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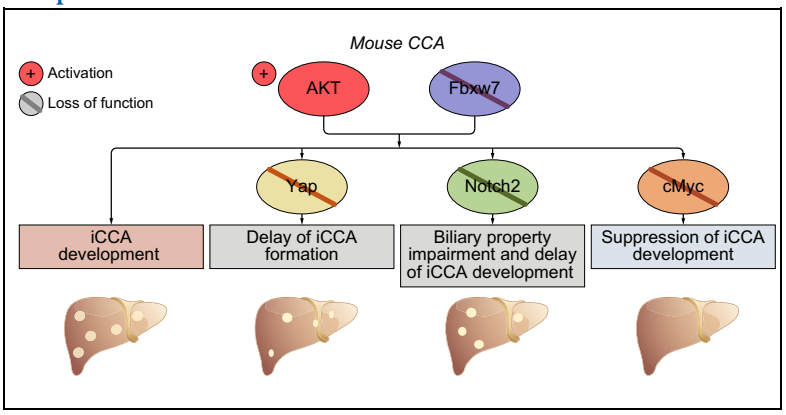
Loss of Fbxw7 synergizes with activated Akt signaling to promote c-Myc dependent cholangiocarcinogenesis

Graphical abstract



Highlights

- Downregulation of the FBXW7 tumor suppressor gene was identified as a universal feature of human intrahepatic cholangiocarcinoma (iCCA).
- Hydrodynamic transfection of inactivated Fbxw7 synergized with an activated form of AKT to induce rapid iCCA in mice.
- Cholangiocarcinogenesis was prevented by c-Myc suppression, while being delayed by either *Yap* or *Notch 2* depletion in these mice.
- Inhibition of c-MYC might represent an innovative therapeutic strategy for the treatment of human iCCA with low FBXW7.

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Lay summary

There is mounting evidence that FBXW7 functions as a tumor suppressor in many cancer types, including intrahepatic cholangiocarcinoma, through its ability to promote the degradation of numerous oncoproteins. Herein, we have shown that the low expression of FBXW7 is ubiquitous in human cholangiocarcinoma specimens. This low expression is correlated with increased c-MYC activity, leading to tumorigenesis. Our findings suggest that targeting c-MYC might be an effective treatment for intrahepatic cholangiocarcinoma.

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Loss of Fbxw7 synergizes with activated Akt signaling to promote c-Myc dependent cholangiocarcinogenesis

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Background & Aims: The ubiquitin ligase F-box and WD repeat domain-containing 7 (FBXW7) is recognized as a tumor suppressor in many cancer types due to its ability to promote the degradation of numerous oncogenic target proteins. Herein, we aimed to elucidate its role in intrahepatic cholangiocarcinoma (iCCA).

Methods: Herein, we first confirmed that *FBXW7* gene expression was reduced in human iCCA specimens. To identify the molecular mechanisms by which FBXW7 dysfunction promotes cholangiocarcinogenesis, we generated a mouse model by hydrodynamic tail vein injection of *Fbxw7*ΔF, a dominant negative form of Fbxw7, either alone or in association with an activated/myristylated form of AKT (myr-*AKT*). We then confirmed the role of c-MYC in human iCCA cell lines and its relationship to FBXW7 expression in human iCCA specimens.

Results: *FBXW7* mRNA expression is almost ubiquitously downregulated in human iCCA specimens. While forced overexpression of *Fbxw7*ΔF alone did not induce any appreciable abnormality in the mouse liver, co-expression with AKT triggered cholangiocarcinogenesis and mice had to be euthanized by 15 weeks post-injection. At the molecular level, a strong induction of Fbxw7 canonical targets, including Yap, Notch2, and c-Myc oncoproteins, was detected. However, only c-MYC was consistently confirmed as a FBXW7 target in human CCA cell lines. Most importantly, selected ablation of c-Myc completely impaired iCCA formation in *AKT/Fbxw7*ΔF mice, whereas deletion of either Yap or Notch2 only delayed tumorigenesis in the same model. In human iCCA specimens, an inverse correlation between the expression levels of FBXW7 and c-MYC transcriptional activity was observed.

Conclusions: Downregulation of FBXW7 is ubiquitous in human iCCA and cooperates with AKT to induce cholangiocarcinogenesis in mice via c-Myc-dependent mechanisms. Targeting c-MYC might represent an innovative therapy against iCCA exhibiting low FBXW7 expression.

Lay summary: There is mounting evidence that FBXW7 functions as a tumor suppressor in many cancer types, including intrahepatic cholangiocarcinoma, through its ability to promote the degradation of numerous oncoproteins. Herein, we have shown that the low expression of FBXW7 is ubiquitous in human cholangiocarcinoma specimens. This low expression is correlated with increased c-MYC activity, leading to tumorigenesis. Our findings suggest that targeting c-MYC might be an effective treatment for intrahepatic cholangiocarcinoma.

