGF-β-induced transactivation of the EGFR,[18] switching the response to TGF-β from cytostatic to tumorigenic in liver tumour cells.[19] Much less is known about the potential role of clathrin in the TGF-β signalling in liver cells. In hepatocytes, Dooley's group described that blocking clathrin trafficking does not alter the canonical-SMAD phosphorylation induced by TGF-β,[20] which indicates that clathrin-dependent endocytosis is not required for the early signals induced by TGF-β. Nevertheless, the role of clathrin in other endocytosis-independent responses, or in the crosstalk between TGF-β and the EGFR pathway, is completely unknown. More knowledge in this area is necessary to understand the role of clathrin in hepatocarcinogenesis.

Therefore, in this study, we aimed to analyse the potential role of clathrin in TGF-β-induced signalling in liver cells and its relevance in liver cancer.

Material and methods

Human HCC tissues and ethics statements

Human tissues were collected with the required approvals from the Institutional Review Board ("Comité Ético de Investigación Clínica (CEIC)", Bellvitge University Hospital) and patient's written consent conformed to the ethical guidelines of the 1975 Declaration of Helsinki. See Table S1 for further details.

DEN-induced hepatocarcinogenesis animal model

C57BL/6JOlaHsd mice were maintained in the Complutense University of Madrid (UCM) animal facility. Male mice aged 15 days received intraperitoneal injection of DEN (10 mg/kg) diluted in saline buffer. The experimental procedure was approved by the Institutional Committee for Animal Care and Use (CEA -UCM 87/2012, Madrid, Spain). At 9 months of age, animals were euthanized, and their livers removed. The samples were used in previous studies of the group.[21] More information and Ethical Statements are provided in the supplementary information.

Cell culture

Mouse hepatocytes, isolated from 3.5–4-day-old neonatal male mice, were immortalized as described.[22] PLC/PRF/5 and SNU449 cell lines were obtained from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK) and American Tissue Culture Collection (ATCC, Virginia, USA), respectively. Cell culture conditions and treatments are provided in the supplementary information.

CLTC targeting knockdown in liver cells

For small interference RNA (siRNA) transient transfection, cells (30–40% confluence) were transfected with TransIT-Quest reagent (Mirus, Madison, WI, USA) at final siRNA concentration of 50 nM. Further details in supplementary information.

Statistical analysis

Statistical analyses were performed as an estimation of the associated probability, using a Student's *t* test or 1/2-way ANOVA method, depending on the conditions involved. Experiments were carried out at least 2–3 independent times with 2–3 technical replicates. Data were represented as mean ± SEM. Normal distribution was assumed.

For further details regarding the materials used, please refer to the CTAT table and supplementary information.

Results

Clathrin expression is increased in tumoural tissues from patients with HCC and in a DEN-induced hepatocarcinogenesis model in mice

Clathrin heavy-chain (*CLTC* gene; CHC17 isoform) mRNA expression was increased in matched samples of tumour compared to adjacent non-tumour tissues in a cohort of 60 patients with HCC, collected in the Bellvitge University Hospital (Fig. 1A-B). Moreover, immunohistochemical analyses revealed that HCC tissues presented stronger clathrin staining than healthy tissue samples, which showed barely perceptible or moderate staining (Fig. 1C). Clathrin localized in tumoural areas, with more intense expression at the tumoural borders. To extend the analysis to a higher number of patients, we used the Chen Liver database (n = 196) and the TCGA database (n = 212), from Oncomine (https://www.oncomine.org).[23] HCC tissues expressed higher levels of *CLTC* mRNA and had higher *CLTC* DNA copy numbers than normal tissue (Fig. S1A,B).

Analysis of clathrin expression in a DEN-induced hepatocarcinogenesis model in mice also revealed that, at 9 months after treatment, clathrin mRNA (*Cltc* gene) and protein levels were higher in tumoural areas in comparison to non-tumoural areas (Fig. 1D). Tumoural areas were identified by Ki67 staining (a proliferative marker).

These data suggest that clathrin could play a role in hepatocarcinogenesis.

CTLC knockdown unbalances pro- and anti-apoptotic signals induced by TGF-β through impairment of the EGFR pathway

To analyse the specific role of clathrin in response to EGFR ligands and TGF-β, we induced *CLTC* knockdown through siRNA technology in mouse hepatocytes and in the HCC PLC/PRF/5 cell line (Fig. S2). First, we analysed whether clathrin is required for cell responses to extracellular EGFR ligands. In response to heparin binding EGF-like growth factor (HB-EGF), an EGFR ligand, clathrin downregulation attenuated EGFR, Akt and ERK (MAPK1) activation in both mouse hepatocytes and PLC/PRF/5 cells (Fig. 2). Furthermore, in a proliferation functional assay, *Ctlc* knockdown hepatocytes exhibited lower proliferation rates,

