Hepatocellular carcinoma (HCC) is a leading cause of cancer deaths, but its molecular heterogeneity hampers the design of targeted therapies. Currently, the only therapeutic option for advanced HCC is Sorafenib, an inhibitor whose targets include RAF. Unexpectedly, RAF1 expression is reduced in human HCC samples. Modelling RAF1 downregulation by RNAi increases the proliferation of human HCC lines in xenografts and in culture; furthermore, RAF1 ablation promotes chemical hepatocarcinogenesis and the proliferation of cultured (pre)malignant mouse hepatocytes. The phenotypes depend on increased YAP1 expression and STAT3 activation, observed in cultured RAF1-deficient cells, in HCC xenografts, and in autochthonous liver tumours. Thus RAF1, although essential for the development of skin and lung tumours, is a negative regulator of hepatocarcinogenesis. This unexpected finding highlights the contribution of the cellular/tissue environment in determining the function of a protein, and underscores the importance of understanding the molecular context of a disease to inform therapy design.
Hepatocellular carcinoma (HCC) is the commonest primary liver malignancy and the fifth most frequent cancer death cause in men. The incidence is highest in developing countries but cases in the Western World are increasing. The 5-year survival rate is poor, biomarkers and molecule-based therapies are lacking, and resistance to currently used chemotherapies is common. HCC correlates with hepatitis virus B or C infection, but also with exposure to aflatoxin B, alcohol abuse and obesity. Liver injury is a strong proliferative stimulus for surviving hepatocytes, which re-enter cell cycle to maintain organ mass and function. Injury/regeneration cycles favour the accumulation of genetic alterations and thus oncogenic hepatocyte transformation, ultimately leading to liver cancer. Activation of the Wnt/beta-catenin pathway combined with oxidative stress metabolism and RAS/ERK pathway, loss of tumour suppressor genes, and mutations in chromatin regulators are most frequently observed; overexpression or activation of receptor tyrosine kinases such as ERB2 and MET, of the mTOR pathway, as well as CMYC receptor tyrosine kinases such as ERB2 and MET, of the mTOR pathway, as well as CMYC (ref. 2) and the transcriptional co-activator YAP1 (ref. 3), are observed with varying frequency. Activating mutations of the interleukin 6 (IL6) receptor subunit gp130 and of the transcription factor STAT3 are frequent in HCC4,5.

Hepatocarcinogenesis can be recapitulated in the mouse, allowing functional analysis of specific signalling pathways. Genetic manipulation of JNK and p38 MAPK or the NF-kB pathway induce hepatocarcinogenesis or accelerate chemically driven tumorigenesis by increasing hepatocyte apoptosis, compensatory proliferation and/or inflammation6; pathways converging on STAT3 promote the progression of premalignant cancer progenitor cells7. Finally, the Hippo pathway and its target YAP1 are key regulators of hepatocyte differentiation in tumourigenesis8.

RAF1 is a kinase best known as the effector linking RAS to MEK/ERK activation. Additional essential functions of RAF1 rely on protein–protein interaction-based cross-talk with other pathways including Hippo, whose function is antagonized by RAF1 (ref. 8). In the mouse, Raf1 ablation causes liver apoptosis9,10, suggesting an essential function in this organ and a potential role in liver cancer development. Contrary to this expectation, patient data show reduced RAF1 expression in human HCCs; based on this, we have investigated the role of RAF1 in HCC using two different mouse models: (1) HCC xenografts and (2) hepatocarcinogenesis induced by the alkylating agent diethylnitrosamine (DEN) and promoted by Phenobarbital (Pb), which mimics human disease in terms of gene expression profiles and critically depends on inflammation11–14. Both models have revealed a tumour suppressor function of RAF1 in HCC, consistent with the reduced RAF1 expression in HCC patients.

Results

Loss of RAF1 promotes HCC development. We analysed RAF1 expression in paired tumour and non-tumour tissue of each of 31 human HCC specimens. RAF1 expression in tumours was significantly lower compared with the matched surrounding non-tumour tissue, and the degree of RAF1 expression in tumour (defined as the ratio of RAF1 expression in matched tumour/non-tumour tissues) negatively correlated with tumour grade (Fig. 1a). This was surprising for us but it is backed up by the data in the protein atlas, showing that RAF1 expression is low or undetectable in HCC samples probed with two different antibodies (http://www.proteinatlas.org/ENSG00000132155-RAF1/cancer/tissue/liver + cancer).

To determine whether RAF1 ablation plays a role in HCCs as suggested by the analysis of these data, we generated an isogenic HCC cell line in which RAF1 can be knocked down by the expression of a shRNA controlled by a doxycycline-inducible promoter, without affecting the expression of A- or BRAF (Hep3B RAF1 KD; Fig. 1b). Addition of doxycycline to the medium strongly increased the proliferation of Hep3B RAF1 KD cells (Fig. 1b). More importantly, a tremendous increase in tumour mass was observed in HCC xenografts in nude mice when RAF1 was knocked down in vivo by adding doxycycline to the drinking water (Fig. 1c).

