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# CRIPTO promotes an aggressive tumour phenotype and resistance to treatment in hepatocellular carcinoma

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## ABSTRACT

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide. Despite increasing treatment options for this disease, prognosis remains poor. CRIPTO (TDGF1) protein is expressed at high levels in several human tumours and promotes oncogenic phenotype. Its expression has been correlated to poor prognosis in HCC. In this study, we aimed to elucidate the basis for the effects of CRIPTO in HCC.

We investigated CRIPTO expression levels in three cohorts of clinical cirrhotic and HCC specimens. We addressed the role of CRIPTO in hepatic tumorigenesis using Cre-*loxP*–controlled lentiviral vectors expressing CRIPTO in cell line derived xenografts. Responses to standard treatments (sorafenib, doxorubicin) were assessed directly on xenograft-derived *ex vivo* tumour slices. CRIPTO-overexpressing patient derived xenografts were established and used for *ex vivo* drug response assays. The effects of sorafenib and doxorubicin treatment in combination with a CRIPTO pathway inhibitor were tested in *ex vivo* cultures of xenograft models and 3D cultures.

CRIPTO protein was found highly expressed in human cirrhosis and hepatocellular carcinoma specimens but not in those of healthy participants. Stable overexpression of CRIPTO in human HepG2 cells caused epithelial-to-mesenchymal transition, increased expression of cancer stem cell markers, and enhanced cell proliferation and migration. HepG2-CRIPTO cells formed tumours when injected into immune-compromised mice, whereas HepG2 cells lacking stable CRIPTO overexpression did not. High level CRIPTO expression in xenograft models was associated with resistance to sorafenib, which could be modulated using a CRIPTO pathway inhibitor in *ex vivo* tumour slices.

Our data suggest that a subgroup of CRIPTO-expressing HCC patients may benefit from a combinatorial treatment scheme and sorafenib resistance may be circumvented by inhibition of the CRIPTO pathway.

**KEYWORDS:** hepatocellular carcinoma; CRIPTO; GRP78; HepG2; sorafenib resistance; organoids; liver cirrhosis, neoplasia, patient-derived xenografts, zebrafish xenograft

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide [1]. In the majority of cases, HCC arises on a background of cirrhosis, which may be caused by chronic exposure to damaging factors, such as chronic alcohol abuse, hepatitis B or C, and various other chronic liver diseases [2]. The invasive and metastatic potential of HCC's is an important factor causing poor prognosis of affected patients. Treatment options include resection of the tumour, liver transplantation, minimal invasive image-guided oncologic therapies such as local ablation and transarterial therapies for the early- and intermediate tumour stages [3].

Targeted systemic treatments available for advanced stage tumours or tumours progressing after loco-regional therapies are the tyrosine kinase inhibitors (TKIs) sorafenib (first-line) [4] and regorafenib (second-line) Sorafenib is a tyrosine kinase (VEGFR, PDGFR, RAF) inhibitor, which delays HCC progression and metastatic spread but is effective in only a minority of patients and has severe side effects [4-7]. Recently, it was shown that nivolumab, a programmed cell death protein-1 (PD-1) checkpoint inhibitor, induces durable objective responses in patients with advanced stage HCC [8]. Due to lack of biomarkers, it remains a challenge to estimate disease progression or responsiveness to therapies [4]. In addition to the high number of non-responders to systemic therapy, there is a high percentage of HCC patients that relapse after surgical resection or minimal invasive oncological therapies. Prognosis of HCC is often dismal with significant risk of tumour recurrence or insufficient response to therapies due to non-specificity of the treatments [9]. Circulating α-fetoprotein (AFP) levels have been explored as biomarkers in HCC [10]. However, detection of high levels of AFP cannot be used for diagnosis or prognosis as it does not predict tumour size, stage and HCC progression and is absent in 30% of HCC cases [10]. Elucidation of the basic mechanisms behind the invasive and migration properties of HCCs and identification of markers that can predict therapeutic response and likelihood of recurrence are needed to identify suitable personalized treatments. For example, identification of sorafenib responders versus non-responders based on biomarker expression and functional *ex vivo* assay would allow better treatment selection.

CRIPTO (Teratocarcinoma-Derived Growth Factor 1; TGDF1) is a GPI-anchored signalling protein and atypical member of the Transforming Growth Factor (TGF) gene family [11-13]. CRIPTO has multiple binding partners and signalling functions [14,15]. It enables the signalling of a subset of TGF-β superfamily ligands including Nodal [16] that require a co-receptor (CRIPTO or Cryptic) to bind and assemble their type I and type II signalling receptors [17]. CRIPTO also inhibits other TGF-β superfamily ligands and attenuates cytostatic TGF-β1 signalling [12]. In addition, CRIPTO can act independently of the TGF-β pathway as a secreted factor that activates c-src/MAPK/Akt signalling, a pathway that is oncogenically mutated in liver cancer [18]. Notably, each of these CRIPTO signalling functions was shown to depend on CRIPTO binding to cell surface glucose regulated protein 78 kDa (GRP78) [19]. GRP78 is strongly induced by endoplasmic reticulum (ER) stress and, like CRIPTO, plays key roles in embryogenesis, stem cell regulation and tumour progression [20]. In addition to its physiological roles in stem cells and embryogenesis, CRIPTO is an oncofetal protein that is silenced postnatally. Re-expression is often associated with pathological conditions such as neoplasia of breast, prostate, ovarian, bladder, colon, skin, lung and brain [21-29]. Recently, CRIPTO expression was correlated to poor survival and tumour recurrence in HCC patients [30]. Moreover, liver-specific deletion of GRP78 promoted maintenance of tissue homeostasis and played a protective role during endoplasmic reticulum-stress response while elevated GRP78 levels were associated with HCC progression [31-33].

In this study, we investigated CRIPTO expression in HCC aiming to elucidate the effects of the CRIPTO pathway while also exploring its potential use as therapeutic target. We identified a potential role for CRIPTO in therapy resistance to sorafenib, suggesting that combination treatment with an inhibitor of CRIPTO pathway might induce a beneficial response in selected patient groups.

## MATERIALS AND METHODS

### Human specimens

Aetiopathological heterogeneity in tumours was taken into account during the selection of HCC patient material; in this study we assessed specimens from HCV infection-driven HCC (HCC-HCV, N=4) and alcoholic liver disease-driven HCC (HCC-ALD, N=4). Non-cirrhotic hepatitis C (HCV) tissues (N=5) were selected as controls. Selection of tissues was performed in agreement with the “code of good practice”. Written informed consent was obtained from each patient included in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the institution's human research committee (B15.006/SH/sh, biobank METC MDL/009/NV/nv). Tissue microarrays (TMAs) used were from the Pathology Department of University Hospital Basel (N=234 tissue samples including 33 HCC-Adjacent tissue matched pairs) and a commercially available TMA (US Biomax, BC03117, N= 69 tissue samples including 7 HCC-Adjacent tissue matched pairs).