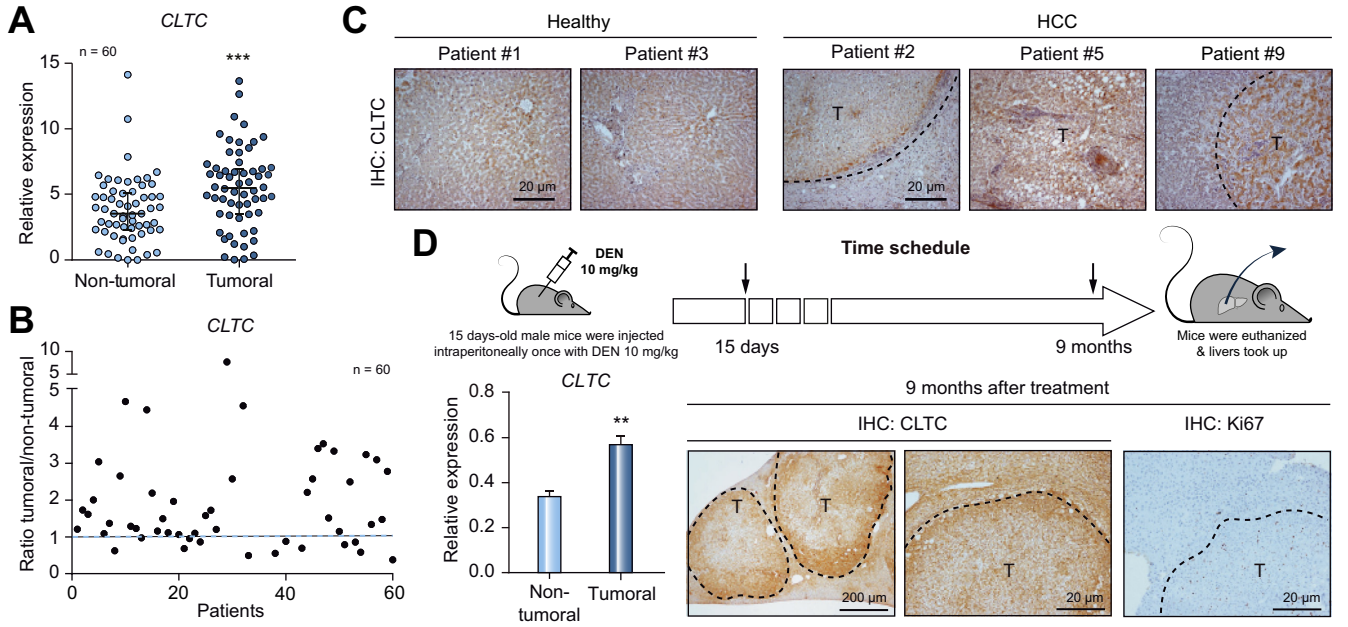

Fig. 1. Clathrin expression levels are higher in tumoural areas in both patients with HCC and in DEN-induced-hepatocarcinogenesis animal model. (A) mRNA levels of *CLTC* in patient tissues. Wilcoxon matched-pairs signed rank test: ***p <0.001. (B) Relative expression of HCC tumour tissues compared to respective surrounding tissue (n = 60). Relative expression of *SRF4* gene. (C) Clathrin immunohistochemistry in HCC and healthy patients. Representative images are shown. (D) Experimental study design and mRNA *Cltc* expression in non-tumoural and tumoural areas in mice. Relative expression to *mL32* gene. Clathrin and Ki67 immunohistochemistry. Data are mean ± SEM. (n ≥3 animals). Student's *t* test: **p <0.01. DEN, diethylnitrosamine; HCC, hepatocellular carcinoma; T, tumour. (This figure appears in colour on the web.)

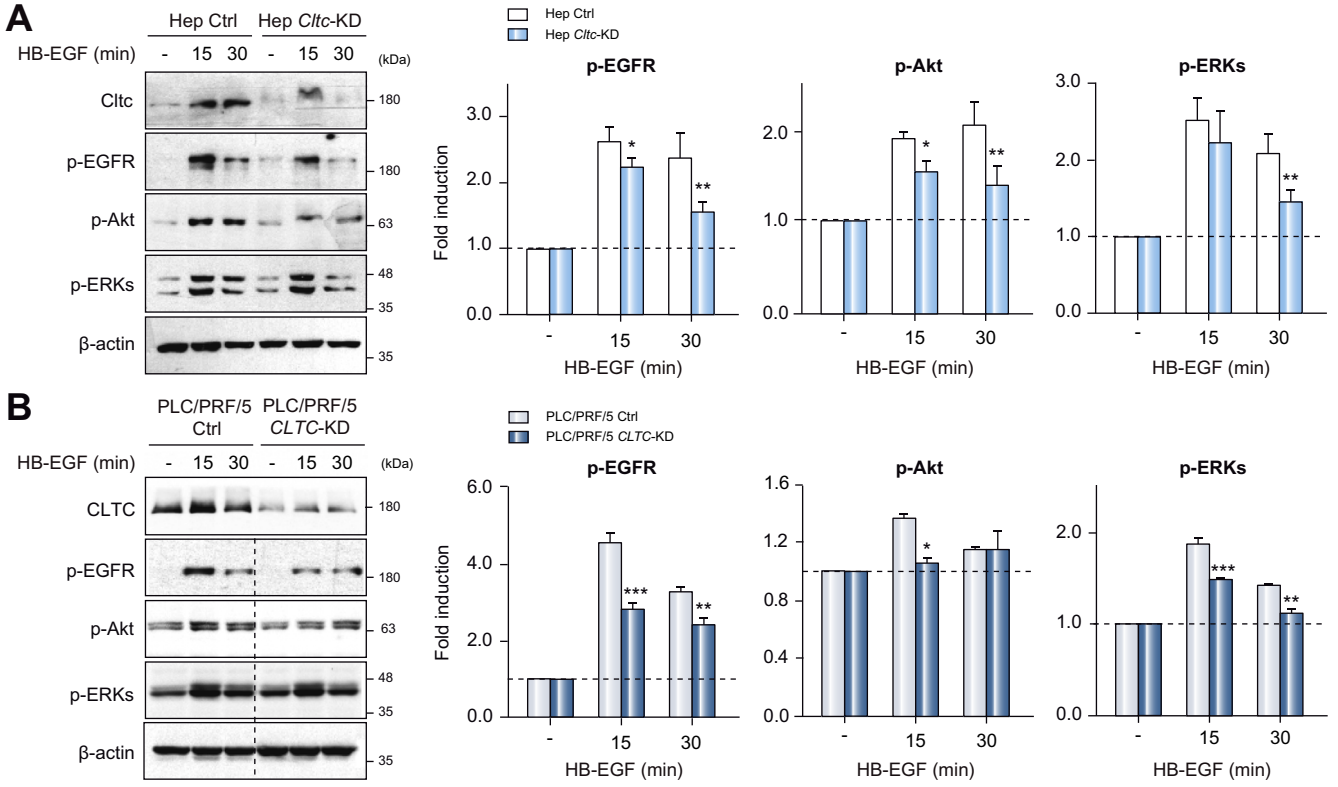

Fig. 2. Activation of the EGFR signalling pathway by HB-EGF is attenuated in *CLTC* knockdown liver cells. Response to HB-EGF in (A) mouse hepatocytes (Hep) and in (B) PLC/PRF/5 cells. Left: western blot of protein extracts: a representative experiment is shown. In (B), dashed lines show where the blots were cut. Samples were run on the same gel but were non-contiguous. Original blots are available in the supplementary information. Right: densitometric analysis: results expressed as fold induction *vs.* each corresponding control (untreated cells). Data are mean ± SEM (n = 3). Two-way ANOVA with Bonferroni *post hoc* test. *p <0.05, **p <0.01, ***p <0.001 compared to siControl cells.

analysed by Ki67 immunofluorescence, in response to HB-EGF or FBS. FACS cell cycle analysis also revealed that the increase in the % of cells in S-phase in response to these mitogens was significantly lower in *Ctlc* knockdown hepatocytes than in control cells (Fig. S3). These results suggest that clathrin could be important for the induction of proliferative and survival signals by EGFR.