Finally, we examined the role of RAF1 in the development of autochthonous liver tumours using conditional RAF1 ablation in hepatocytes (parenchymal cells) and bile duct cells (AlfpCre-Raf1f/f mice, hereafter referred to as Δhep) as well as global deletion of RAF1 by injecting MxCre;Raf1f/f mice with Poly I:C (ref. 15) (termed Δp;np; RAF1 ablated in parenchymal and non-parenchymal liver cells). Deletion was efficient and did not affect the expression of other RAF kinases (Supplementary Fig. 1a and b). The mice did not develop spontaneous liver tumours but were more sensitive than controls to DEN/Pb-induced hepatocarcinogenesis. A significant increase in macroscopic tumours was obvious in Δhep mice 30 weeks after DEN treatment; liver:body weight ratio, tumour numbers and tumour-occupied area were significantly higher in Δhep mice than in controls (Fig. 1d). Most lesions were adenosomas comprising hepatocytes with a high nuclear:cytoplasmic ratio and compressing the surrounding liver parenchyma; however, 4 out 6 Δhep animals developed HCC (showing trabecular growth patterns, high cellularity and low nuclear:cytoplasmic ratio), compared with 1 out of 6 controls. Consequently, the survival rate of Δhep mice was decreased (Supplementary Fig. 1c). In Δp;np mice, liver:body weight ratios and tumour-occupied areas were only slightly increased; however, RAF1 ablation increased tumour multiplicity (Fig. 1e) and malignancy (7 out of 10 Δp;np animals developed HCC, compared with 3 out of 8 F/F mice). Thus, RAF1 suppresses chemical hepatocarcinogenesis.

Analysis of both models revealed low, similar numbers of apoptotic cells in control and Δhep or Δp;np tumour-bearing livers (Supplementary Fig. 1d). Mitotic indexes were similar in tumours of all genotypes (Fig. 1e). However, more cycling cells, mostly non-parenchymal (Np) cells, were present in the non-tumour tissue of Δhep, but not Δp;np livers (Fig. 1f, Supplementary Fig. 1f). The dominant non-parenchymal cell type in livers of all genotypes were F4/80+ cells, mostly concentrated around portal veins; Δhep livers contained more macrophages, granulocytes and CD3+ cells than controls (Fig. 1g), while this increase was not observed in Δp;np livers (Supplementary Fig. 1h). Numbers of F4/80+ cells were similar in tumours of all genotypes (Supplementary Fig. 1g). Thus, RAF1 ablation in parenchymal cells led to increased inflammation in tumour-bearing livers. Consistent with this, the monocyte chemoattractant CCL2 was elevated in the blood of Δhep animals (Fig. 1h), and Δhep livers contained increased amounts of chemokines (CCL2, 4, 5 and 7; CXCL1) and cytokines (IL2, IL4, IL5, IL6, IL10, IL27, TNFz, IL1β, IFNγ; Fig. 1i). In contrast, the levels of both serum chemokines and liver chemokines/cytokines detected in Δp;np mice were comparable to those of controls, except CCL2 which was slightly elevated (Supplementary Fig. 1i and j). As in most chemical models of hepatocarcinogenesis16, fibrosis or cirrhosis were not detected (Supplementary Fig. 1k). Thus, RAF1 ablation in hepatocytes increased tumour multiplicity, whereas lack of RAF1 in non-parenchymal cells restrained inflammation leading to reduced tumour size.

Carcinogenesis in the DEN model critically depends on the interplay between hepatocytes and inflammatory macrophages15. These cells express FSP1, a protein involved in macrophage recruitment and chemotaxis in vivo17, as well as high amounts of...
chemokines and cytokines. FSP1^+ cells increased during carcinogenesis in livers of all genotypes; however, the increase in Δhep organs was significantly higher than in control and Δp/np livers (Supplementary Fig. 2a and b). Consistent with the slightly elevated CCL2 levels in tumour-bearing Δp/np mice, RAF1-deficient hepatocytes expressed higher basal and LPS-induced levels of this chemokine (Supplementary Table 1). However, RAF1-deficient macrophages failed to migrate into matrigel plugs containing CCL2 in vivo (Supplementary Fig. 2c) or in a transwell assay in culture (Supplementary Fig. 2d); treatment with low concentrations of a chemical inhibitor of the RAF1 interaction partner ROKα (ref. 8) restored migration.
(Supplementary Fig. 2d and e). RAF1-deficient and proficient macrophages produced similar amounts of chemo- and cytokines (Supplementary Table 1). These data imply that widespread RAF1 ablation impairs the recruitment of macrophages to the tumour-bearing Δp/np livers, limiting the inflammatory reaction and restraining the growth, but not the onset of DEN/Pb-induced tumours originating from RAF1-deficient hepatocytes.