**Animal models**

Animal protocols were approved by the Committee for Animal Experimentation and the Veterinary Authorities of the Canton of Bern, Switzerland (BE55/16). Mice received food and water *ad libitum* and were housed in individually ventilated cages. NOD.Cg-Prkdc$^{scid}$ Il2rg$^{tm1Wjl}$/SzJ (NSG) mice were injected subcutaneously with 0.5x10E6 HepG2-CRIPTO (n=4) or HepG2-MOCK (n=3) cells in Matrigel (354234, Corning, Kaiserslautern, Germany). Tumour growth was monitored weekly. At week 12 mice were sacrificed and tumours collected for further analysis.

Patient derived xenograft (PDX): A tumour needle biopsy from an anonymized advanced stage HCC patient was subcutaneously implanted in NSG mice and routinely passaged *in vivo*. A zebrafish line (Tg(fli1:GFP)i114) was handled and maintained according to local animal welfare regulations to standard protocols (http: //www.ZFIN.org). Two days post fertilization (dpf), dechorionated zebrafish embryos were anesthetized and injected with approximately 200 HepG2-MOCK or HepG2-CRIPTO cells fluorescently labelled as described previously [34]. Two days after injection the embryo were imaged and clumps of cells (Foci) counted. Zebrafish embryos (including non-injected controls) were maintained at 33 °C, to compromise between the optimal temperature requirements for fish and mammalian cells. Data are representative of/from at least two independent and blind experiments with ⩾30 embryos per group. Foci were counted using Leica Application Suite X software (version 1.1.0.12420, Leica Biosystems B.V. Amsterdam, the Netherlands).

### *Ex vivo* tumour tissue culture and organoid generation

Tissue from HCC PDX or HepG2-CRIPTO derived tumours were maintained in *ex vivo* cultures. Tissue slices (150–200 µm) were cultured using Transwell plates with an attached nitrocellulose membrane (ThinCert #662640 inserts for 24well plates, 0.4 µm pore size, Greiner Bio-One, Kremsmünster, Austria) that allowed contact of the tissue with the growth medium but not the plastic in a manner that prevented alteration of the tissue [35,36]. Culture plates were placed in a sealed container saturated with oxygen 40–50% at 37 C. Cultures were maintained for 7 days. Organoids were derived from the bulk of PDX tumours similarly to previously developed methods [37,38]. Single cell suspensions were obtained by enzymatic homogenization of the tissue by collagenase type II (Gibco, 5 mg/ml) and Accutase (Sigma), followed by red blood cell lysis. Organoids were maintained in low attachment plates (Corning, Germany), in defined media (supplementary material, Table S1). Tumour slices and organoids were incubated for 7 d and 2 d respectively, with dimethylsulphoxide (DMSO 0.1%), DMSO plus Control Fc antibody (2 µg/ml), sorafenib (1 µM), doxorubicin (1 µg/ml), GRP78 N20 blocking peptide (sc1050P, 2 µg/ml, Santa Cruz,). After treatment, tissues were processed for histology.

### Cell lines and CRIPTO overexpression

The HepG2 cell line was maintained in Dulbecco’s Modified Eagle Media (DMEM) supplemented with 10% fetal calf serum (FCS; Gibco) and 1% penicillin/streptomycin (P/S; Invitrogen Corp., Paisley, UK). Lentiviral pTomo-mock [39] and pTomo-CRIPTO constructs (provided by Dr. P. Gray; sequence from [12]) were used for HepG2 cell transduction. Selection of positive clones was based on red fluorescent protein (RFP)-based cell sorting (FACS). Activation of CRIPTO transcription was induced by lentivirus-CRE transduction, which switched off RFP expression and induced green fluorescent protein (GFP) expression.

Further information is provided in supplementary material, Materials and methods.

## RESULTS

### CRIPTO promotes a proliferative and mesenchymal phenotype in *in vitro* HCC cells.

CRIPTO has been shown to play important role in tumour development and progression in various cancer types, however its role in hepatic pathologies, such as in HCC remains understudied.

Firstly, we studied the functional effects of CRIPTO *in vitro*, by stable overexpression of CRIPTO in HepG2, an HCC-derived cell line with low tumorigenicity *in vivo* [40]. We used a lentiviral red-to-green pTOMO-CRIPTO construct in which CRE recombinase activity excises a floxed RFP cassette, which turns on CRIPTO expression and increases expression of GFP [39]. A pTOMO-MOCK lentivirus lacking the CRIPTO insert was used to generate a control cell line (HepG2-MOCK). HepG2-CRIPTO cells transduced with pTOMO-CRIPTO and CRE virus lacked RFP expression and had GFP expression as predicted **(Figure 1A)**. HepG2-MOCK cells were also generated in the presence of viral-mediated CRE and maintained expression of RFP and weak GFP upon transduction, whereas non- transduced cells (HepG2) showed no fluorochrome **(Figure 1A)**. CRIPTO overexpression in the HepG2-CRIPTO cells was confirmed at both the mRNA **(Figure 1B)** and protein levels **(supplementary material, Figure 1A).** The mRNA levels of *NODAL* and *GRP78*, encoding CRIPTO interaction partners, were both induced in HepG2-CRIPTO cells **(Figure 1C-D)**. GRP78 protein levels were reduced **(supplementary material, Figure 1B)** and phosphorylated AKT (pAKT) levels were increased indicating downstream pathway activation **(supplementary material, Figure 1C)** in HepG2-CRIPTO cells. PCNA protein level differences were minor **(supplementary material, Figure 1D).** HepG2-CRIPTO cells acquired a more mesenchymal phenotype relative to control cells (HepG2 WT and HepG2-MOCK) as indicated by the downregulation of *E-CADHERIN* **(Figure 1E)** and upregulation of EMT markers such as *VIMENTIN (VIM)*, *ZEB-1*, *ZEB-2*, *TWIST1* and *SNAIL-2* **(Figure 1F-J)**. HepG2-CRIPTO cells exhibited higher expression of the cancer stem cell (CSC) markers *BMI-1* and *CD44* than control cells **(Figure 1L-M)**. EPCAM levels were also higher in HepG2-CRIPTO cells although this difference did not reach statistical significance **(Figure 1K)**. Additional stemness marker expression was assessed at the mRNA level; *GLI-1*, *av INTEGRIN* (*ITGAV*), β3 *INTEGRIN* (*ITGB3*), *ALDH1A*1, *SOX2* and *CD24* **(supplementary material, Figure 2A-F)**. HepG2-CRIPTO cells also had higher proliferation rate **(Figure 1N)**, enhanced migration **(Figure 1O)** and wound closure properties **(Figure 1P)**, compared to control cells in line with EMT-associated gene expression **(Figure 1E-J)**.