In foetal hepatocytes and liver cancer cells, TGF-β simultaneously induces both pro- and anti-apoptotic signals whose final balance determines cell fate.[11,24] TGF-β binding to specific TGF-β receptors induces phosphorylation of SMAD2/3. At short time (30 min) after TGF-β treatment, phospho-SMAD2 did not show lower levels in *Cltc* knockdown mouse hepatocytes, compared to siControl cells (Fig. 3A). Similarly, no differences were found in PLC/PRF/5 cells (Fig. 3B). Indeed, clathrin is not necessary for TGF-β-mediated SMAD phosphorylation. Next, we wondered whether *CLTC* knockdown cells showed alterations in the survival signals triggered by TGF-β, which involve EGFR phosphorylation. Western blot analysis showed a decrease in EGFR and Akt phosphorylation in both TGF-β-treated hepatocytes and PLC/PRF/5 cells (Fig. 3A-B). Curiously, levels of p-EGFR appeared to be higher in *CLTC* knockdown cells. We previously described a SRC-dependent transactivation of the EGFR under serum withdrawal conditions in liver tumour cells.[25] Since our experiments are performed in the absence of serum, the results might indicate that *CLTC* knockdown would favour this auto-transactivation of the EGFR. However, higher phosphorylation of the EGFR and its downstream signals was not observed in response to TGF-β.

In order to know whether the absence of clathrin could be affecting the membrane cell trafficking of EGFR, we performed EGFR confocal microscopy analysis. We observed that in PLC/PRF/5 cells, clathrin colocalized with EGFR at the cell membrane and after TGF-β treatment both were internalized (Fig. S4A). Cytosolic clathrin colocalized with the Golgi reticular system and the Pearson correlation coefficient increased in

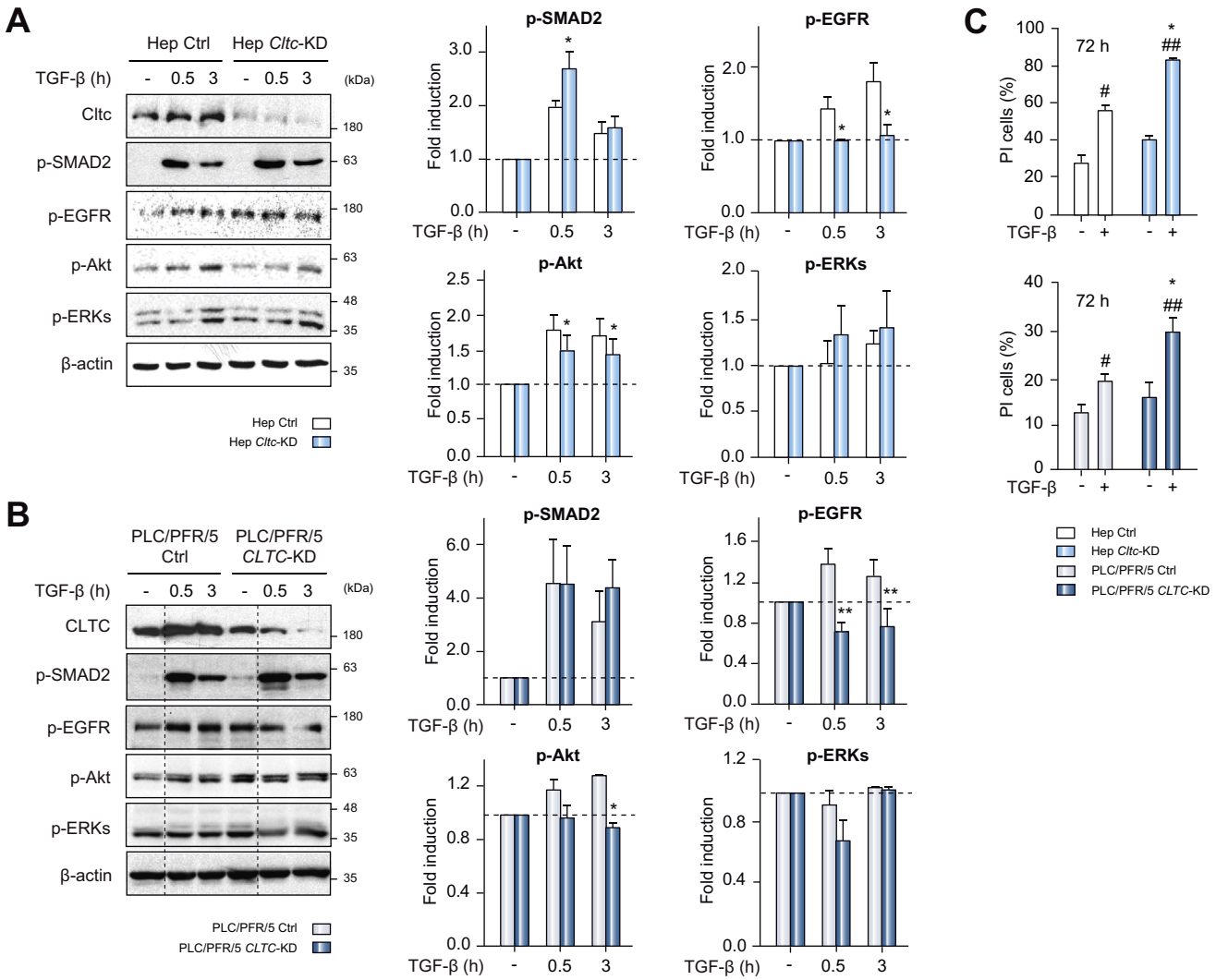

Fig. 3. *CLTC* knockdown attenuates TGF-β-mediated anti-apoptotic signals in mouse hepatocytes. Response to TGF-β in (A) mouse hepatocytes (Hep) and in (B) PLC/PRF/5 cells. Left: western blot of protein extracts: a representative experiment is shown. Dashed lines show where the blots were cut. Samples were run on the same gel but were non-contiguous. Original blots are available in the supplementary information. Right: densitometric analysis: results expressed as fold induction *vs.* each corresponding control (untreated cells). (C) Cell viability was measured by flow cytometric analysis of PI incorporation and expressed as percentage of PI-positive cells. Data are mean ± SEM (n = 3). Two-way ANOVA with Bonferroni *post hoc* test. $^{*}p$ <0.05, $^{**}p$ <0.01 compared to siControl cells. $^{\#}p$ <0.05, $^{\#\#}p$ <0.01 compared to untreated condition. PI, propidium iodide.

TGF-β-treated cells (Fig. S4B). In *CLTC* knockdown cells, EGFR localized mainly in cytosolic compartments and no significant changes were observed after TGF-β treatment (Fig. S5A). A similar pattern was observed in cells treated with dynasore (GTPase inhibitor of dynamin activity, which prevents endocytosis) (Fig. S5B). These results together suggest that clathrin and endocytotic processes are required for the cell membrane trafficking of EGFR in liver tumour cells.