**RAF1 ablation increases the number of cancer progenitor cells.** We next determined the impact of RAF1 on the early stages of chemical hepatocarcinogenesis. Liver damage and the number of apoptotic and cycling cells following DEN administration were indistinguishable in Δhep, Δp/np and F/F organs (Supplementary Fig. 3a–c). Later during tumourigeneis, however, more cycling hepatocytes could be detected in RAF1-deficient livers (8 weeks after carcinogen application in Δhep and 12 weeks in Δp/np livers; Fig. 2a). In addition, foci of altered hepatocytes19 (http://www.niehs.nih.gov/research/resources/liverpath/hyperplast/index.cfm#fociellar) were observed in all genotypes. These foci contained Ki67 + cells and cells expressing the liver cancer progenitor marker CD44 (ref. 7), and increased YAP1, a key negative regulator of hepatocyte differentiation20 (Fig. 2b). The foci were more frequently observed in RAF1-deficient than in F/F livers. Thus, conditional ablation of RAF1 by two different Cre transgenes accelerates hepatocarcinogenesis. The faster proliferative reaction of Δhep mice is likely due to increased inflammation (exemplified by macrophage recruitment, Supplementary Fig. 2a). To investigate the role of hepatocyte RAF1 in an inflammatory environment similar to that of F/F mice (Supplementary Fig. 2b, Supplementary Fig. 1i), we concentrated on the Δp/np model and quantified the number of liver cancer progenitor cells 16 weeks after DEN treatment. Progenitors express high levels of the adhesion molecule CD44, which promotes cell–cell adhesion, and are therefore mostly found in aggregate-containing liver fractions7. The number of liver cancer progenitor cells (defined as CD44 +/CD31 ~/Ter119 ~/CD45 ~) was significantly increased in RAF1-deficient organisms (Fig. 2c).

**Molecular characterization of RAF1-deficient lesions.** We next analysed the state of signalling pathways connected to RAF1 and HCC development in tumour-bearing livers and in xenografts. In F/F tumour-bearing livers, ERK phosphorylation was observed in non-tumour, but not in tumour tissue; in Δp/np livers, ERK phosphorylation could also be detected in tumours, implying that RAF1 is dispensable for ERK activation under these conditions (Fig. 3a). RAF1 interacts with the mammalian Hippo pathway21, a prominent suppressor of hepatocarcinogenesis and of YAP1 activity3. Expression of the RAF1 binding partner MST2 and phosphorylation of MST1/2 were comparable in both genotypes; LAT3 was expressed at slightly higher levels in tumours of both genotypes, and no correlation could be established between RAF1 expression and the phosphorylation of LAT3 on T1079 in the hydrophobic motif. Consistently, although YAP1 expression was higher in tumours and highest in the Δp/np organs, phosphorylation on the Hippo target site S127 was not significantly altered (Fig. 3a). STAT3 Y705 phosphorylation was consistently higher in Δp/np than in F/F tumours and correlated with the expression of the GP130 subunit of the IL6 receptor (Fig. 3a), which has been implicated in gastrointestinal tumourigenesis22. The expression of βcatenin, frequently activated in HCC, was slightly elevated in tumours of both genotypes (Fig. 3a); in addition, its subcellular localization was similar in RAF1 deficient and proficient tumours (Supplementary Fig. 4a). Increased YAP1 and GP130 expression, STAT3 and ERK phosphorylation were also observed in Δhep tumour-bearing livers (Supplementary Fig. 4b). In this setting, increased STAT3 phosphorylation could also be observed in the Δhep non-tumour tissue, likely a result of the inflammatory reaction in these organs.

The biochemical phenotypes observed in the autochthonous tumours could be secondary, that is, RAF1 ablation could promote the development of tumours with these molecular characteristics. To investigate this, we performed biochemical analysis of xenograft lysates, which confirmed RAF1 knockdown and recapitulated the increased expression of YAP1 and GP130 as well as the higher levels of STAT3 phosphorylation observed in the autochthonous tumours. ERK phosphorylation, however, was reduced in this setting, suggesting that the increased growth of RAF1 xenografts is ERK independent (Fig. 3b).

We next interrogated the non-selected HCC patient cohort for YAP1 expression. In contrast to RAF1 (Fig. 1a), YAP1 protein levels were higher in tumours compared with the surrounding tissue, and the degree of YAP1 expression in tumours (ratio of YAP1 expression in matched tumour/non-tumour tissue) positively correlated with tumour grade (Fig. 3c). In addition, the ratio of RAF1/YAP1 expression in the same tumour negatively correlated with histological grade in the whole cohort (Fig. 3c), indicating that tumours with low RAF1, high YAP1 protein levels are found in the most malignant group. This is consistent with the higher percentage of HCC-like tumours observed in the chemical carcinogenesis models (see above). We also determined STAT3 expression and nuclear localization (as a proxy for phosphorylation, to avoid possible misrepresentation due to the different fixation/storage conditions23,24) in the archival sections at our disposal. STAT3 expression was significantly lower in tumours than in non-tumour tissue (Fig. 3d); this was surprising for us but it is again consistent with the data in the protein atlas.
showing that STAT3 expression is weak in cancer in general and undetectable in 40–83% of HCC samples with 4 out of 5 antibodies used (http://www.proteinatlas.org/ENSG00000168610-STAT3/cancer/tissue/liver).