### CRIPTO overexpression induces pro-tumorigenic *in vivo* effects.

We investigated possible effects of CRIPTO on the cancer phenotype *in vivo*. HepG2-CRIPTO and HepG2-MOCK cells were implanted subcutaneously in Matrigel plugs in immunodeficient mice. Tumour formation was observed at 5 wk post-implantation; HepG2-MOCK cells formed smaller tumours compared to the HepG2-CRIPTO bearing mice **(Figure 2A)**. We isolated single cells from the HepG2-CRIPTO tumour tissues and selected CRIPTO-transduced cells based on GFP expression (HepG2-CRIPTO-p1) **(Figure 2B)**. The single cells were then injected in the duct of Cuvier of 2 dpf zebrafish embryos along with the parental HepG2-CRIPTO and HepG2-MOCK lines **(Figure 2C-D)** in order to determine their potential to migrate and generate tumour foci *in vivo*. HepG2-CRIPTO and HepG2-CRIPTO-p1 both showed significantly more foci at 6 dpf relative to HepG2-MOCK **(Figure 2C)**. Histological analysis of HepG2-MOCK and HepG2-CRIPTO tumour tissues, grown as subcutaneous xenografts in mice, showed different morphological structures **(Figure 2E-F)**; only HepG2-CRIPTO tumours resembled HCC morphology. Both tumours showed similar levels of proliferation (PCNA) **(Figure 2G-I)** and HNF4a liver marker expression **(Figure 2J-K)**, however, different levels of CRIPTO were observed **(Figure 2L)**. HepG2-MOCK xenograft tumours were distinguished by staining for the epithelial marker E-CADHERIN **(Figure 2M)** and absence of mesenchymal VIMENTIN expression **(Figure 2O)**, in contrast with the HepG2-CRIPTO tumours that are E-CADHERIN negative **(Figure 2N)** and have VIMENTIN positive areas **(Figure 2P)**. HepG2-CRIPTO tumours were cultured *ex vivo* for 7 d, during which they were treated with doxorubicin or sorafenib, two compounds which are currently widely used in clinical practise for HCC treatment, in the setting of transarterial chemoembolization and oral therapy respectively. Based on H&E staining, both treatments **(Figure 3C-D)** lead to tissue damage compared to the untreated and vehicle (DMSO) group **(Figure 3A-B)**. Immunofluorescence analysis of proliferating cells (PCNA+; Proliferating Cell Nuclear Antigen positive), indicated that doxorubicin treatment had a negative impact on tissue viability exhibited by absence of PCNA positive cells **(Figure 3G,T).** Sorafenib treatment showed areas of sustained proliferation **(Figure 3H,T)**.

The gene expression profile of tumours treated *ex vivo* with doxorubicin and sorafenib showed reduced CRIPTO expression **(Figure 3I)** in both conditions compared to control groups. However, CRIPTO expression was higher in the sorafenib condition compared to doxorubicin **(Figure 3I)**. *GRP78* levels **(Figure 3J)** were not affected, if compared to the vehicle control. *EPCAM* and *E-CADHERIN (CDH1)* levels were also reduced following doxorubicin and sorafenib treatment **(Figure 3K, N)**, while *CD44* was increased significantly in both drug treatments **(Figure 3M)**. Interestingly, only sorafenib induced expression of stem cell marker *BMI-1* **(Figure 3L)**, and EMT-associated genes *VIM* and *TWIST1* **(Figure 3O-P)**. Expression of the liver cancer stem cell marker *CD24* was significantly decreased upon sorafenib treatment **(supplementary material, Figure 2G)**. The induction of mesenchymal (*VIM, TWIST1*) and stem cell markers (*CD44, BMI-1*) by sorafenib in the HepG2-CRIPTO tumour slices, may indicate an acquired aggressiveness due to a CRIPTO related activation of a resistance mechanism to sorafenib. To understand whether sorafenib resistance pre-exists in the HepG2 or is linked to CRIPTO overexpression, we assessed the proliferation rate of the HepG2 parental, control MOCK line and HepG2-CRIPTO in response to different sorafenib or doxorubicin concentrations. Proliferation, as assessed using an MTS assay, was reduced in the doxorubicin and sorafenib treated HepG2, HepG2-MOCK and HepG2-CRIPTO cell lines. Interestingly, HepG2, and HepG2-MOCK cells proliferated less in the presence of sorafenib (1 µM) when compared to HepG2-CRIPTO (**Figure 3Q-S**, 96 h).

### CRIPTO may confer sorafenib resistance

To further investigate the role of CRIPTO in HCC, we established a patient derived xenograft (PDX) from a needle biopsy obtained from a CRIPTO-expressing advanced cancer stage HCC prior to patient treatment with Sorafenib (**Figure 4A-B**, original tumour and PDX1). The PDX tissue (PDX2 and PDX3 tumour) maintained both HNF4a and CRIPTO expression over time **(Figure 4A-B)**, showing persistent proliferation **(Figure 4C-D)** and minimal apoptosis **(Figure 4C,E)** and HCC morphology **(Figure 4F-G)**.

To address whether CRIPTO inhibition increases tumour responsiveness to sorafenib, we employed an *ex vivo* tissue slice culture system. Tumour slices and organoids derived from the PDX (from three different passages) were treated with sorafenib, N20 (GRP78 blocking peptide), which blocks CRIPTO/GRP78 binding and CRIPTO signalling, or both in combination. Treatment with N20 blocking peptide inhibited AKT signalling activity as shown by increased FOXO-luc activity **(supplementary material, Figure 1E)**. H&E staining showed necrotic regions in all treatments in various degrees **(Figure 5C-F)** compared to the original and untreated condition respectively **(Figure 5A-B)**. Proliferation (PCNA levels) were affected in both single and combination treatments **(Figure 5G-M)**. However, treatment with sorafenib and N20 in combination reduced cell proliferation **(Figure 5L,M)** to a greater extent than treatment with either of the single compounds alone. In organoid cultures derived from the PDX tumour tissue, viability, measured by CellTiterGlo assay, was also significantly reduced in the combination treatment **(Figure 5N-S)** indicating a higher susceptibility of sorafenib-resistant cells when CRIPTO-GRP78 signalling was blocked.

### CRIPTO expression in human HCC

Expression of CRIPTO is present in embryonic tissues and becomes silenced in postnatal tissues. Reactivation of CRIPTO in adult tissues has been associated with various cancer types and thus far, only one study has investigated the role of CRIPTO in hepatic malignancy. Considering the low expression of CRIPTO at mRNA and absence of protein levels in human liver tissues (both obtained from www.proteinatlas.org) the role of CRIPTO at homeostatic and pathological liver conditions merits further investigation.

We determined the transcript levels of *CRIPTO* (*TDGF1*) in normal human liver, tumour and tumour-adjacent tissues by RT-qPCR. Expression was low to undetectable in normal samples (N=8) compared to both tumour (N=7, **Table 1**) and tumour-adjacent (N=7, Cirrhotic, **Table 1**) tissues, which showed similar levels indicating that increased *CRIPTO* expression is possibly associated with pre-existing chronic liver disease.