Since *CLTC* knockdown cells showed alterations in the survival signals triggered by TGF-β, we analysed whether these cells were more sensitive to the pro-apoptotic effects of TGF-β. *CLTC* knockdown liver cells showed an increased sensitivity to TGF-β in terms of cell death. These differences were clearly visualized under microscopy (Fig. S6). Furthermore, *CLTC* knockdown cells, after TGF-β treatment, exhibited a higher percentage of non-viable cells, compared to control cells, after 72 h of treatment, measured by flow cytometric analysis of propidium iodide incorporation (Fig. 3C). These results suggest that clathrin levels, through favouring the response to EGFR ligands, could unbalance the response to TGF-β in liver cells in favour of anti-apoptotic signals.

TGF-β upregulates clathrin expression in liver tumour cells

The analysis of *CLTC* and *TGFB*1 expression in different HCC cell lines revealed a positive correlation between *CLTC* and *TGFB1* mRNA levels (Fig. 4A), correlating with the cell phenotype. Indeed, epithelial HCC cells showed lower expression of both genes, while mesenchymal HCC cells expressed higher levels. Likewise, the same result was observed for clathrin by western blot (Fig. 4B). These results suggested a possible regulation of clathrin expression by TGF-β. Corroborating this, both hepatocytes and PLC/PRF/5 cells showed higher protein levels of clathrin after TGF-β treatment, which in the case of PLC/PRF/5 significantly correlated with higher mRNA levels of *CLTC* at 72 h after TGF-β treatment (Fig. 4C,D). These results indicate a positive correlation between clathrin and TGF-β.

CLTC knockdown sensitizes liver tumour mesenchymal HCC cells to the pro-apoptotic effects of TGF-β

HCC cell lines used in this work had already been described to respond with different gene expression profiles to TGF-β treatment: the early gene signature defined a pro-apoptotic and cytostatic response, whereas the late TGF-β signature defined an anti-apoptotic and invasive response.[26] PLC/PRF/5, Huh7 and Hep3B cell lines belong to the early TGF-β signature group, while mesenchymal HLF and SNU449 cell lines belong to the late TGF-β signature group. We previously found that autocrine TGF-β expression is high in mesenchymal-like HCC cells, which are resistant to its pro-apoptotic signals and respond to it inducing EMT, migration and invasion. Thus, we wondered whether the high expression of *CLTC* could be responsible for the lack of pro-apoptotic response to TGF-β in the mesenchymal cell lines. We chose SNU449 due to its mesenchymal phenotype and its higher expression levels of both clathrin and TGF-β, in comparison with PLC/PRF/5 which expressed lower levels of both proteins and had an epithelial phenotype. *CLTC* knockdown in SNU449 cells (Fig. 5A) attenuated the phosphorylation of EGFR in response to either HB-EGF or TGF-β (Fig. S7) and sensitizes them to the pro-apoptotic effects of TGF-β, analysed by propidium iodide incorporation and % of cells with a DNA content lower than 2C – SubG1 cells (Fig. 5B-D). Interestingly, TGF-β receptor I (TβRI, *ALK5* gene) knockdown cells showed a decrease in basal *CLTC* expression (Fig. S8), which suggests that autocrine production of TGF-β in mesenchymal-invasive cells

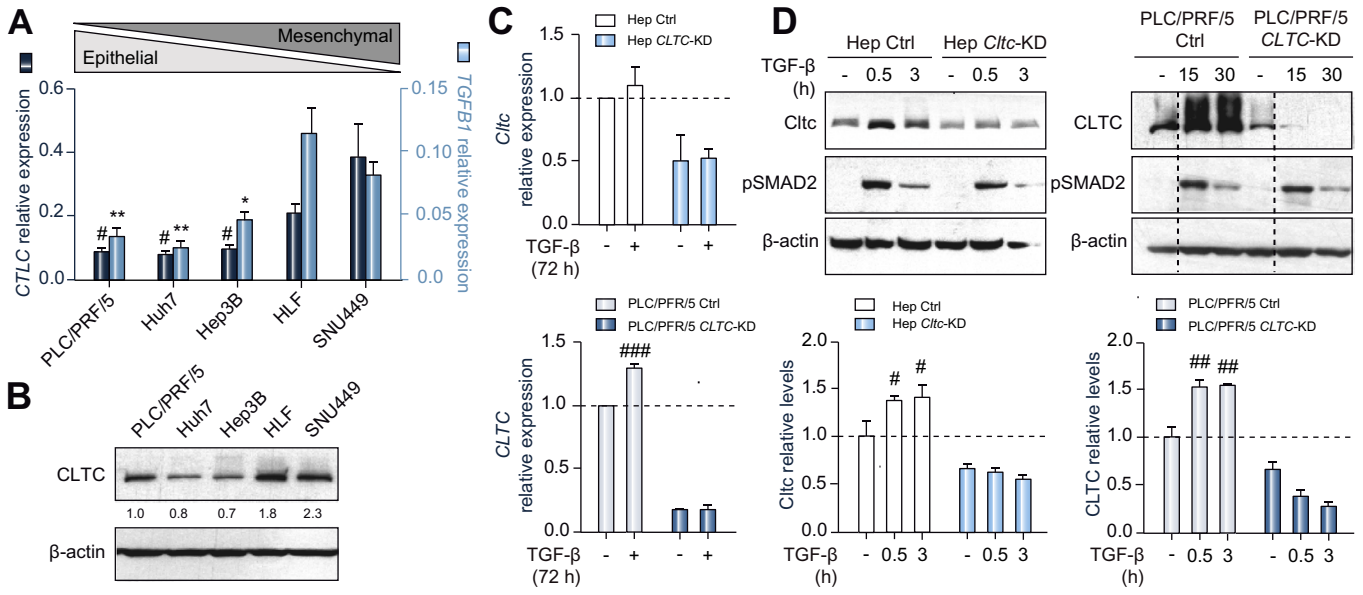

Fig. 4. Positive correlation between clathrin and TGF-β in liver cells. (A) Analysis of mRNA expression in different HCC cells (n = 3). Relative expression to *hL32* gene. (B) Parallel western blot of clathrin and densitometric analysis (n = 2). In (C) and (D), cells either untreated or treated with TGF-β at the indicated times. (C) Analysis of *CLTC* mRNA levels. Relative expression to *mL32* or *hL32* gene. *(D) Upper*: western blot: representative experiment is shown. Dashed lines show where the blots were cut. Samples were run on the same gel but were non-contiguous. Original blots are available in the supplementary information. *Bottom*: densitometric analysis. Data are mean ± SEM. One-way ANOVA with Bonferroni *post hoc* test in A: $^{*}p$ <0.05, $^{**}p$ <0.01 (*TGFB1* expression) and $^{\#}p$ <0.05 (*CLCT* expression) compared to SNU449 cells. Two-way ANOVA with Bonferroni *post hoc* test in C: $^{\#}p$ <0.05, $^{\#\#}p$ <0.01 and $^{\#\#\#}p$ <0.001 compared to untreated cells.