As previously shown for pSTAT3 (ref. 25), STAT3 nuclear staining was restricted to few cells in most of the samples; however, the ratio of RAF1/YAP1 expression in the same tumour negatively correlated with the presence of medium to large clusters of STAT3 nuclear staining. Thus, tumours with low RAF1, high YAP1 expression contain larger clusters of nuclear STAT3.

**RAF1 knockout cells have a competitive proliferation advantage.** Hep3B cells in which RAF1 was silenced by shRNA proliferated better than control cells (Fig. 1b) and showed increased YAP1 and GP130 expression as well as STAT3 activation when grown as xenographs. Similar results were obtained by silencing RAF1 in cultured Hep3B, HuH-7 and HepG2 cells with siRNAs targeting regions distinct from the one targeted by the shRNA (Fig. 4a). Thus, RAF1 silencing impacts the proliferation and signalling in transformed liver cells in vivo (Fig. 1c) and in culture (Figs 1b and 4a). To test whether RAF1 ablation confers a competitive proliferation advantage to premalignant liver cells, we established DEN-induced hepatocyte (DIH) lines from Raf1F/F and MxCre;Raf1F/F mice. Following immortalization, both lines were treated with IFNβ, leading to RAF1 deletion in the MxCre;Raf1F/F DIH (∆/∆ DIH). As already observed in livers, RAF1 deletion was efficient and did not affect the expression of A- or BRAF (Fig. 4b). RAF1-deficient and control DIH were much smaller than primary hepatocytes (P-HEPS) and expressed CD44 and CD31—present in non-aggregate and aggregate fractions of F/F and ∆/np livers, as determined by FACS analysis. Data are represented as mean ± s.e.m., *P≤0.05, **P<0.01, ***P<0.005 according to Student’s t-test. See also Supplementary Fig. 3.

To gain insight into the molecular mechanisms underlying the proliferation phenotype of RAF1-deficient cells, we treated F/F and ∆/np P-HEPS and F/F or ∆/∆ DIH with IL6, a cytokine pivotal for hepatocarcinogenesis6. RAF1-deficient cells showed decreased ERK phosphorylation (slight decrease in P-HEPS), but constitutively higher YAP1 and GP130 expression (AFP) instead of albumin (ALB) (Fig. 4c and d). ∆/∆ DIH had a clear proliferation advantage, particularly in media with low serum (Fig. 4e). They also attracted macrophages and produced CCL2 and CXCL1 more efficiently than F/F DIH (Supplementary Fig. 6).

Figure 2 | RAF1 ablation increases the number of cancer progenitor cells. (a) Quantification of Ki67+ liver cells 8 (top panel) or 12 weeks (bottom panel) after DEN treatment. (b) Foci of altered hepatocytes (FAH) in F/F and ∆hep or ∆/np livers isolated 12 weeks after DEN injection. Sections were stained with H&E or with the indicated antibodies. FAH are delimited by dotted circles (n=3 per genotype). Scale bars, 50 μm. (c) Percentage of cancer progenitor cells (CD44+/CD31–Ter119–CD45–) present in non-aggregate and aggregate fractions of F/F and ∆/np livers, as determined by FACS analysis. Data are represented as mean ± s.e.m., *P≤0.05, **P<0.01, ***P<0.005 according to Student’s t-test. See also Supplementary Fig. 3.
Figure 3 | Molecular characterization of RAF1-deficient lesions. (a) Immunoblotting of F/F and Δp/np livers collected 30 weeks after DEN treatment. The plots represents a densitometric quantification of the immunoblot performed using ImageJ. The data are expressed as relative band intensity adjusted to TUBA or ACTB, which serve as loading controls (upper plot). Phosphorylation is expressed as the ratio between the phosphospecific antibody signal and the signal obtained with the protein-specific antibody. In both cases, the data are normalized to the F/F non-tumour samples, which were arbitrarily set as 1. (b) Immunoblot analysis of signaling pathways in xenograft samples (n = 3, analysed 40 days after transplant). The plots show a quantification of the immunoblots performed as described in (a). (c) YAP1 expression in the same patient cohort examined in Fig. 1a. Scale bar, 50 μm. Left panel, representative IHC image. Middle panel, comparison of YAP1 expression in matched tumour and non-tumour tissue. Right panel, YAP1 expression in tumours correlates positively with tumour grade and the ratio of RAF1/YAP1 expression in the same tumour negatively correlates with histological grade. (d) STAT3 expression in the same cohort. Left panel, representative IHC image. Middle panel, comparison of STAT3 expression in matched tumour and non-tumour tissue. Right panel, RAF1/YAP1 expression in the same tumour negatively correlated with the presence of medium-large clusters of STAT3 nuclear staining. Scale bar 50 μm. In (a,b), the data are represented as mean ± s.e.m., *P ≤ 0.05, **P < 0.01, ***P < 0.005 according to Student’s t-test. (c,d) Middle panels, In the box and whiskers plots (Tukey method), the box represents interquartile range, the middle bar the median, and the whiskers extend to 1.5 times the interquartile range. Statistical analysis was done using Wilcoxon signed rank test; the analysis in the right panels represents the Spearman correlation. r_s and P values are indicated. See also Supplementary Fig. 4.
Taken together, the results show that the molecular defects observed in RAF1-deficient tumour-bearing livers and in human HCC lines were already present in P-HEPS and did not arise during transformation or immortalization.