To better understand the expression pattern of CRIPTO in disease states, we determined the protein expression in a tissue microarray (TMA Basel university cohort, 234 tissue samples) by immunohistochemistry and found that HCC tissue (N=128) had significantly (p=0.0389) less CRIPTO expression than non-HCC tissue (N=106) **(Figure 6B, Table 2)**. The non-HCC cases reflect unpaired, tumour-adjacent tissues from fibrosis, cirrhosis, low grade and high-grade dysplastic nodule cases **(supplementary material, Figure 3A)**, thus not healthy liver tissues. Within this TMA, we also analysed paired, matched cases of tumour and adjacent non-tumour tissue (N=33) corresponding to the same patients and found that CRIPTO levels were similar in both cases **(Figure 6H, Table 3)**. A second TMA (US Biomax) was used for the validation of these results and we confirmed CRIPTO expression in both tumour-adjacent cirrhotic tissues and tumour (HCC) tissue, as in the first TMA **(supplementary material, Figure 3B, Table 2-3).**

Next, we evaluated CRIPTO and GRP78 expression by immunofluorescent staining in tumours of resected human livers from HCC and adjacent cirrhotic (non-tumour) tissues (N=8) and in non-cirrhotic tissues (N=5, HCV infection). Clinical and tumour characteristics are shown in **Table 2** (all cases) and **Table 3** (matched cases). CRIPTO expression was detected at higher levels in the tumour compared to adjacent cirrhotic non-tumour tissue in 2 out 4 patients with HCV-related disease **(Figure 6D-E)** and in 2 out of 4 ALD-related HCC patients (N=4) **(Figure 6G-H)**. In non-cirrhotic HCV samples, we observed that expression levels of CRIPTO are lower than the tumour-adjacent groups **(Figure 6E)**.

The CRIPTO binding and signalling partner, GRP78, was expressed in a similar pattern with CRIPTO in hepatocytes. Quantification indicated that higher GRP78 expression in tumour tissue, as compared to adjacent non-tumorous tissue, was detected in one HCV-related **(Figure 6D,F)** and two ALD-related HCC patients **(Figure 6G,I)**. In matched cases in all three TMA data sets we have analysed here, we detected CRIPTO expression in HCC specimens both in the tumour and tumour-adjacent tissue, however at different levels, and identified three subgroups of CRIPTO expressing HCC **(Table 4)**.

Given the low expression of *CRIPTO* in non-cirrhotic samples **(Figure 6A, E)** and high expression already in tumor-adjacent cirrhotic tissue, we accessed the transcript levels of *CRIPTO* in three microarray datasets [41-43] (publicly available data from the Oncomine database). Comparison among normal liver, cirrhosis, HCC and Liver Cell Dysplasia indicated that HCC cases had statistically significantly higher CRIPTO mRNA levels versus normal liver tissues in all datasets **(Figure 6J-L; P≤0.01)**. Cirrhotic cases showed non-significant changes **(Figure 6J, L)**, while Liver Cell Dysplasia showed significantly higher levels versus normal liver expression levels **(Figure 6L)**.

The data above highlight that in individual HCC cases, evaluation of the tumour-adjacent tissue is informative but cannot be considered as control. CRIPTO expression is upregulated in many cases of pathological conditions (cirrhosis, dysplasia), thus absolute expression levels of CRIPTO in liver tissues should be compared to reference levels from healthy tissues, and not cirrhotic, tumour-adjacent counterparts.

## DISCUSSION

CRIPTO is a cell surface protein that regulates signalling of TGF-β superfamily ligands and also has EGF-like activity. It is a small glycosylphosphatidylinositol (GPI)-anchored cell surface/secreted oncoprotein that plays important roles in regulating stem cell differentiation, embryogenesis, tissue growth and remodelling [14]. The tumour-promoting role of CRIPTO has been documented in multiple malignancies, including those characterized by osteotropism in their metastatic stage, such as breast and prostate cancer [25,44,45]. Recently, higher CRIPTO expression was detected in a cohort of HCC patients (49.8%, N=205), and which correlated with poor prognosis [30].

We showed that CRIPTO expression in tumours was mainly detected in areas lining the stromal compartment. This is not surprising given its role in promoting EMT that we and other groups have shown for other cancer types [46]. The CRIPTO-positive tumour cells adjacent to the stroma may enter the circulation and be responsible for metastatic spread. However, further studies are necessary to corroborate this hypothesis.

Interestingly, in the TMAs (Basel and US Biomax) we analysed, CRIPTO protein expression was higher in the non-tumour tissue as compared to tumour tissue. Same trend was observed in smaller cohort of adjacent and tumour tissue in matched cases of the TMAs, although the number of available matched pairs in the present study may be too small to draw firm conclusions.

Given the fact that the non-tumour tissues have a cirrhotic background and CRIPTO is not expressed during homeostasis, it could be speculated that CRIPTO correlates to disease progression [30,40]. Moreover, it should be noted that tissue sections (cores) available in the TMAs have small size, without information of the exact location of where it was derived from within the tumour tissue. The LUMC cohort of matched cases contained large tissue areas derived from a histopathological confirmed area (tumour, tumour-adjacent) as evaluated by a certified pathologist. It needs to be emphasized that HCCs are heterogeneous tumours and therefore the results of this study should be validated in larger numbers of matched larger tissue sections. Using a transcriptomic approach, we showed that *CRIPTO* mRNA expression is progressively elevated in pathological hepatic conditions such as HCC and liver cell dysplasia compared to normal state, as assessed in three distinct datasets (Oncomine).

In line with the human data, stable overexpression of CRIPTO in HepG2 cells led to a more aggressive tumour phenotype *in vitro, ex vivo* and *in vivo* characterized by EMT, mesenchymal phenotype, as well as stem cell characteristics as determined by histology and transcript level alterations. Our data support the notion that CRIPTO plays a role in cirrhosis as well as tumour initiation and aggressiveness by increasing cellular plasticity and stem cell properties similar to what we have also shown recently for prostate cancer [34].

Elevated CRIPTO levels in both HCC and the cirrhotic, potentially premalignant, state suggest that targeted inhibition of CRIPTO could be beneficial in combination with chemical compounds currently used in clinical practice. Doxorubicin is a single-agent drug that has been the most studied chemotherapy agent for advanced HCC [47]. Despite initial studies showing high response rates, subsequent studies showed only a small survival advantage. It is currently widely used for trans-arterial chemoembolization. The development of combination therapy using molecularly targeting drugs such as sorafenib might be useful for the prevention of early HCC metastasis. Given that sorafenib is the standard of care in advanced stage HCC but provides only a 3-month median survival benefit in advanced stage HCC [46] and no survival benefit in combination doxorubicin (Clinical trial phase III NCT01015833, [48]), we explored the possible role of CRIPTO in sorafenib resistance. A low dose of sorafenib did not affect the proliferation of HepG2-CRIPTO cells. These results suggest that CRIPTO causes a differential drug response and refractoriness to sorafenib. This possibility was supported by our finding that HepG2-CRIPTO tumours cultured *ex vivo* possessed areas of proliferating cells following sorafenib treatment.

