PI3Kδ activity controls plasticity and discriminates between EMT and stemness based on distinct TGFβ signaling

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The stem cells involved in formation of the complex human body are epithelial cells that undergo apicobasal polarization and form a hollow lumen. Epithelial plasticity manifests as epithelial to mesenchymal transition (EMT), a process by which epithelial cells switch their polarity and epithelial features to adopt a mesenchymal phenotype. The connection between the EMT program and acquisition of stemness is now supported by a substantial number of reports, although what discriminates these two processes remains largely elusive. In this study, based on 3D organoid culture of hepatocellular carcinoma (HCC)-derived cell lines and AAV8-based protein overexpression in the mouse liver, we show that activity modulation of isoform δ of phosphoinositide 3-kinase (PI3Kδ) controls differentiation and discriminates between stemness and EMT by regulating the transforming growth factor β (TGFβ) signaling. This study provides an important tool to control epithelial cell fate and represents a step forward in understanding the development of aggressive carcinoma.
The class I phosphoinositide 3-kinases (PI3Ks) are the best-studied enzyme of PI-metabolism and frequently deregulated in cancer. The class I PI3Ks consist of four isoforms (α β γ δ), however most of the fundamental or clinical studies were performed using pan-PI3K inhibitors targeting all isoforms. The isoform-specific roles just started to be investigated this last decade due to the generation of gene-targeted mice and commercially available isoform-selective inhibitors. Isoform δ of phosphoinositide 3-kinase (PI3Kδ) is the latest member of class I PI3Ks identified more than 20 years ago as predominantly expressed in the spleen and thymus while almost undetectable in other tissues.

PI3Kδ plays a major role in the immune system, and extensive work devoted to understanding this protein led to establishment of the first PI3K inhibitor, idelalisib (CAL-101), an ATP-competitive kinase inhibitor that targets PI3Kδ with a high potency and selectivity, approved for the treatment of lymphomas. Nevertheless, the fundamental role of this enzyme in non-hematopoietic cells, notably those in the epithelium, remains enigmatic. Several recent studies have shown the level of this protein to be elevated in solid cancers but also stem cells that polarize during embryonic development and stem cells grown in 3D in Matrigel and stem cells grown in 3D in Matrigel. Nevertheless, the fundamental role of this enzyme in non-hematopoietic cells, notably those in the epithelium, remains enigmatic. Several recent studies have shown the level of PI3Kδ to be elevated in solid cancers but also stem cells that polarize during embryonic development and stem cells grown in 3D in Matrigel. Thus, we analyzed the expression of PI3Kδ in Huh7 + PI3Kδ cells impaired rosette formation, indicating that rosette formation was dependent on PI3Kδ activity (Supplementary Fig. 1d).

PI3Kδ reprograms Huh7 cells into stem-like cells. This rosette-like structure consisting of Huh7 + PI3Kδ cells, surrounded by a dense ECM visible by laminin-111 labeling (Fig. 2a), is reminiscent of not only liver progenitor cells/small cholangiocytes but also stem cells that polarize during embryonic development and stem cells grown in 3D in Matrigel. Thus, we analyzed the expression of PI3Kδ in Huh7 + PI3Kδ cells impaired rosette formation, indicating that rosette formation was dependent on PI3Kδ activity (Supplementary Fig. 1d).