YAP1 and STAT3 drive proliferation in RAF1 knockout cells.

In siRAF1 Hep3B cells, both YAP1 and pSTAT3 were found in the nucleus; accordingly, the target genes CTGF (YAP1 target) and BIRC5 (common YAP1 and STAT3 target) were expressed at higher levels in these cells (Fig. 5a,b). Reducing YAP1 levels by siRNA (Fig. 5b) or, respectively, treating cells with the potent JAK kinase inhibitor Pyridone 6 (ref. 26) (P6; Fig. 5c), reduced both the expression of the target genes and cell proliferation. Importantly, P6 abrogated STAT3 phosphorylation but did not alter YAP1 expression or ERK phosphorylation (Fig. 5c).

siRNA-mediated YAP1 silencing and P6 treatment also efficiently impaired target gene expression and proliferation of Δ/Δ DIH cultured in medium with 5% fetal bovine serum (FBS) (Fig. 5d and e), under which conditions ERK phosphorylation was clearly lower in Δ/Δ than in control DIH. Thus, the YAP1/STAT3 activation observed in siRAF1 Hep3B and Δ/Δ DIH contributes to proliferation. In intestinal epithelia, GP130 participates in IL6-driven STAT3 phosphorylation27 as well as in YAP1 activation through phosphorylation of Y357 by Src (ref. 28). Knockdown of GP130 in premalignant hepatocytes and Hep3B cells reduced STAT3 phosphorylation but did not alter YAP1 expression or phosphorylation, indicating that GP130 is not required for YAP1 activation in these cells (Fig. 5f).

The results in Fig. 5d and e indicated that reduced ERK phosphorylation in Δ/Δ DIH cells grown in 5% FBS medium did not impair proliferation. To more directly assess the relevance of
ERK activation in the proliferation of DIH, we treated F/F and Δ/Δ DIH with GDC-0879, a potent and specific RAF inhibitor, or with the multikinase inhibitor Sorafenib, used in the treatment of advanced HCC. GDC-0879 completely inhibited ERK activation in DIH of either genotype, but affected the proliferation of the control much more than that of the Δ/Δ cells (Supplementary Fig. 7). In contrast, Sorafenib at the concentration used activated rather than inhibited ERK, but effectively reduced STAT3 phosphorylation as previously described as well as DIH proliferation, independently of the genotype (Supplementary Fig. 7).
Thus, ERK phosphorylation status did not correlate with proliferation in DIH. We next treated DIH cells with PP2 to determine whether SFK inhibition had an impact on YAP1 phosphorylation on Y357, as recently described for intestinal cells\textsuperscript{39}, and whether this correlated with reduced proliferation. PP2 had a profound effect on cell proliferation, abolished the phosphorylation of YAP1 in DIH of both genotypes, and reduced STAT3 phosphorylation in RAF1 knockout DIH (Supplementary Fig. 7). Collectively, the data indicate that the inhibitors affect their expected targets; in addition, the differences observed in the inhibitors' effects in RAF1-proficient or -deficient DIH suggest a rewiring of signalling pathway in the RAF1 knockout DIH.

Our data so far are consistent with a model in which the regulation of YAPI and STAT3 signalling underlies increased proliferation of RAF1-deficient Hep3B and DIH. This correlates with increased expression of YAPI itself and of the STAT3 activator GP130. This increased expression did not correlate with higher mRNA amounts in Hep3B, P-HEPS, or DIH (Fig. 6a–c), suggesting a regulation at the translational or post-translational level. Blocking protein translation with cycloheximide revealed that YAPI and even more so GP130 were subject to rapid turnover, and that both proteins were more stable in all three RAF1-deficient cell types (Fig. 6d–i).

Discussion

Our study defines a tumour suppressor role of RAF1 in hepatic carcinogenesis. Firstly, RAF1 is found downregulated in a non-selected cohort of human HCC samples; secondly, modelling RAF1 downregulation in human HCC cells, in culture or in xenografts, increases cell proliferation; and thirdly, the same result was obtained in two independent genetic models (AlpCre and MxCre-induced RAF1 ablation in the DEN/PB-treated mice) and in premalignant hepatocytes derived from these models. This consistency is remarkable given the molecular heterogeneity of human HCC as well as of the human cell lines studied\textsuperscript{30} and the transgenic models used. The finding was entirely unexpected as the existing literature unanomously points to pro-tumourigenic functions of RAF1. RAF1 antagonizes apoptosis in both embryonic\textsuperscript{9} and adult liver\textsuperscript{10}, and is required to promote proliferation in RAS-driven skin and lung carcinogenesis\textsuperscript{31–33}.