PDX tissue slices cultured *ex vivo* showed no sensitivity to sorafenib. However, the combination treatment with the N20 peptide, which blocks CRIPTO signalling, showed a significant reduction of proliferation and no effect on apoptosis. This suggests that it might be beneficial to employ a combination of treatments that either target CRIPTO directly or one of its downstream signalling mediators, e.g. ERK and AKT pathways, in order to achieve an inhibition of proliferation in the HCC tumour cells. Similarly, organoids derived from the PDX tumour tissue, treated in the same conditions as the *ex vivo* cultures, showed reduction in viability when treated with both N20 and sorafenib. This suggests that inhibition of CRIPTO/GRP78 signalling specifically enhances the response to sorafenib. Mechanistically this effect can be explained by the fact that PI3K/AKT kinase, which is downstream of CRIPTO/GRP78, is also involved in acquisition of resistance after long exposure to sorafenib [49] while inhibition of AKT may re-sensitize tumour cells [50]. CRIPTO has been implicated in therapy resistance in lung cancer with studies showing that high CRIPTO expression correlates with lower sensitivity to treatment with EGFR kinase inhibitors [51,52]. Modulation of CRIPTO expression or downstream (SRC, AKT) signalling reverses the resistance to EGFR inhibitors [52].

The results presented in this study show that CRIPTO signalling increases proliferation and seems to be required for tumour progression, as suggested for both prostate and breast cancer. Our findings suggest that blocking CRIPTO signalling may have therapeutic benefit in combination with existing therapies for HCC.

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## Author contributions statement

SK designed experiments, acquired data, interpreted data and wrote manuscript. DvH designed experiments, acquired data, interpreted data, revised manuscript. PG provided materials, designed experiments, interpreted data and wrote manuscript. LC, IK and JG acquired and interpreted data. BESJ interpreted data and revised manuscript. MCB, DS, BvH, AFS, SO and GNT provided clinical support, interpreted data and revised manuscript.

AFS acquired data and provided technical support. LT provided materials and revised the manuscript. HV and MC designed experiments, interpreted data, revised manuscript. MKdJ designed concept of the study and experiments, interpreted data and wrote manuscript.

## TABLES

**Table 1.** Clinical parameters of specimens used from the LUMC cohort for mRNA analysis. The list corresponds to Figure 6A.

| **qPCR cases** | LUMC (N=15) |
|---|---|
| Gender, male, N(%) | 11 (73.3%) |
| Age | 72 (59-74.5) |
| Control (no underlying liver disease) | 8 (53.3%) |
| **Adjacent and HCC matched samples** | |
| Fibrosis-Cirrhosis (yes, N(%)) | 7 (46.6%) |
| HCC | 7 (46.6%) |
| T1 (N) | 5 |
| T2 (N) | 1 |
| T3 (N) | 1 |
| T4 (N) | n.a. |

**Table 2.** Baseline characteristics of patients (staining samples).

Patient samples correspond to Figure 6B, supplementary material, Figure S3A (TMA Basel), Figure 6D-I (LUMC) and supplementary material, Figure S3B (TMA US Biomax). Median (IQR). n.a. = not applicable. Only the cases with known clinical background are included in this table.

| **Staining cases** | LUMC (N=13) | TMA Basel (N=163) | TMA US Biomax (N=62) |
|---|---|---|---|
| **Cohort with clinical background, N(%)** | 13 (100%) | 76 (46.6%) | 62 (100%) |
| Gender, male, N(%) | 13 (100%) | 58 (35.6%) | 52 (83.9%) |
| Age | 54 (47-63) | 76 (67-80) | 53 (45-59) |
| Fibrosis-Cirrhosis (Yes / Undefined, N(%)) | 8 (61.5%)/ n.a. | 38 (23.3%) / 9 (5.5%) | 62 (100%)/ n.a. |
| Patients with HCC, N(%) | 8 (61.5%) | 46 (28.2%) | 48 (77.4%) |
| T1 (N) | 1 | 15 | 1 |
| T2 (N) | 5 | 12 | 18 |
| T3 (N) | 2 | 18 | 25 |
| T4 (N) | n.a. | 1 | 4 |
| **Unknown clinical background** | n.a. | 87 (53.4%) | n.a. |
| Patients with HCC, N | n.a. | 82 (50.3%) | n.a. |
| Fibrosis-Cirrhosis (Yes/Undefined, N(%)) | n.a. | 49 (30.1%) / 10 (6.1%) | n.a. |

**Table 3.** Baseline characteristics. Patient samples correspond to the matched tumour versus tumour-adjacent tissues of Figure 1A-F (LUMC), Figure 1H (TMA Basel) and supplementary material, Figure S3 (TMA US Biomax). Median (IQR). n.a. = not applicable

| **Matched cases** | LUMC (N=8) | TMA Basel (N=33) | TMA US Biomax (N=7) |
|---|---|---|---|
| Gender (male), N(%) | 8 (100%) | 26 (78.8%) | 6 (85.7%) |
| Age | 56.5 (51-63) | 77 (68-80.5) | 52 (48-54) |
| Fibrosis-Cirrhosis (Yes/Undefined, N(%)) | 8 (100%) | 27 (81.8%) / 6(18.1%) | 7 (100%) |
| TNM stage | | | |
| T1 | 1 (12.5%) | 11 (33.3%) | n.a. |
| T2 | 5 (62.5%) | 7 (21.2%) | 2 (28.6%) |
| T3 | 2 (25%) | 15 (45.5%) | 4 (57.1%) |
| T4 | n.a. | n.a. | 1 (14.3%) |

**Table 4. Comparison of CRIPTO expression in the different cohorts of tumour versus tumour-adjacent tissue (Matched cases).**

| Matched tissue samples | LUMC (N=8) | TMA Basel (N=33) | TMA US Biomax (N=7) |
|---|---|---|---|
| Cripto tumour < Cripto adjacent tissue, N(%) | 2 (25%) | 16 (48.5%) | 5 (71.4%) |
| Cripto tumour = Cripto adjacent tissue, N(%) | 0 (0%) | 8 (24.2%) | 1 (14.3%) |
| Cripto tumour > Cripto adjacent tissue, N(%) | 6 (75%) | 9 (27.3%) | 1 (14.3%) |