PI3Kδ is enriched in stem cells. We then performed a series of bioinformatics analyses to obtain more information about the potential involvement of PI3Kδ in stemness. Using bioinformatics approaches based on GSE26093, we showed high PI3Kδ expression in human embryonic stem cells (hESCs), induced pluripotent stem cells (iPSCs), and mesenchymal stem cells (MSCs) (Supplementary Fig. 4a). Furthermore, using GSE23034, we analyzed the expression of PI3Kδ during the generation of iPSCs from mature human hepatocytes.
Fig. 1 PI3Kδ is required for bile canaliculi formation and its overexpression induces formation of rosette-like structures. a Time-course analysis of lumen formation in Huh7 and Huh7 overexpressing PI3Kδ (Huh7 + PI3Kδ) organoids plated in 3D Matrigel-matrix and stained after 2, 4 or 6 days for Zona-occludens 1 (ZO-1, green), actin microfilaments using phalloidin (red) and nuclei using Hoechst (blue). Scale bar: 10 μm. b Quantification of the phenotypes percentage over the days of culture of Huh7 (n = 117 organoids) and Huh7 + PI3Kδ (n = 123 organoids). c Quantification of Huh7 and Huh7 + PI3Kδ circularity index, organoids area and nuclei per organoid. Each dot of the graph corresponds to an organoid. All values are expressed as mean ± S.E.M.
expression significantly increased during the reprogramming process, while PI3Kα expression decreased, but no significant change in PI3Kβ or PI3Kγ was observed (Supplementary Fig. 4b). Subsequently, we hypothesized that PI3Kδ expression is important for embryonic development and needs to be downregulated to allow differentiation. To test this hypothesis, we analyzed the expression of PI3Kδ during the differentiation of hESCs into hepatocyte-like cells in vitro (GSE7074136). We validated the efficiency of the differentiation protocol described by the authors to generate hepatocyte-like cells and showed that they were capable of polarizing in Matrigel and forming bile canaliculi (Supplementary Fig. 5a, b).
from cells collected at different time points during hESC differentiation, we observed a gradual decrease in PI3Kδ expression, which was correlated with expression of the pluripotency factors nanog and oct4 and inversely correlated with expression of the differentiation genes albumin and HNF4a (Supplementary Fig. 5c, d). Thus, suggesting that PI3Kδ could have a role in development.

PI3Kδ-induced reprogramming is dependent on TGFβ/Src. To gain further insight into the mechanisms involved in PI3Kδ-dependent reprogramming, we performed transcriptomic analysis of Huh7 + PI3Kδ cells versus control cells in triplicate, and a fold change in PI3Kδ of +53 was observed (Supplementary Fig. 6a). The Pavlidis template matching algorithm was applied, enabling the identification of 660 Affymetrix probes for genes co-regulated with PI3Kδ (312 positively regulated and 348 negatively regulated) (Supplementary data 1). Unsupervised classification of this expression profile enabled discrimination between the two conditions, as represented by a heatmap (Supplementary Fig. 6b) and confirmed by unsupervised principal component analysis (Supplementary Fig. 6c). Among the genes downregulated in Huh7 + PI3Kδ cells, BAX, CYP2D6, and FOXO1 are involved in hepatocyte-specific processes such as xenobiotic metabolism and hormone and steroid synthesis (Fig. 3a, c), confirming that overexpression of PI3Kδ led to Huh7 cell dedifferentiation. Among the upregulated genes, most of them are related to ECM and thus reinforced the reported effect of PI3Kδ on ECM assembly in MDCK cells and the data from Fig. 2a. They included the non-receptor tyrosine kinase protein Src and connective tissue growth factor (CTGF) which is involved in most cellular responses to TGFβ and particularly those leading to ECM remodeling. EMT and SMAD7 are also highlighted (Fig. 3b, Supplementary Fig. 7a). Interestingly, processes such as cellular response to TGFβ, extracellular signal-regulated kinase 1 (ERK1) and 2 (ERK2) cascade and the response to mechanical stimuli were highlighted in Huh7 + PI3Kδ cells (Fig. 3d). The cues from mechanical stimuli arising from the ECM surrounding cells played important role in biological processes such as proliferation and differentiation. Subsequently, we wondered if the effects of PI3Kδ on rosette formation were dependent of ECM signaling. First, we confirmed ERK, AKT and Src phosphorylation and the increase of Src (Fig. 3e–g, Supplementary Fig. 7b–d). We then investigated whether the PI3Kδ-induced phenotype depends on Src-mediated ECM signaling. Pharmacological inhibition of Src with herbimycin A impaired rosette formation and resulted in the formation of organoids that formed multiple lumens ( Supplementary Fig. 7e). The expression of phosphorylated Src (pSrc), Notch2 and CK19 decreased, as did the laminin surrounding the rosettes (Fig. 3h, Supplementary Fig. 7f and Supplementary Fig. 8a). Additionally, we assessed the involvement of TGFβ signaling in the PI3Kδ-induced rosette phenotype. Inhibition of the TGFβ receptor with SB431542 promoted the formation of organoids with multiple lumens and decreased Notch2 signal, similar to the effects of src inhibition (Fig. 3i). Interestingly, treatment with SB431542 strongly decreased p-SMAD2, vimentin and Src, indicating that TGFβ signaling regulated PI3Kδ-induced Src expression, which was required for the reprogramming of Huh7 cells into stem-like cells (Fig. 3j, Supplementary Fig. 8b).