The molecular correlate of RAF1 ablation/downregulation is also remarkably consistent: lack of RAF1 results in the increased expression of YAPI and GP130 and in STAT3 phosphorylation/activation in all models tested. These include P-HEPS treated with IL6, showing that the defect is directly related to RAF1 deletion and does not arise during the transformation of RAF1-deficient cells. The phenotype is in line with the role of the IL6 pathway in the development and progression of cancer progenitor cells\textsuperscript{7} and of YAPI as antagonist of hepatocyte differentiation\textsuperscript{20}.

In contrast to models of transgenic YAPI expression or of YAPI activation by Hippo pathway disruption\textsuperscript{2}, RAF1 ablation does not lead to spontaneous hepatocarcinogenesis. This milder phenotype may be due to the Hippo pathway, which is functional in RAF1-deficient livers and cells and can counteract hepatocyte proliferation driven by mitogens\textsuperscript{34}. In the autochthonous tumour models, the severity of the phenotype also correlates with the extent of liver inflammation, being stronger in Δhep than in Δnp animals. This is consistent with the fact that increased YAPI expression promotes hepatocyte proliferation \textit{in vivo} only upon liver injury or inflammation, that IL6 cooperates with YAPI in this setting\textsuperscript{35}, and that GP130 is necessary for fulfilled DEN-induced tumourigenesis in the mouse\textsuperscript{14}.

The ERK activation status, on the other hand, did not correlate with proliferation in autochthonous tumours, xenografts, or cultured cells, implying that in the absence of RAF1 proliferative signalling is rewired to rely on the activation of YAPI and STAT3 rather than ERK. While ERK activation is widely regarded as pro-tumourigenic, it was recently shown to inversely correlate with stem cell self-renewal in mammary tumours\textsuperscript{36} and with the maintenance of stem cell identity in mouse intestine\textsuperscript{37}. Also noteworthy in this context, activation of the STAT3 pathway by IL6 (ref. 38) or EGFR/SFK (ref. 39) can render BRAF mutant cancer cells resistant to RAF/ERK inhibition.

Mechanistically, our data are consistent with a model in which RAF1 ablation promotes the expression of YAPI and GP130, which in turn supports the activation of STAT3 by JAK, engendering a positive feedback loop supported by the inflammatory environment in which hepatocarcinogenesis occurs. Increased GP130 expression selectively supports the activation of STAT3 by proinflammatory cytokines of the IL6 family, but not by those of the IL10 family, broadly speaking anti-inflammatory in nature; the importance of the GP130/STAT3 axis in epithelial inflammation and gastrointestinal tumourigenesis\textsuperscript{42} and of GP130 in liver tumours\textsuperscript{4,7,14,40,41} has been amply documented. Besides the cell-autonomous proliferation phenotype, RAF1-deficient DIH are much more efficient than controls in attracting macrophages; they also produce higher amounts of CCL2, a STAT3 target gene\textsuperscript{43}, and of CXCL1, upregulated by YAPI in breast cancer cell lines\textsuperscript{42}. This is consistent with the increased numbers of inflammatory cells and the rich chemokine/cytokine milieu observed in Δhep tumour-bearing livers, and is reminiscent of the inflammation and macrophage accumulation caused by liver-restricted Hippo pathway inactivation\textsuperscript{44,45}. In Δnp animals, the failure of RAF1-deficient macrophages to migrate in response to chemokines and infiltrate the tumour-bearing livers correlates with limited inflammation and tumour load. This is consistent with the tumour-promoting role of inflammation in the DEN/Pb model, and implicates the immigrant macrophages as source of...
inflammatory mediators. The competitive advantage of RAF1-deficient initiated hepatocytes is still evident in the Δp/np animals in the form of increased tumour multiplicity.

One key question is how RAF1 ablation increases YAP1 and GP130 expression at the protein level. YAP1 turnover is regulated by at least two ubiquitylation-dependent mechanisms: degradation by the βTrCP-SCF ubiquitin ligase complex, promoted by the Hippo pathway46; or by Elongin B/C/Cullin-5, antagonized by oncogenic RAS47. RAF1 is a Hippo pathway antagonist and a RAS effector; therefore, if its ablation impinged on one of these mechanisms, it should decrease, not increase YAP1 expression. YAP1/TAZ are also integral components of the βcatenin destruction complex, to which they recruit βTrCP, promoting βcatenin degradation in the absence of Wnt signals48. Considering that βcatenin expression and nuclear localization are not altered by RAF1 ablation (Fig. 3a, Supplementary Fig. 4a, Fig. 5a), it is unlikely that RAF1 regulates this complex; alternatively, RAF1 may regulate YAP1 turnover by ubiquitin-independent mechanisms, such as autophagy49. GP130 degradation is less well investigated. In the absence of IL6, basal turnover of GP130 is mainly maintained by the proteasome system, whereas after IL6 stimulation GP130 is monoubiquitinated by the E3 ligase c-Cbl and undergoes internalization, endosomal sorting and lysosomal degradation50. RAF1 can be recruited to endosomal membranes51 and may therefore affect GP130 turnover by controlling either GP130 internalization or endosome trafficking.