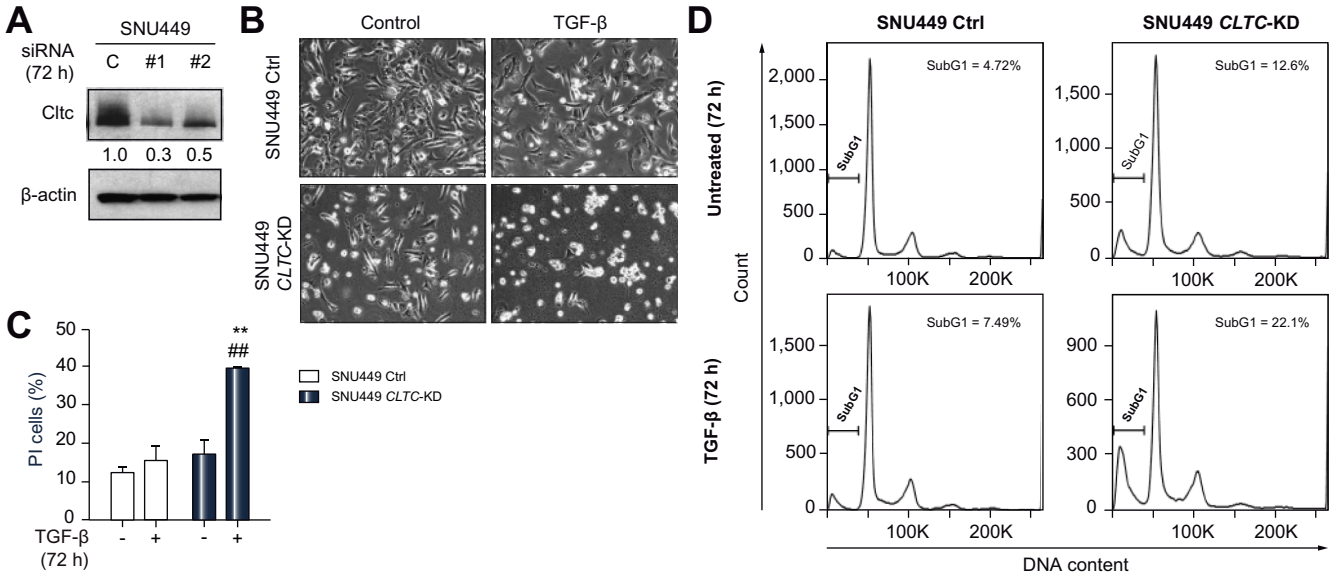

Fig. 5. *CLTC* knockdown sensitizes SNU499 HCC cells to TGF-β in terms of apoptosis. (A) Western blot and densitometric analysis (n = 2). A representative experiment is shown. (B) Representative images after TGF-β treatment (72 h) (n = 3) (C) FACS analysis of the % of PI-positive cells (+PI). Data are mean ± SEM. Two-way ANOVA with Bonferroni *post hoc* test: $^{**}p$ <0.01 compared to siControl cells; $^{\#\#}p$ <0.01 compared to untreated cells. (D) FACS Cell Cycle Analysis, highlighting the % of subG1 cells. A representative experiment (n = 3) is shown. PI, propidium iodide.

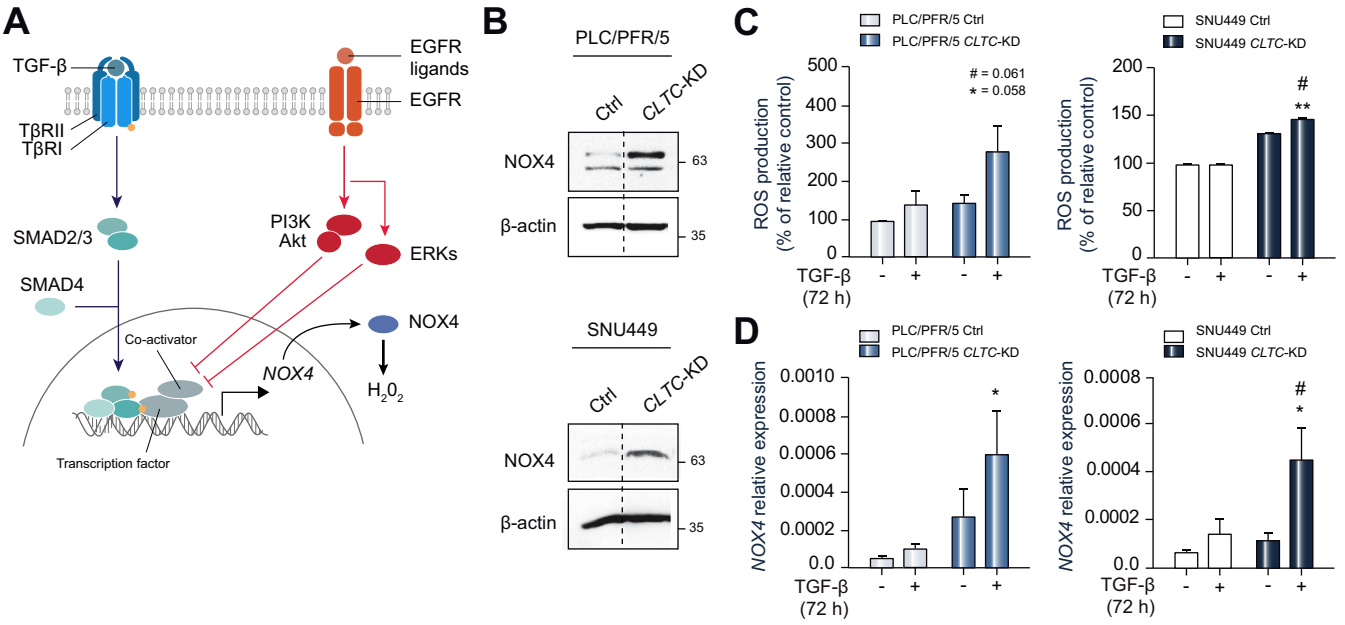

Fig. 6. *CLTC* knockdown in liver cancer enhances the response to TGF-β in terms of ROS production and upregulation of *NOX4* mRNA levels. (A) Graphical scheme: NOX4 is a downstream TGF-β effector negatively regulated by EGFR pathway.[29] (B) Effect of *CLTC* knockdown on NOX4 protein levels (western blot) in PLC/PRF/5 and SNU449. Dashed lines show where the blots were cut. Samples were run on the same gel but were non-contiguous. Original blots are available in the supplementary information. (C) Extracellular ROS production expressed as relative percentage *vs.* untreated siControl cells (n = 3). (D) *NOX4* mRNA levels (n = 3). Relative expression to *hL32* gene. Data are mean ± SEM. Two-way ANOVA with Bonferroni *post hoc* test. $^{*}p$ <0.05 compared to siControl cells. $^{\#}p$ <0.05 compared to untreated cells. ROS, reactive oxygen species. (This figure appears in colour on the web.)

mediates upregulation of clathrin expression, favouring its pro-tumorigenic actions.