PI3Kδ reprograms hepatocytes in the mouse liver. To study the relevance of our in vitro observations, we injected 8-week-old C57BL/6 mice with adeno-associated vector serotype 8 (AAV8), which has high affinity for mouse hepatocytes and has been suggested to transduce >90–95% of hepatocytes, via intraportal vein injection. We used a plasmid encoding EGFP or mouse PI3Kδ under the liver-specific thyroid-binding globulin (TBG) promoter (pAAV TBG-EGFP (named AAV-control), pAAV TBG-PI3Kδ (named AAV-PI3Kδ)). Overexpression of PI3Kδ did not alter weight gain in the mice (Supplementary Fig. 9a). The infection of murine livers was verified by visualization of GFP fluorescence (Supplementary Fig. 9b). Hematoxylin and eosin staining revealed that PI3Kδ overexpression induced subtle ductular reaction characterized by numerous and disorganized small ductular structures around the portal vein (PV) (Fig. 4a, b) known to be associated with hepatocyte reprogramming. PI3Kδ was assessed by immunohistochemistry and the signal was observed in sinusoids in line with its description in blood cells. Nevertheless, in control mice a faint signal was observed around the PV (Fig. 4a, b); in AAV-PI3Kδ mice liver the PI3Kδ staining increased in all the liver and here again the signal was intense in sinusoids and around the PV. Although no major changes were observed at the central veins (Fig. 4a, b). Together, these data suggested an enhancement of PI3Kδ localization in the PV area of mouse liver. We thus performed RT-qPCR on the liver samples and data revealed the increase of epithelial and pluripotency genes, as well as the Notch2, Src, TGFβ and Smad7 genes, associated with a decrease in expression of genes involved in hepatocyte differentiation were observed in AAV-PI3Kδ mouse livers (Fig. 4c).

Transcriptomic analysis revealed 73 upregulated genes in the livers of AAV-PI3Kδ mice, allowing discrimination of the two conditions (Supplementary data 2, Supplementary Fig 10a, b). Gene set enrichment analysis of the two conditions revealed that PI3Kδ regulates different cell functions, such as the response to hypoxia, apical junctions, mitotic spindle function and myogenesis (Supplementary Fig 10c–e). Gene set enrichment analysis of
PI3Kδ-induced signatures revealed the enrichment of liver bipotency and stem cell phenotypes, as in liver cancer, which exhibits high levels of H3K27me3 marks, EpCAM+ bile duct cells, and hepatoblastoma, which exhibits the properties of hepatoblasts (Fig. 4d, e). Taken together, these results show that overexpression of PI3Kδ in the mouse liver induced the dedifferentiation of mouse hepatocytes, as observed in Huh7 + PI3Kδ cells (Fig. 2g). Thus, PI3Kδ overexpression reprograms hepatocytes into stem-like cells with polarity and epithelial features.