Irrespective of the precise mechanism, our data consistently show that reduced RAF1 expression confers on liver cells the double selective advantage of higher STAT3 activation (by GP130-dependent stimuli) and higher YAP1 expression. This two-birds-with-one-stone mechanism can apparently cooperate with a range of oncogenic mutations.

Methods
Animal studies. MxCre;Raf1F/F mice have been previously described15 and were used to generate mice with a global RAF1 deletion (Δp/np mice; RAF1 deletion in vivo was induced by Poly I:C treatment). Mice lacking RAF1 in the hepatocyte compartment (Δhep mice), were generated by mating Raf1F/F to AlfpCre transgenic mice52. All strains were on a Sv/129 background. To induce carcinogenesis, male
mice were injected with DEN (Sigma; 100 mg kg⁻¹ body weight i.p.) at 4 weeks of age, and received a Pb diet to promote tumour growth (Sniff; 0.07% Pb, Sigma) from 8 weeks of age until killed. Animal experiments were authorized by the Austrian Ministry of Science, Research and Economy.

Phe3b xenografts (1 × 10⁵ in 0.1 ml of PBS) were inoculated in the flank of nude mice. shLuc or shRAF1 expression was induced by adding doxycycline to the drinking water (1.5 mg ml⁻¹ in dark bottles renewed every other day). Tumours were collected 40 days after injection and their volumes were determined according to the formula V = (a × b²)/2, where a is largest diameter and b is the perpendicular diameter.

**Human HCC samples.** 3-μm-thick sections of formaldehyde-fixed and paraffin-embedded samples of a non-selected cohort of surgically resected HCCs were obtained from the Biobank of the Medical University of Graz. All samples that fulfilled basic quality criteria (tumour cell content and absence of necrosis) were included in the study and in the data analysis. Collection and use of the samples was approved by the Ethical Committee of the Medical University of Graz (approval no. 27-334 ex14/15).

**Immunohistochemistry and immunoblotting.** H&E, TUNEL staining, immunohistochemistry and immunoblotting were carried out as described. The following antibodies were used for immunohistochemistry: mouse, CD44 (550538, BD Biosciences, 1:50), Ki67 (Novocastra, 1:1,000), YAPI (4912, Cell Signaling, 1:1,000), H Amp (2986, Cell Signaling, 1:1,000), GFAP (600196, EMD Millipore, 1:1,000), PLK1 (207043, Cell Signaling, 1:1,000), PCNA (2586, Cell Signaling, 1:800), pStat3Y705 (9145), STAT3 (9139), ERK1/2 (9102), pMST1(T183)/MST2(T180) (3681), MST2 (3952), pERK1/2 (9101), Fibroblast growth factor receptor 3 (FGFR3) (52314, Cell Signaling), ACTB (sc-1616), pE-Christopson (1:50), Ki67 (Novocastra, 1:1,000), YAP1 (12395, Cell Signaling, 1:1,000), RAF1 (154754, Abcam, 1:500) and STAT3 (9139, Cell Signaling, 1:800). Granulocytes were visualized using Naphthol AS-D chloroacetate (specific esterase) kit (91-1CT, Sigma). Images were acquired with a laser scanning confocal microscope (Olympus FLUOVIEW 1000, with 60×/1.4 Plan-Neofluar objectives) equipped with ZEISS AxioCam MRc5 and ZEISS Axiovision Release 4.8.1 software. RAF1 and YAP1 expression in human samples was quantified by measuring reciprocal chromogen intensity with the Image J software as previously described.

For immunoblotting, P-HEPS, DIH and organs were lysed in RIPA buffer (150 mM NaCl, 5 mM EDTA pH 8, 1 mM EGTA pH 8, 0.1% Triton X-100, 50 mM sodium deoxycholate 0.5%, 1 mM phenylmethyl sulphonyl fluoride, 10 mM NaF, 10 mM Na3VO4 and Roche protease inhibitors cocktail. EGTA. Both buffers were supplemented with 1 mM phenylmethyl sulphonyl fluoride, 10 mM NaF, 10 mM Na3VO4 and Roche protease inhibitors cocktail. To obtain nuclear extracts, Hep3B were lysed in nucleus buffer (1 mM K2HPO4, pH 7.4, 5 mM MgCl₂, 0.5% NP-40, 120 mM HEPES pH 7.4, 1 mM EDTA, 1.5 mM EGTA with buffers supplemented with phosphatase and protease inhibitors. Nuclear fractions were collected by centrifugation at 14,000 g for 5 min, resuspended in RIPA buffer and clarified by centrifugation at 14,000 g for 15 min. Protein concentrations were determined with a BCA assay (Pierce) followed by absorbance selection. Silencing was induced by adding 2 μg ml⁻¹ of doxycycline to the medium.