Levels of clathrin in HCC cells affect the response to TGF-β in terms of upregulation of the pro-apoptotic mediator NOX4

TGF-β requires reactive oxygen species (ROS) production to induce apoptosis in liver cells,[27,28] an effect that is counteracted by the EGFR pathway[28] (scheme in Fig. 6A). ROS are mainly produced through the NADPH oxidase NOX4, which is upregulated by TGF-β, an effect that is counteracted by the EGFR pathway.[29,30] In fact, NOX4 plays a liver tumour suppressor role.[31] Interestingly, *CLTC* knockdown PLC/PRF/5 and SNU449 cells showed higher protein levels of NOX4 compared to control cells (Fig. 6B). Analysis of ROS production and *NOX4* mRNA expression in TGF-β-treated *CLTC* knockdown cells showed higher ROS production, correlating with upregulation

of *NOX4* mRNA levels in both cell lines (Fig. 6C-D) compared to control cells.

We next wondered whether the overexpression of *CLTC* in the PLC/PRF/5 cells could confer resistance to the pro-apoptotic effects of TGF-β. *CLTC* overexpressing cells (Fig. S9) showed higher basal mRNA levels of *TGFB1* and lower expression of *NOX4* compared to control cells. Furthermore, clathrin overexpression provided resistance to the pro-apoptotic effects of TGF-β, preventing cell death.

These results suggest that clathrin, due to its role in favouring the EGFR anti-apoptotic pathway, impairs NOX4 upregulation and ROS production by TGF-β in liver tumour cells, which would in turn inhibit its pro-apoptotic effects and favour its pro-tumorigenic response.

Relevance of the expression of *CLTC* and *TGFB1* on the prognosis and overall survival of patients with HCC

Next, we decided to evaluate expression of *CLTC*, *TGFB1* and *EGFR* genes in the HCC patient samples cohort (n = 60). Notably, *CLTC* expression positively correlated with *TGFB1* and *EGFR* expressions (Fig. 7A). To expand the analysis to a higher number of patients we used the TCGA database (n = 369), with the aim of performing a bioinformatic analysis of the importance of *TGFB1* and *CLTC* gene expression in HCC prognosis and overall survival. We found that higher expression of *TGFB1* predisposes patients with HCC to lower overall survival (hazard ratio 1.84; 95% CI 1.13–2.98; $p = 0.019$). High *CLTC* expression tends to indicate a poor prognosis (hazard ratio 1.33; 95% CI 0.78–2.28; $p = 0.312$). Next, we defined a cohort of patients where the expression of both genes was high (average expression + 1SD) (Fig. S10A). In this patient cohort, a positive correlation between *TGFB1* and *CLTC* was also found (Fig. S10B). Interestingly, patients with a high combined scored for both genes had a significantly lower overall survival (3.22; 95% CI 1.45–7.13; $p = 0.013$) (Fig. 7C), compared with the effect of high expression of each gene separately. Finally, we decided to analyse the overall survival for *CLTC* expression in patients (n = 358) stratified according to high ($N_{High} = 54$) or low ($N_{Other} = 304$) *TGFB1* expression (Fig. 7C). It is worth mentioning that *CLTC* expression had no prognostic value in those patients where *TGFB1* expression was not high, whereas high *TGFB1* expression significantly decreased the overall survival of patients that expressed high levels of *CLTC*. Interestingly, when we focused on the analysis of some mesenchymal genes whose expression was related to TGF-β in HCC cells, such as *CXCR4* or *CD44*,[32,33] patients with higher expression of both *TGFB1* and *CLTC* had higher expression levels of these mesenchymal genes in comparison to patients who expressed lower levels of *TGFB1* and *CLTC* (Fig. S11). All these results indicate that *CLTC* expression influences the prognosis and overall survival of patients with HCC and high expression of *TGFB1*.

Discussion

HCC is one of the most common types of cancer and is associated with a very poor prognosis, mainly due to the heterogene-