**PI3Kδ discriminates between EMT and stemness.** To further investigate how PI3Kδ reprograms Huh7 cells, we studied the
impact of PI3Kδ enzymatic activity. Treatment of Huh7 cells withidelisib /CAL-101 at different doses altered the formation of canaliculi and resulted in the dose-response formation of organoids with an inverted polarity in which the apical domain stained by ZO-1 faced the ECM, whereas in control cells, ZO-1 stained the apical membrane facing the lumen of the tubules (Fig. 5a). This was reminiscent of our data from MDCK cells12. Subsequently, we analyzed the expression of several genes, as done upon PI3Kδ overexpression (Fig. 2g). Overall, PI3Kδ inhibition using 5 µM CAL-101 or siPI3Kδ significantly decreased Src and NOTCH2/3 genes, epithelial genes and pluripotency factors and their target genes as expected. Strikingly, mesenchymal markers were significantly increased, and the effect was more pronounced than that upon PI3Kδ overexpression. Here, again, these changes were associated with the loss of hepatocyte markers (Fig. 5b). Similar data were observed at different concentration of CAL-101 (Supplementary Fig. 10a–c), suggesting a differentiation and EMT processes with a decrease of Src and p-AKT level (Supplementary Fig. 11d, e). Comparing the control and siPI3Kδ condition in RT-qPCR, we didn’t detect significant changes regarding the other isoforms of class I PI3Ks, confirming that the effects seen is due to the δ isoform (Supplementary Fig. 11g). We also confirmed the changes in CDH1, VIM and EpCAM gene expression by immunofluorescence analyses of the encoded proteins (Fig. 5c, d, Supplementary Fig. 11f). Notably, the localization of E-cadherin at cell–cell contacts reinforced the notion that Huh7 + PI3Kδ cells are highly polarized epithelial cells (Fig. 5c). Therefore, PI3Kδ inhibition induced epithelial to mesenchymal transition (EMT), characterized by decreased epithelial gene expression and increased mesenchymal marker expression, consistent with the inverted polarity phenotype of the organoids (Fig. 5a). Indeed, EMT has been shown to reverse the apicobasal polarity axis47.

Different TGFβ signaling pathways control EMT and stemness. TGFβ is a master regulator of the EMT process48. We therefore investigated whether TGFβ signaling was also involved in the polarity inversion of organoids generated upon PI3Kδ inhibition. The addition of SB431542 to inverted polarized organoids restored the canaliculi-like structure of the Huh7 organoids, and the cells no longer expressed vimentin (Supplementary Fig. 11h). We therefore sought to validate these observations in MDCK cells, a well-established model for the study of epithelial cell plasticity. As in Huh7 cells, inhibition of PI3Kδ induced polarity inversion and the increase of N-cadherin in MDCK organoids (Supplementary Fig. 12a), whereas PI3Kδ overexpression increased src signal both in a TGFβ signaling-dependent manner (Supplementary Fig. 12a–c). Interestingly RT-qPCR data confirmed the changes in induced pluripotency factors, polarity gene PRKCZ, the transcripts for E-cadherin, vimentin and N-cadherin (Supplementary Fig. 12d). TGFβ signaling controls plasticity and was involved here in the formation of both the rosette structure and inverted polarity organoids; thus, it contributes to EMT and stemness. To distinguish these processes, we studied signaling downstream of TGFβ receptors upon the modulation of PI3Kδ activity. Mothers against decapentaplegic homologs (SMADs) are the main transducers of TGFβ receptor signaling. These proteins are notably involved in the regulation of EMT induced by TGFβ. While SMAD3 is one of the main effectors of TGFβ-induced EMT49, SMAD7 is a known inhibitor of this process50 and was recently found to promote stemness31. Strikingly, SMAD3 was upregulated in Huh7 + CAL-101 and Huh7 + siPI3Kδ cells (Fig. 5e), whereas SMAD7 was upregulated in Huh7 + PI3Kδ cells (Figs. 3b, 5e) and downregulated in Huh7 + CAL-101 cells (Fig. 5e). We also measured SMAD3 activity following the addition of TGFβ using the luciferase reporter CAGA52. We observed enhanced TGFβ-induced SMAD3 activity in Huh7 + CAL-101 cells and conversely, the stimulation of Huh7 + PI3Kδ cells with TGFβ decreased SMAD3 activity (Fig. 5f). Overall, these data reveal that PI3Kδ activity regulates different TGFβ-dependent pathways, ultimately leading to different cellular responses, and highlights the differences between stemness and EMT, as illustrated in Fig. 5g.