## FIGURE LEGENDS

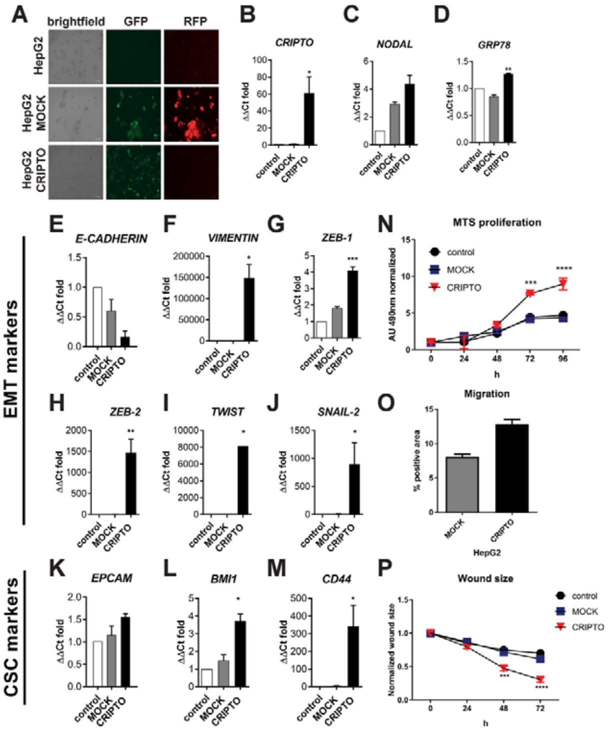

**Figure 1.** ***In vitro*** **effects of stable overexpression of CRIPTO in HepG2 cells**

(A). Cell morphology of wild type HepG2 cells; control, stably overexpressing pTomo MOCK construct (MOCK, GFP+, RFP+) and stably overexpressing CRIPTO (CRIPTO, GFP+); both cell lines were transduced with pTOMO and CRE lentivirus. (B-D). RT-qPCR for mRNA expression of CRIPTO-associated members of the NODAL and GRP78 pathways in (B) *CRIPTO* (*TDGF1*), (C) *NODAL*, (D) *GRP78* mRNA expression (n=3, ±SEM). Values are normalized to *ACTB* and to control sample (ΔΔCT fold expression). (E-J). Levels of mRNA expression of epithelial-to-mesenchymal (EMT) markers in control, MOCK and overexpressing CRIPTO HepG2 cells was assessed by RT-qPCR; (E). *E-CADHERIN* (*CDH1*), (F). *VIMENTIN* (*VIM*), (G). *ZEB1*, (H). *ZEB2*, (I). *TWIST1*, (J). *SNAIL-2*. Unpaired t-test, p value < 0.05 (*). (K-M). mRNA expression levels of cancer stem cell (CSC) markers; (K). *EPCAM*, (L). *BMI-1*, (M). *CD44*. All values are normalized to *ACTB* and to control sample (ΔΔCT fold expression), n=3, ±SEM. Unpaired t-test, p value < 0.05 (*). (N). Metabolic activity MTS assay (24, 48, 72, 96 h) was performed in control, MOCK and CRIPTO overexpressing HepG2 cells. Accumulation of MTS was measured based on absorbance at 490 nm. Values are normalized to the basal measurements at 0 h after cell seeding. Graph represents values for three independent experiments (n=3). Error bars indicate ±SEM. Two-way ANOVA, p value < 0.001 (***), < 0.0001 (****). (O) Transwell migration assay of MOCK and CRIPTO overexpressing HepG2 cells; quantification of percentage positive area of migrated cells (Crystal violet cell dye) was performed in two independent experiments. Error bars indicate ±SEM. (P). Cell motility was assessed in wound healing (scratch) assay. Wound size was quantified in a time-dependent manner (0, 24, 48 and 72 h) in three independent experiments. Data was normalised to the 0 h time point; error bars indicate ±SEM. Unpaired t-test, p value < 0.05 (*).

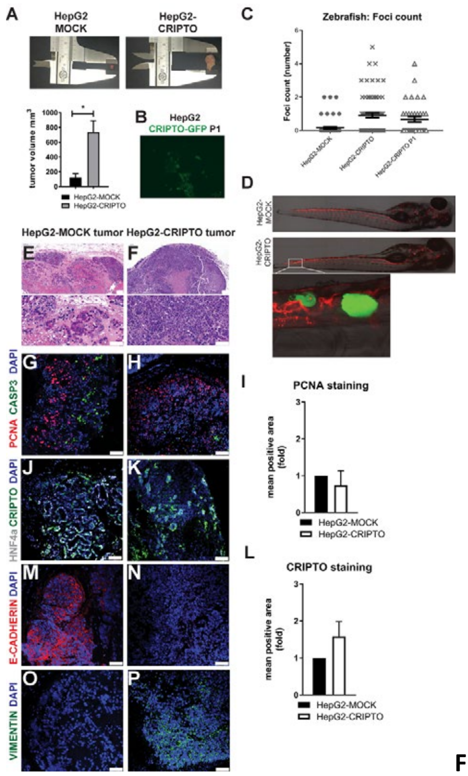

**Figure 2. *In vivo* tumour formation is induced by overexpression of CRIPTO**

(A) Subcutaneous tumour growth of HepG2-MOCK and HepG2-CRIPTO cells in immunocompromised mice. Tumour volumes at endpoint calculated with calliper measurement and using the formula V = (L x W x W)/2.

Average values from HepG2-MOCK (N=3) and HepG2-CRIPTO (N=4) are shown. Error bars indicate ±SEM. Unpaired t-test, p value < 0.05 (*). (B-D) GFP-positive HepG2-CRIPTO cells (p1) from the tumours were collected and injected in zebrafish to monitor cell migration and tumour growth (C-D) along with HepG2-MOCK and HepG2-CRIPTO cells. (E-F) Haematoxylin and eosin staining representative of the HepG2-MOCK and HepG2-CRIPTO tumours. Scale bars: 100 µm (top), 50 µm (bottom). (G-I). Immunofluorescence for PCNA (red) and cleaved Caspase-3 (green) in HepG2-MOCK (G) and HepG2-CRIPTO (H) tumour sections. Scale bars: 50 µm (I). Quantification of PCNA-positive area normalized to nuclei surface area, and represented as fold-change over the HepG2-MOCK samples. (J-K) Immunofluorescence for HNF4a (grey) and CRIPTO (green) in HepG2-MOCK and HepG2-CRIPTO tumour sections. Scale bars: 50 µm. (L) Quantification of CRIPTO expression (positive stained area normalized to nuclei surface area); fold over HepG2-MOCK values. (M-N). Immunofluorescence for epithelial marker E-CADHERIN (red) in HepG2-MOCK and HepG2-CRIPTO tumour sections. Scale bars: 50 µm. (O-P). Immunofluorescence for mesenchymal marker VIMENTIN (green) in HepG2-MOCK and HepG2-CRIPTO tumour sections. Scale bars: 50 µm.