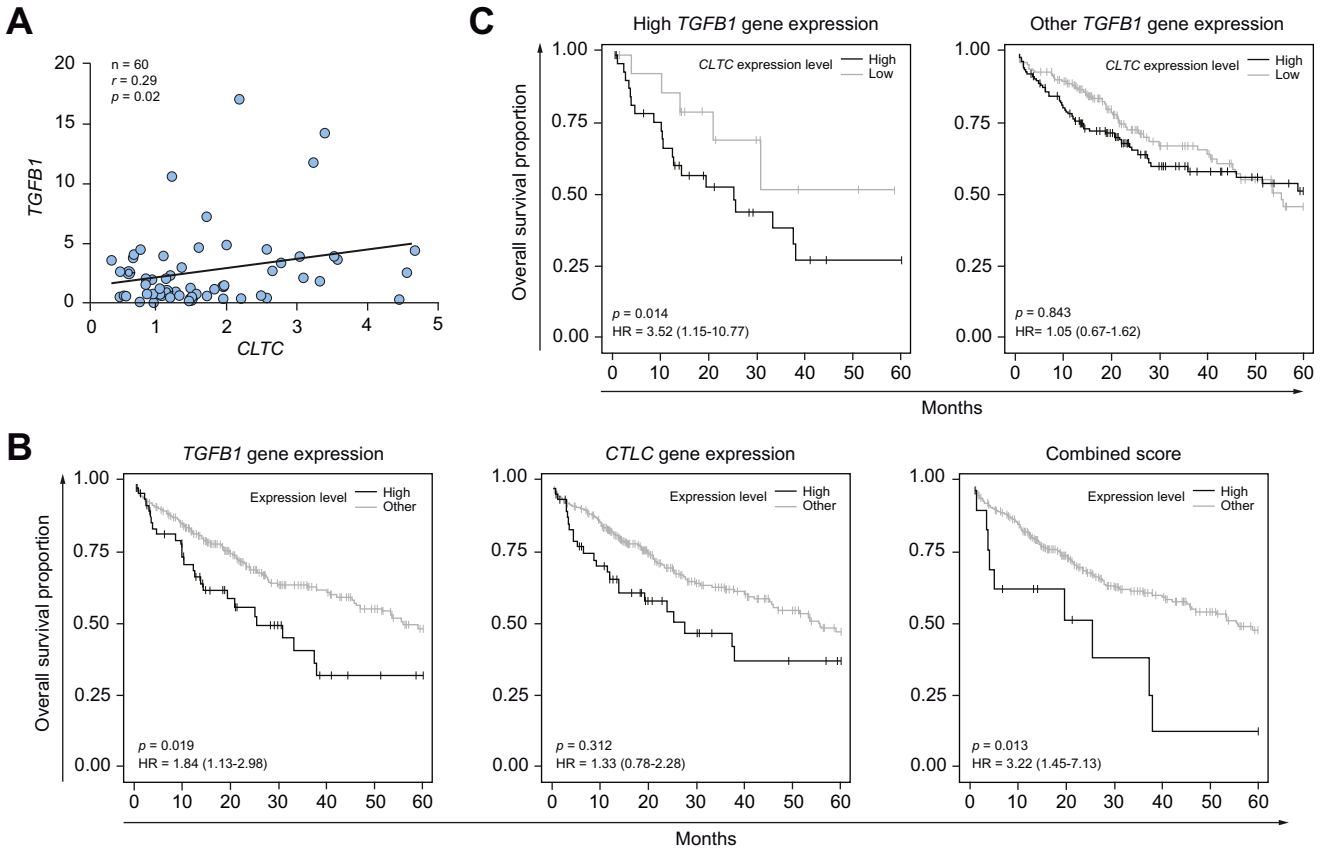

Fig. 7. Prognostic value of *CLTC* expression, in combination with *TGFB1*, in patients with HCC. (A) Pearson correlation analysis of patients with HCC (n = 60). Each dot represents relative expression of each HCC tumour tissue compared to its respective adjacent tissue. Relative expression to *SRF4* gene. (B, C) Kaplan-Meier curves of overall survival of patients with HCC from the TCGA-LIHC cohort (n = 358). (B) Overall survival and *TGFB1* expression, *CLTC* expression, and a score combining both categorizations. (C) Overall survival and *CLTC* expression stratified by *TGFB1* expression ($N_{High} = 54$, $N_{Other} = 304$).

ity of the tumours and the lack of effective targeted therapy. Drugs with a wide action, such as sorafenib or other multikinase inhibitors, have shown only modest efficacy. Considering this, it seemed rational to focus on crucial cellular mechanisms, common to different intracellular signals, as potential therapeutic targets. This was the initial idea behind this work, which aimed to study the role of clathrin, an essential protein involved in endocytosis and signalling of multiple tyrosine kinase receptors, in liver tumorigenesis. Initial analysis indicated that *CLTC* gene expression was significantly increased in a large percentage of patients with HCC (Fig. 1, Fig. S1). Furthermore, we confirmed an increase in the protein levels of clathrin in tumours, particularly in the peritumoural area, compared to tissues from healthy patients or the non-tumoural areas of the same patients. In agreement with this result, in the model of DEN-induced hepatocarcinogenesis in mice, we observed higher expression of the gene (*Cltc*) and increased levels of clathrin in tumoural areas compared to non-tumoural areas (Fig. 1). This encouraging result that, surprisingly, had not been explored before, pushed us to analyse the relevance of clathrin expression in the response of an immortalized cell line of mouse hepatocytes and an HCC cell line, PLC/PRF/5, both of them used in our previous studies to analyse EGFR and TGF-β signalling.[14,15,18]

According to our data, clathrin is essential for the hepatocyte and liver tumour cell response to EGFR ligands, such as HB-EGF, in terms of full EGFR/Akt/ERKs phosphorylation and cell proliferation (Fig. 2 and Fig. S3). After ligand binding, EGFRs are recruited to clathrin-coated pits and their phosphorylation is amplified by clustering platforms that promote the dimerization and activation of unliganded EGFRs[34] and protect the signalling complex from membrane phosphatases.[35] Endocytosis to early endosomes seems to be a requirement for full ERK activation.[36] Phosphorylation of Gab1 and Akt following EGFR activation also requires clathrin.[2] These data suggest that clathrin downregulation might reduce active phosphorylated EGFR at the plasma membrane and endosomes, as previously suggested.[37]

The anti-apoptotic response induced by TGF-β in hepatocytes and HCC cells requires the transactivation of EGFR through increases in the expression and shedding of EGFR ligands.[11,12,15] Due to clathrin's role in EGFR signalling, we hypothesized that it would also be required for the non-canonical signalling of TGF-β. Attenuation of clathrin expression could also potentiate the canonical pathways and their functions, with increased and/or longer phosphorylation of SMAD2/3. Both effects would contribute to an increase in the pro-apoptotic actions of TGF-β. We observed only a slight increase in the levels of phospho-SMAD2 in *CLTC* knockdown hepatocytes and no changes in PLC/PRF/5 cells (Fig. 3). In contrast, an impairment in TGF-β-induced EGFR and Akt phosphorylation, which correlated with an increase in TGF-β-induced apoptosis, was observed in both *CLTC* knockdown hepatocytes and PLC/PRF/5 cells (Fig. 3 and Fig. S6). Clathrin would be required for a correct membrane traffic, dynamin-dependent, of the EGFR between Golgi and the cell membrane (Fig. S4 and S5).

Increases in TGF-β-induced apoptosis in knockdown cells correlated with higher levels of *NOX4* and ROS production after TGF-β treatment. Upregulation of *NOX4* by TGF-β is required for its pro-apoptotic activity (Fig. 6). The EGFR pathway inhibits *NOX4* expression, acting at the transcriptional level on the *NOX4* promoter.[29] In this sense, clathrin downregulation could impair the inhibition of NOX4 by EGFR and could promote the pro-apoptotic role of TGF-β. Inhibition of NOX4 in liver cells might lead to pro-tumorigenic processes. NOX4 plays a role in regulating liver cell proliferation either under physiological conditions or during tumorigenesis. NOX4 silencing increases the tumorigenic potential of human HCC cells in xenografts in mice, resulting in earlier onset of tumour formation and increases in tumour size.[31] The loss of NOX4 increases actomyosin levels and favours an epithelial to amoeboid transition, contributing to tumour aggressiveness.[38] Indeed, here we propose a new unknown, interesting and essential role for clathrin in the regulation of the crosstalk between the TGF-β and EGFR pathways. Indeed, clathrin is necessary for the EGFR transactivation that prevents TGF-β-induced ROS production required for cell death (Fig. 6). This hypothesis is further confirmed in the experiments of *CLTC* overexpression in PLC/PRF/5 cells, where we found that *NOX4* expression decreases and TGF-β-induced apoptosis is inhibited in *CLTC* overexpressing cells (Fig. S9).