PI3Kδ activity controls plasticity and the fate of different HCC cells. In order to gain further insights regarding the role of PI3Kδ in liver cell polarization and differentiation, we used CAL-101 to treat HCC cells with high expression level of PI3Kδ including HepG2, Hep3B, another hepatoblastoma derived cell lines and the hepatic bi-progenitor cell line HepaRG (Fig. 6a, b). Unlike Huh7 cells, these cells were not able to form bile canaliculi on 3D culture after 6 days rather HepG2 and Hep3B could form rosette structure (Supplementary Fig. 13). We then inhibited PI3Kδ activity in those cells with increasing doses of CAL-101. Surprisingly, we observed a rescue of bile canaliculi formation with a maximum effect at 1 and 5 µM for all the cell lines (Fig. 6c, d). Interestingly, the presence of canaliculi in the different cell lines was associated with the increase of the expression of differentiated hepatocyte markers such as albumin and the cytochrome P450 1A2 (CYP1A2), cytochrome P450 2D6 (CYP2D6) (Fig. 6e and supplementary Fig. 14a, b). Concomitantly, the induced pluripotent transcription factors Nanog and SOX2 significant decreased in a dose dependent manner while CD44 and CDH2 used as EMT markers were very low and abruptly increased significantly at 10 µM where organoids adopted inverted polarity phenotype with no canaliculi formation (Fig. 6d, e). Interestingly, we also observed a decrease of SMAD7 transcripts while we detected a significantly increases at 10 µM of CAL-101 for SMAD3 (Fig. 6e). All together, these data strongly suggested that high PI3Kδ activity controlled stemness and its modulation...
induced hepatocytes cell differentiation as validated by immuno-fluorescence staining of albumin (Fig. 7a, b) and its full inhibition promoted EMT (Fig. 6e). These data also provided evidence that these plastic events are regulated by different TGFβ-dependent pathways and highlighting the differences between stemness and EMT in different hepatic cell lines.

Finally, we performed a well described method for 2D culture by which HepaRG cells were able to acquire the differentiated hepatocyte phenotype when treated with DMSO 53 (Fig. 7c). HepaRG cells were grown to confluence for 7 days and treated with 1.8% DMSO or different concentrations of CAL-101 for 7 days. We observed a more than two folds increase of albumin
staining in HepaRG + 1 µM CAL-101 comparing to HepaRG + DMSO (Fig. 7c, d). These data provide a distinct role of PI3Kδ in hepatocyte differentiation and may serve as a new tool to acquire differentiated hepatocyte in vitro and both in 2D and 3D.

Discussion

In this study and as summarized in Fig. 7e, we showed that PI3Kδ controls plasticity in epithelial cells and that its balance is required to maintain epithelial cell polarity and differentiation. In the liver, this results in the formation of bile canaliculi and the functional differentiation of hepatocytes. Inhibition of PI3Kδ induced EMT, revealed by an increase in mesenchymal genes and a decrease in epithelial and polarity genes in cells that formed inverted polarized organoids. Conversely, PI3Kδ overexpression in cells promoted stemness, characterized by an increase in both mesenchymal and epithelial markers and pluripotency factors.

To note that the stemness was described as a broad window on the halfway to EMT, a stage named hybrid EMT54–56. Here, we propose that distinct routes promote the stemness which repre- sents a gain of epithelial and pluripotency factors, while EMT, as it is largely defined, is accompanied by the loss of these genes57. Importantly, PI3Kδ activity allows the discrimination of these two processes, both of which require TGFβ-dependant plasticity. We identified SMAD3 and SMAD7 as the main downstream effectors of TGFβ that control the divergence between EMT and stemness, respectively.