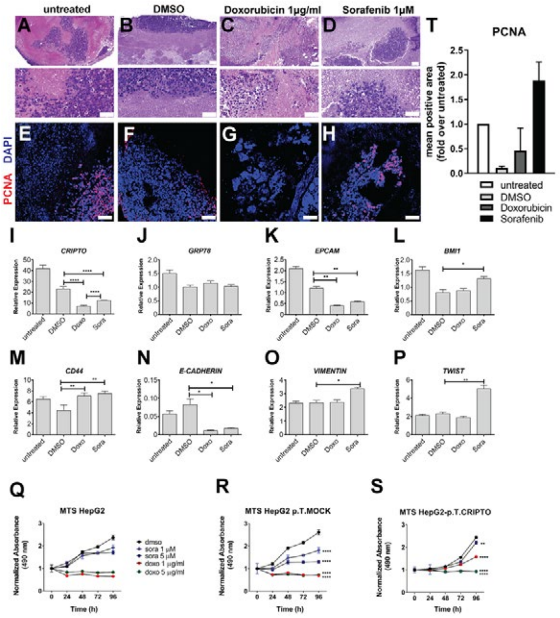

**Figure 3.** ***Ex vivo*** **drug response to doxorubicin and sorafenib treatments indicates differential proliferation,**

**EMT and CSC marker expression in CRIPTO$^{high}$ tumour slices *ex vivo***

(A-D) *Ex vivo* culture of HepG2-CRIPTO tumour slices, H&E staining of untreated part (A), DMSO vehicle (B), doxorubicin (1 µg/ml) (C), and sorafenib (1 µM) (D) treated. Scale bars: 100 µm (top), 50 µm (bottom). (E-H). Immunofluorescence of PCNA (red) staining on *ex vivo* cultured tissue parts; untreated part (E), DMSO vehicle (F), doxorubicin (G), and sorafenib (H) treated. DAPI marks the nuclei (blue). Scale bars: 50 µm. (I-P). Levels of mRNA for (I) *CRIPTO*, (J) *GRP78*, (K) *EPCAM*, (L) *BMI1*, (M) *CD44*, (N) *E-CADHERIN*, (O) *VIMENTIN* and (P) *TWIST1* in HepG2-CRIPTO tumours (untreated) exposed to vehicle (DMSO), doxorubicin (1 µg/ml) (C), and sorafenib (1 µM). Unpaired t-test, p value < 0.05 (*), < 0.01 (**). (Q-S) Metabolic activity MTS assay (24, 48, 72, 96 h) was performed in control (Q), MOCK- (R) and CRIPTO-overexpressing HepG2 cells (S). Cells were exposed to sorafenib (1 or 5 µM) and doxorubicin (1 or 5 µg/ml). Values are normalized to the basal measurements at 0 h after cell seeding. Graph represents values for three independent experiments (n=3). Error bars indicate ±SEM. Two-way ANOVA, p value < 0.001 (***), < 0.0001 (****). (T) Quantification of immunofluorescence staining. Mean percentage of PCNA-positive area, normalized to the nuclei (DAPI positive area). Error bars indicate ±SD.

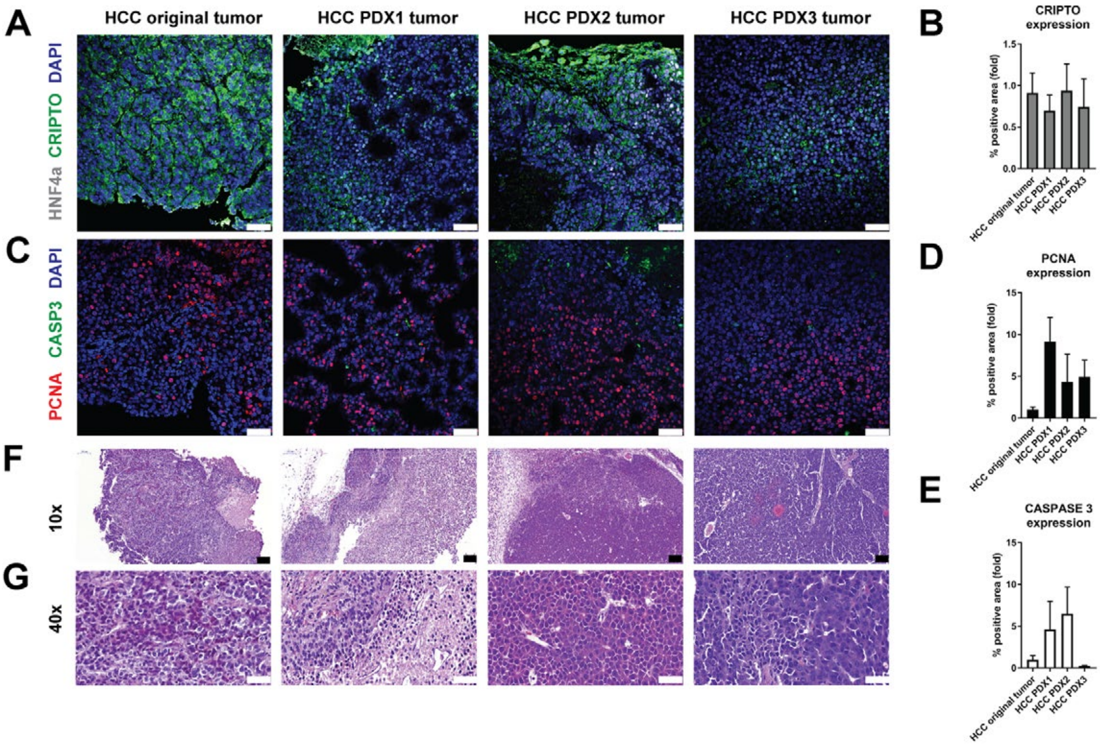

**Figure 4.**

**Establishment of CRIPTO-positive patient-derived xenograft model**

(A). Immunofluorescence staining for HNF4a (grey) and CRIPTO (green) expression in an HCC tumour sample (left), HCC PDX 1st passage (PDX1), 2nd passage (PDX2, centre) and 3rd passage (PDX3, right). (B) Quantification of CRIPTO immunofluorescence staining in the PDX tumours represented as fold over the original tumour tissue expression values. Error bars indicate ±SEM. (C) Immunofluorescence staining for PCNA (red) and cleaved CASP3 (green) expression in an HCC tumour sample (left), HCC PDX 1st passage (PDX1), 2nd passage (PDX2, centre) and 3rd passage (PDX3, right). (D) Quantification of PCNA and (E) cleaved CASPASE 3 (CASP3) immunofluorescence staining in the PDX tumours represented as fold-change over the original tumour tissue expression values. Error bars indicate ±SEM. (F-G) H&E staining at 10x (F) and at 40x objective (G) magnification HCC tumour sample (left), HCC PDX 2nd passage (PDX2, centre) and 3rd passage (PDX3, right). Scale bars: 50 µm, 100 µm.