Our results also show that the more aggressive mesenchymal-like HCC cells express higher levels of both *TGFB1* and *CLTC* (Fig. 4). Interestingly, after TGF-β treatment, clathrin expression is upregulated in both hepatocytes and PLC/PRF/5 cells, the effect being much more pronounced in the liver tumour cells, where upregulation is observed at both mRNA and protein levels (Fig. 4). We have recently described that mesenchymal cells overexpress TGF-β, which have acquired mechanisms to escape from its suppressor effects and respond to it undergoing EMT, which facilitates migration and invasion.[32] Here we hypothesized that the high levels of clathrin could be responsible for the lack of pro-apoptotic response to TGF-β in these mesenchymal-like cells. Results support this hypothesis, since targeting *CLTC* knockdown in SNU449 cells, a mesenchymal HCC cell line that is unresponsive to the pro-apoptotic effects of TGF-β, sensitize them to cell death (Fig. 5).

From these results, we decided to further improve the analysis in our cohort of patients with HCC, finding that *CLTC* expression positively correlates with *TGFB1* expression (Fig. 7), suggesting that clathrin levels would be high when their signalling pathways are activated. Interestingly, clathrin seems to be overexpressed in the tumour's border. TGF-β, expressed by stromal cells, could activate the expression of clathrin in tumoural cells enhancing its pro-tumorigenic effects and favouring the mesenchymal phenotype, migration/invasion, which would contribute to tumour expansion and/or dissemination. Analysis in public databases allowed us to demonstrate that expression of *CLTC* by itself has no prognostic value for overall survival in patients with HCC, but the combination of high *TGFB1* and *CLTC* expression clearly decreased overall survival compared to high expression of *TGFB1* alone.

In summary, we describe a novel role for clathrin in liver tumourigenesis, wherein it mediates EGFR signalling and favours non-canonical anti-apoptotic TGF-β pathways. *CLTC* expression is increased in a large percentage of patients with HCC and high expression of *CLTC* worsens the overall survival in patients with high expression of *TGFB1* (Fig. 8). Targeting the TGF-β pathway has been proposed as a new promising therapeutic tool in HCC.[39] However, the identification of new biomarkers that indicate when TGF-β is acting as pro-tumourigenic is essential to help in the selection of those patients most likely to benefit from therapy aimed at inhibiting its pathway. Our results indicate that high expression of TGF-β concomitant with high levels of clathrin would identify those

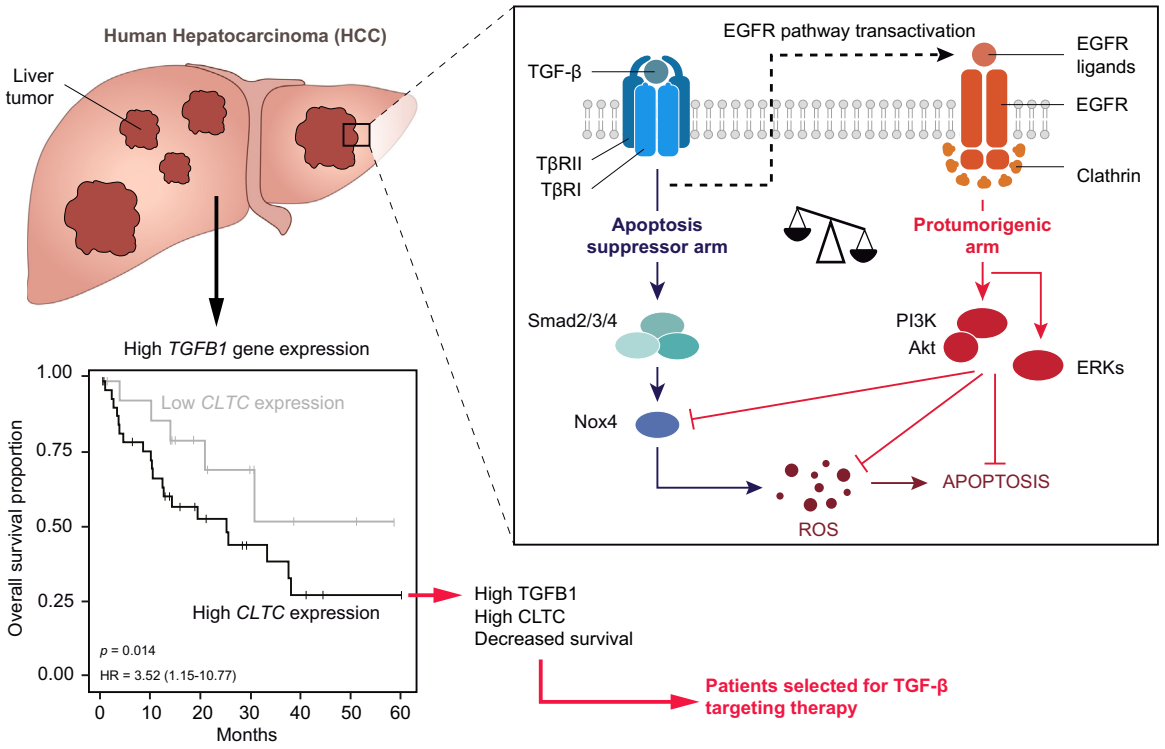

Fig. 8. Summary diagram. *Left*: In liver cancer cells, clathrin favours non-canonical pro-tumorigenic TGF-β through EGFR pathway transactivation. *Right*: HCC patients who express higher levels of both *TGFB1* and *CLTC* show lower overall survival. *CLTC* expression could help select those patients that benefit from a TGF-β targeting therapy. HCC, hepatocellular carcinoma. (This figure appears in colour on the web.)

patients with HCC who are likely to benefit from TGF-β targeted therapies. Moreover, targeting clathrin would potentiate the suppressor actions of TGF-β in HCC.

Abbreviations

ATCC, American Tissue Culture Collection; ECACC, European Collection of Authenticated Cell Cultures; CLTC, clathrin heavy-chain; DEN, diethylnitrosamine; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; HB-EGF, heparin binding EGF-like growth factor; HCC, hepatocellular carcinoma; PI, propidium iodide; ROS, reactive oxygen species; TGF-β, transforming growth factor-β.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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

Authors' contributions

Study concept and obtained funding: IF; design of experiments: IF and DCD; acquisition of data: DCD, with the help in some experiments of EB, IPH, JMC, AM and JLL; experiments in DEN model of hepatocarcinogenesis: AA, BH, AS; collection of patient samples: ER, TS, EB; TCGA bioinformatic analysis: XS, AA.; analysis and interpretation of data: IF, DCD, EB, ER, TS, XS, AA.; writing of the manuscript: IF, DCD with the critical revision of all the authors.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2019.09.012.

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Author names in bold designate shared co-first authorship

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