Interestingly, our data also showed that PI3Kδ expression was elevated in different stem cells, including hESCs, thus strongly supporting its role in the developmental stemness. Furthermore, the profile of each of the class I PI3K isoforms appeared different and indeed, the PI3Kδ expression was elevated in the stem cells and was found correlated to pluripotency factors expressions. By contrast the PI3Ka expression decreased in stem cells and its expression increased along with differentiation process. Thus, it will be important to decipher the spatio-temporal regulation of stemness and plasticity by PI3Kδ and the contribution of other isoforms which remained enigmatic58. The crucial role of sig- naling pathways such as Notch in liver plasticity has been reported59. Interestingly, we were able to demonstrate in our study that the Notch pathway is activated by PI3Kδ.

Moreover, we studied the effects of CAL-101 treatment on different HCC-derived cells grown on 3D and data showed dif- ferent scenarios regarding drug concentration which are also related to PI3Kδ expression. Indeed, using HuH7 with low expression of PI3Kδ, treatment induced loss of differentiation and promoted EMT. However, cells such as HepG2, Hep3B and HepaRG which have a high expression of PI3Kδ are able to form canaliculi and expressed hepatocyte differentiation markers. However, at the highest dose of CAL-101 formed inverted polarized structures and with EMT features. Thus PI3Kδ appeared as a central regulator of epithelial cells plasticity in liver as well as in MDCK cells. Furthermore, the establishment of relevant in vitro culture systems is a challenge for the toxicology assessment of drugs by the pharmaceutical industry and for the study of liver cell biology. In this context, CAL-101 appeared here as an efficient tool for in vitro hepatic differentiation.

We noticed that PI3Kδ overexpression creates a dense layer of ECM around the rosettes as previously described with MDCK cysts52 and furthermore the bioinformatic data (Fig. 3b) high- lighted an increase of ECM upon PI3Kδ overexpression. Therefore, we demonstrated here that Src is an important regulator of the signaling from the cell-ECM interactions required for PI3Kδ-dependent morphogenetic effects. ECM plays important role during differentiation of liver cells as well as in carcinoma development. Furthermore, nearly 80% of HCC are established on cirrhotic liver presenting ECM alteration. Together, these findings open new perspectives for investigating the role of PI3K isoforms in the plasticity of epithelial cells in both development and cancer conditions which remained open questions58.

Methods

Cells and 3D culture. HuH7, Hep3B and HepG2 cell lines (from ATCC) were cultured in Dulbecco’s modified Eagle’s Medium containing 4.5 g/L glucose supplemented with 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids, 1% sodium pyruvate and 1% of penicillin/streptomycin at 37 °C in 5% CO₂. HepaRG cell line (from Biopredic) was cultured in William’s E medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 5 µg/mL insulin and 3 × 10⁻⁵ M hydrocortisone hemisuccinate. To obtain HepaRG differentiation, cells were cultured in the same medium as above for 2 weeks supplemented with 1.8% dimethylsulfoxide (DMSO). MDCK (Madin Darby Canine Kidney from Keith Mostov, UCSF, San Francisco) cells were cultured in minimal essential medium (MEM) supplemented with 5% fetal bovine serum and 1% of penicillin/ streptomycin. For the 3D culture, cells were trypsinized at 10,000 cells/mL in 2% Matrigel (BD Biosciences). 500 µL of cells were plated in each well of eight-well Lab-Tek II chamber slides (Thermo Fisher Scientific) coated with Matrigel and grown for up to 6 days.

Human embryonic stem cells maintenance, differentiation and 3D culture. Undifferentiated human H1 ES cells (WiCell) were maintained in monolayer culture on Matrigel (BD Biosciences) in mTeSR1 medium (STemcell Technologies, Vancouver, Canada). H1 ES cells were passaged manually at 1:4 to 1:6 split ratios. For 3D culture, cells were dissociated as small patch at day 16 using accutase and a Matrigel (BD Biosciences)-coated eight-well Lab-Tek II chamber slides (Thermo Fisher Scientific) coated with Matrigel and grown for up to 6 days.