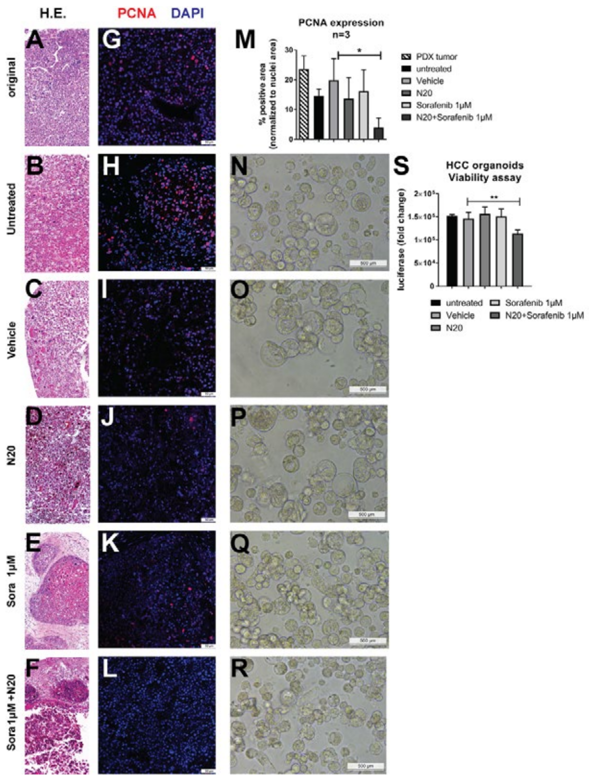

**Figure 5. Sorafenib resistance may be circumvented by CRIPTO pathway inhibitor in an *ex vivo* culture model of HCC PDX**

(A-F). *Ex vivo* tissue culture of HCC tumour tissue slices from the PDX4 of the established PDX. Drug treatments were performed during the 7 d of *ex vivo* cultures; (A) original tissue (non-cultured), (B) untreated, (C) vehicle (DMSO 0.1 %) plus Control-Fc (2 µg/ml)), (D) N20 blocking peptide (2 µg/ml), (E) sorafenib (1 µM), (F) sorafenib (1 µM) plus N20 (2 µg/ml). (G-L) Immunofluorescence staining for PCNA (red) and cleaved caspase-3 (green) expression. DAPI: nuclear dye. (G) original tissue (non-cultured), (H) untreated, (I) vehicle (DMSO 0.1%) plus Control-Fc (2 µg/ml), (J) N20 blocking peptide (2 µg/ml), (K) sorafenib (1 µM), (L) sorafenib (1 µM) plus N20 (2 µg/ml). (M) Quantification of PCNA immunofluorescence staining. Mean percentage of PCNA-positive area, normalized to the nuclei (DAPI positive area). Error bars indicate ±SEM, n=3 independent experiments. Paired t-test, p value < 0.05 (*). (N-R). Bright field images showing the morphology of organoids derived from the HCC PDX, after 48 h of culture (N, untreated), and treatments with (O) DMSO plus ControlFc, (P) N20 (2 µg/ml), (Q) sorafenib (1 µM) or (R) sorafenib plus N20. (S) CellTiter Glo viability luciferase-based assay measuring ATP content in organoids derived from HCC PDX tumour. Organoids were treated with DMSO plus CFc, N20 (2 μg/ml), sorafenib (1 μM) or sorafenib plus N20 for 48 h. Error bars indicate ±SD. Ordinary one-way ANOVA, p value < 0.01 (**).

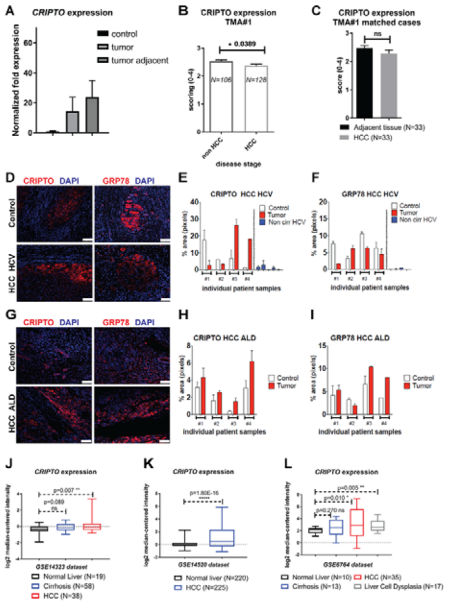

**Figure 6. Expression of CRIPTO and its signalling partner GRP78 in human HCC liver tissues.**

(A). CRIPTO mRNA levels in tissue from healthy liver tissues (N=8), tumour (N=7) and tumour-adjacent (N=7) matched cases; values are normalized to average of the control samples (ΔΔCt fold). (B). CRIPTO staining scoring in tissue microarray (TMA#1) samples; non-HCC (N=106) versus HCC cases (N=128). (C). CRIPTO protein expression in cohort of 19 matched (tumour versus tumour-adjacent tissue) cases of the TMA#1. (D-F). Representative immunofluorescence images of CRIPTO and GRP78 staining in human liver sections from HCV-derived HCC tissue (HCV infection-driven) and adjacent non-tumour control tissue from the same patient. Nuclei are stained with TO-PRO-3 (blue). Scale bars 75 μm. Quantification of protein expression of CRIPTO (E) and GRP78 (F), determined by immunofluorescence in HCC HCV tumour (Tumour) or adjacent non-tumour tissue (Control) from the same patient (N=4). Liver tissue from patients with HCV infection but absence of fibrosis (non-cirrhotic HCV, N=4) was used for comparison. The percentage of positive pixel area was an average from two four focal areas per section. Each bar represents values from each patient. Error bars indicate ±SD. (G-I) Representative immunofluorescence images of CRIPTO and GRP78 protein expression in human liver sections from alcoholic liver disease (ALD)-derived HCC tissue and adjacent non-tumour control tissue from the same patient. Nuclei are stained with TO-PRO-3 (blue). Scale bars 75 µm. Quantification of (H) CRIPTO and (I) GRP78 protein expression as assessed by immunofluorescence in tumour HCC ALD (Tumour) and adjacent non-tumour tissue (Control) from the same patient (N=4). The percentage of positive area (pixels) was the average from two-four focal areas per section. Each bar represents values from patient. Error bars indicate ±SD. (J-L). Transcript levels for CRIPTO in liver tissues from normal, cirrhosis, HCC and liver dysplasia conditions. Data obtained from three distinct publicly available datasets (GSE14323 [42], GSE14520 [43], GSE6764 [41]), accessed through the Oncomine database (www.oncomine.org). Statistical analysis and p values were obtained from the Oncomine plots.

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SUPPLEMENTARY MATERIAL ONLINE

**Supplementary materials and methods YES**

**Supplementary figure legends YES**

**Figure S1.** *In vitro* characterisation of CRIPTO downstream pathway activation

**Figure S2.** Expression of stem cell markers in CRIPTO-overexpressing cells

**Figure S3.** CRIPTO staining in HCC versus non-HCC cases of two TMAs

**Table S1.** Organoid media composition

**Table S2.** Primer sequences used in this study