Cell proliferation, determined by absorbance quantitation, was assayed by MTT (555128, Sigma, 1:50) or BrdU proliferation assay (2750, Millipore, 500 μM). 5 × 10⁵ cells per well were plated in 96-well plates in 5% (IH) or 10% FBS medium (human HCC lines). In selected experiments, cells were treated with the chemical inhibitors P6 (Calbiochem), GDC-0879 (Selleckchem), Sorafenib (Selleckchem), PP2 (Sigma) or cycloheximide (Abcam; 25 μg ml⁻¹ for HEP3B cells, 100 μg ml⁻¹ for P-HEPS and 200 μg ml⁻¹ for MxCre;Raf1F/F). Stimulations with 10 mg/ml or 1 ng/ml IL1 (ProSpec) was carried out in 5% FBS (P-HEPS) and 20% FBS (DIH) for 30 min.

**Flow cytometry of liver fractions.** P-HEPS were isolated from perfused livers by FACS analysis 4 months after DEN injection. Non-aggregate and aggregate fractions were separated based on the ability of the cell suspensions to pass through a 40 μm cell strainer. Each fraction was mechanically dispersed and cells were stained with Fixable Viability Dye eFlour 520 (65-0867-10, 1:500) and mouse CD44 (17-0441-81, 1:1000) and human CD45 (550787, BD Biosciences) and plated on collagen-coated dishes. The following antibodies were used: mouse: CD44 (550538, BD Biosciences, 1:50), Ki67 (Novocastra, 1:1,000), YAPI (4912, Cell Signaling, 1:1,000), EGFR (2292, Cell Signaling, 1:1,000), FAK (2292, Cell Signaling, 1:1,000), LAP (207043, Cell Signaling, 1:1,000), PCNA (2586, Cell Signaling, 1:1,000). Immunoblot analysis was performed using Go Taq qPCR Master mix (Promega). Relative expression was calculated using the DDCT method using GAPDH as the housekeeping gene. The primers used were mouse: Actb (5′-GGGTGGAGAGGACCTGA3′/5′-GTTTGGTGTCATGTTGCAGT3′); reverse (5′-GACAATCATCGTTGCGATG3′); Ctgf forward (5′-GGGCGTGCTCTGTACGCTGAG3′), Ctgf reverse (5′-GGGCGTGCTCTGTACGCTGAG3′); reverse (5′-CTCTGGTTCTGTCCTGTTGCAGT3′); gp130 forward (5′-CTTTGGGACAGTGGAGGACAGA3′), reverse (5′-CTCTGGTTCTGTCCTGTTGCAGT3′); Ctgf forward (5′-GGGCGTGCTCTGTACGCTGAG3′), Ctgf reverse (5′-GGGCGTGCTCTGTACGCTGAG3′); reverse (5′-CTCTGGTTCTGTCCTGTTGCAGT3′); gp130 forward (5′-CTTTGGGACAGTGGAGGACAGA3′), reverse (5′-CTCTGGTTCTGTCCTGTTGCAGT3′); Ctgf forward (5′-GGGCGTGCTCTGTACGCTGAG3′). All primers were from Sigma.

**Migration assays.** BMDM (1 × 10⁴ per well, triplicates) were allowed to migrate towards CCL2 (10 ng ml⁻¹, 479E, R&D Systems) or DIH (7 × 10⁴ per well in 0.5% FBS DIH medium) through a transwell membrane (pore size 8 μm, BD Falcon) in DMEM containing 0.5% FBS. Where indicated, cells were pre-treated with ROK inhibitor (Y27632, Calbiochem, 10 μM, 30 min). Six hours after plating, the cells were fixed (4% paraformaldehyde (PFA), stained with crystal violet and counted. The live migration assays, 30 μg CCL2 in 200 μl matrigel plugs (35623), BD Biosciences) were injected into the flanks of 8–12 weeks old F/F and Δp/n mice, removed and analysed 5 days later.
Statistical analysis. Animal experiments were performed comparing littermates, the evaluators were aware of animal identity throughout the experiments and outcome assessment. The log-rank test was used to evaluate the significance of the difference in survival (Supplementary Fig. 1c). Where applicable, power calculations were used to determine the sample size necessary to obtain significant results (P < 0.05) with a power of > 0.80, assuming twofold changes and a s.d. of 10%. Histological samples were analysed by counting or measuring at least five microscopic fields/section. The investigator was blinded to group allocation. For experiments involving cultured cells, unless otherwise stated values are expressed as means ± s.e.m. of three independent experiments; P values were calculated with the two-tailed Student’s t-test, hetero- or homoscedastic as determined by a previous F-test of equality of variances. The human immunohistochemical data was analysed using Wilcoxon signed rank test and Spearman correlation. A P value ≤ 0.05 is considered statistically significant.

Data availability. The authors declare that all data supporting the findings of this study are available within the paper and its Supplementary information files.

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Author contributions
I.J., G.M., J.R., E.D. and A.L.C. designed, carried out and interpreted experiments. M.P. and B.T. helped with the acquisition of data. I.J. prepared the data for publication. I.F. helped with the histology, K.Z. contributed to the design and interpretation of the human studies, M.B. designed and supervised the project, helped with data interpretation, and wrote the manuscript.

Additional information
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