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Plasmids, siRNA and cell transfection. Human pl106 and siRNAs were obtained from Dr. Xiaokun Li (Washington University, St. Louis, USA). siRNAs used with HepG2 were: siRNA-1: 5'-CCCAAGAAAGCGGCGAGCUCC-3' and siRNA-2: 5'-GGACAGACUGGGGUCCUGUAAG-3'. For transfection, the cells were seeded at the density of 1.10⁵ cells/well of a 12-well-plate and transfected with 100 pmol of specific siRNA or 2 µg cDNA using JetPRIME (Ozyme), according to the
manufacturer’s instructions. For 3D culture, 24 h after the transfection, cells were detached using trypsin and plated on Matrigel as indicated above.

**Immunoblot.** Cells were lysed in Laemmli sample buffer and denatured at 100 °C for 5 min before separation on 10% SDS–PAGE and then electrotransferred onto nitrocellulose blotting membrane (Amersham Protran). After transfer, the membrane was saturated in DPBS containing 0.1% Tween 20 and 5% milk. Primary antibodies (appropriate dilution) were added overnight at 4 °C. After washes in the presence of DPBS, appropriate secondary antibodies coupled with peroxidase were added. Immunoblotting was revealed with chemiluminescent peroxidase substrate (Chemiluminescent Peroxidase Substrate-3; Sigma-Aldrich) and exposure was observed with G: box (Syngene).

**Immunofluorescence staining.** The cells were rinsed with ice-cold Dulbecco’s PBS (DPBS) and fixed with 4% paraformaldehyde for 20 min at 4 °C. The samples were then permeabilized and saturated with DPBS supplemented with 0.7% fish gelatin.
Fig. 5 PI3Kδ activity discriminates EMT from stemness acquisition based on different TGFβ signaling. a Immunofluorescence staining for ZO-1 (green), actin microfilaments using phallolidin (red) and nuclei (blue) of 3D culture in organoids of Huh7 treated or not with PI3Kδ specific inhibitor idelalisib (CAL-101) at different concentrations after 6 days of 3D and quantification of the canaliculus and inverted polarity phenotype percentage in the different conditions (n > 49 for each condition). b RT-qPCR analysis of different genes expression performed in organoids of Huh7, Huh7+CAL-101 (5 µM) and Huh7+siPI3Kδ after 6 days of 3D culture in two independent experiments performed in duplicate for Huh7 + 5CAL and in triplicate for Huh7 + siPI3Kδ. Data are presented as log₁₀ mRNA fold change in Huh7+CAL-101 or Huh7 + siPI3Kδ compared to Huh7. c Immunofluorescence staining for the different markers indicated in the panel in organoids of Huh7, Huh7 + PI3Kδ, Huh7+CAL-101 (5 µM) and Huh7 + siPI3Kδ. Scale bar: 10 µm. The right panels present E-cadherin (green) and actin microfilaments using phallolidin (red) line profile blots of the white lines. d Quantification of relative intensity of these markers. Each dot of the graph corresponds to an organoid. e RT-qPCR analysis of different genes expression performed in organoids of Huh7, Huh7 + PI3Kδ, Huh7+CAL-101 (5 µM) and Huh7 + siPI3Kδ after 6 days of 3D culture in two independent experiments performed in duplicate. f Smad3 transcriptional activity, measured using CAGA-luciferase reporter, in Huh7, Huh7+CAL-101 (5 µM) and Huh7 + PI3Kδ treated with TGFβ (2 ng/ml) and Huh7 treated with TGFβ and SB431542 (2 µM). Data represent a typical experiment performed in triplicate. g Schematic representation of the PI3Kδ-dependant plasticity with the EMT and stemness routes. All values are expressed as mean ± S.E.M.
Fig. 6 Inhibition of PI3Kδ activity improves canaliculus formation and differentiation in HepG2, Hep3B and HepaRG cells associated with the regulation of EMT and stemness markers. a Immunoblot analysis of PI3Kδ protein level in Huh7, HepG2, Hep3B and HepaRG with the quantification of its relative intensity (right, n = 3 experiments). b RT-qPCR analysis of PI3Kδ expression in Huh7, HepG2, Hep3B and HepaRG cells; RPLP0 was used as the housekeeping gene for normalization. c Immunofluorescence staining for ZO-1 (green), actin microfilaments using phalloidin (red) and nuclei (blue) in HepG2, Hep3B and HepaRG cells plated in 3D cultures for 6 days and treated with the PI3Kδ specific inhibitor (CAL-101) at different doses. Scale bar: 10 µm d Quantification of the percentage of the different phenotypes seen in the conditions above (n = 40 organoids). e RT-qPCR analysis of several markers expression in HepG2, Hep3B and HepaRG cells plated in 3D culture and treated with different doses of CAL-101 for 6 days in two independent experiments performed in duplicate; RPLP0 was used as the housekeeping gene for normalization. All values are expressed as mean ± S.E.M.
**Fig. 7** The inhibition of PI3Kδ activity increases albumin protein level in different hepatic cell lines. 

**a** Immunofluorescence staining for Albumin (green), actin microfilaments using phalloidin (red) and nuclei (blue) in HepG2, Hep3B and HepaRG cells plated in 3D cultures for 6 days and treated or not with the PI3Kδ specific inhibitor (CAL-101) at different doses. Scale bar: 10 μm.

**b** Quantification of albumin relative intensity in the different cell lines treated or not with CAL-101 at different doses.

**c** Experiment plan for HepaRG differentiation using DMSO or CAL-101.

**d** Immunofluorescence staining for Albumin (green), actin microfilaments using phalloidin (red) and nuclei (blue) in HepaRG cells treated with DMSO or CAL-101 at different doses after 7 days of treatment. Scale bar: 100 μm. Quantification of albumin relative intensity in HepaRG within the different conditions above.

**e** Proposed PI3Kδ dependant pathway discriminating EMT from stemness. All values are expressed as mean ± S.E.M.
Raw data from affymetrix microarray have been deposited under the following code: GSE128202 for the transcriptomes of Huh7 and Huh7-5 PI3K. GSE113839 for the transcriptomes of AAV and AAV-PI3K. All the original full blots for the cropped images are shown in the supplementary Fig. 15-18. Source data behind the graphs are available in Supplementary Data 5.

Received: 17 August 2021; Accepted: 24 June 2022; Published online: 25 July 2022

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Acknowledgements
We thank Bart Vanhaesebroeck, University College London, UK, for providing plasmids encoding for PI108 isoform. We acknowledge, Larbi Amazit from assistance at the imaging core facility (UMS44, Hôpital Paul Brousse, France). We thank Clarisse Moncheucret and Ambre Leleu for helping in experiments. We thank Samira Benhamoucet-Trouillet for advices in experiments using mouse model injected with AAV 8 constructs. We thank Shenghao Li, Xiaodong Shu and Duanqing Pei for helping in the experiments on the 3D culture of human embryonic stem cells. We gratefully acknowledge Ministère de l’Éducation Nationale for fellowship to J.A, A.S.C. s.a.r.l, Lebanon for fellowship to V.B.M. and funding of the Association la ligue contre le cancer, and ANRS France to A.G.D.

Author contributions
J.A. designed and performed most of the experiments and bioinformatics analysis on stem cells. C.D. designed and performed all the bioinformatic studies and the related supplemental data and wrote the comments. V.B.M. performed colony formation assays and most of the experiments. J.A., V.B.M. and C.D. prepared the figures and legends and discussed the project and contributed to writing the manuscript. J.P.W. participated in experiment using cell culture. N.B designed and performed CAGA-luc, HepaRG experiments and immunohistochemistry analysis. S.I. and S.R. injected mice with AAV. E.D.V. designed AAV-PI3Kδ. T.Z.T. participated in bioinformatic analyses on stem cells. J.P.T. contributed to the study design, data analysis and critical reading of the manuscript. A.G.D. conceived, designed and supervised the study and organized the figures and wrote the original draft of the manuscript. All of the authors read and provided feedback on manuscript and figures.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s42003-022-03637-w.

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Peer review information Communications Biology thanks Julie Guillermet-Gaubert and the other anonymous reviewer(s) for their contribution to the peer review of this work. Primary Handling Editors: Margareta Wilhelm and Christina Karlsson Rosenthal.

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