Introduction

Cholangiocarcinoma (CCA) is the second most common primary liver cancer.[1,2] Depending on the anatomical site, CCA is classified into intrahepatic (iCCA) and extrahepatic cholangiocarcinoma (eCCA).[3] iCCA incidence has been rising over the last decade, while that of eCCA slightly decreased.[4] For iCCA detected at early stage, curative surgical resection is the optimal treatment strategy.[5,6] However, less than one-third of patients achieves negative tumor margins, and recurrence rates are high.[5,7] Furthermore, in patients not meeting the narrow criteria for surgical treatment, therapeutic options are limited.[8] Therefore, substantial efforts should be devoted to unravelling the molecular mechanisms of iCCA development and progression.

Keywords: FBXW7; Cholangiocarcinogenesis; c-Myc; Cholangiocarcinoma murine model; Yap; Notch2.
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This would lead to novel and more effective therapeutic strategies against this pernicious disease.

The E3 ubiquitin ligase F-box and WD repeat domain containing protein 7 (FBXW7) is a *bona* fide tumor suppressor gene.[9,10] Being a substrate recognition component of the Skp1-Cul1-F-box protein-type complex, FBXW7 is responsible for the binding to and the degradation of several oncogenes, including c-MYC,[11,12] YAP,[13] NOTCH1,[14,15] mTOR,[16] CCNE1,[11] and CCND1.[12] Among them, YAP, NOTCH, and c-MYC proteins play a pivotal role in cholangiocarcinogenesis.

In human CCA, ~35% specimens were found to harbor *FBXW7* mutations, resulting in failure of substrate recognition.[17] In addition, the *FBXW7* gene is downregulated in human CCA cells when compared with intrahepatic biliary epithelial cells.[18] Also, *FBXW7* mRNA expression negatively correlates with tumor stage, metastasis, and differentiation in human iCCA specimens.[18,19] Furthermore, low *FBXW7* levels are associated with poorer prognosis and shorter survival in patients with CCA and hepatocellular carcinoma (HCC).[19,20]

Inspired by these previous data, we analyzed a collection of human iCCA and confirmed the reduction of *FBXW7* gene expression in this tumor type, which occurred in the absence of *FBXW7* mutations. Moreover, to define the molecular mechanisms whereby FBXW7 disruption contributes to cholangiocarcinogenesis, we generated a new mouse model by hydrodynamic tail vein injection (HTVi) of myr-*AKT* (an activated/myristylated form of AKT) and *Fbxw7*ΔF (*AKT*/*Fbxw7*ΔF), a dominant negative form of Fbxw7. In this iCCA model, a robust induction of Yap, Notch2, and c-Myc oncoproteins occurred during tumorigenesis. Subsequent *in vitro* and *in vivo* studies identified c-MYC as the prominent FBXW7 target responsible for cholangiocarcinogenesis in experimental and human iCCA.

Materials and methods

Constructs and reagents

Constructs applied include PT3-*EF1α*, pT3-*EF1α*-HA-myr-*AKT* (human), pT3-*EF5α*-HA-*Fbxw7*ΔF (human), PT3-*EF1α*-*MadMyc* (human), pCMV, pCMV-*Cre* and pCMV-sleeping beauty (SB) transposase.

Animals and hydrodynamic tail vein injection

Mice were housed, fed, and monitored according to protocols approved by the Committee for Animal Research at the University of California, San Francisco (San Francisco, CA). Hydrodynamic tail vein injection was performed in 5.5- to 6.5-week-old mice, as described before.[21,22]

Human tissue samples

DNAs from 8 normal livers, 120 frozen iCCAs and corresponding non-tumorous surrounding livers were used for sequencing analysis of the *FBXW7* gene as reported.[23] Among them, 82 iCCA specimens were available as frozen specimens and used for loss of heterozygosity (LOH) and promoter methylation analyses, as described.[24,25] The iCCA samples were collected at the Medical Universities of Greifswald (Greifswald, Germany) and Sassari (Sassari, Italy). Clinicopathological features of iCCA samples are reported in Table S1. Institutional Review Board approval was obtained at the local Ethical Committee of the Medical Universities of Greifswald and Sassari. Informed consent was obtained from all individuals.

Statistical analysis

The Prism 7.0 software (GraphPad, San Diego, CA) was used to analyze the data, presented as Means ± SD. Comparisons between 2 groups were performed with 2-tailed unpaired *t* test when dataset achieve Gaussian distribution or non-parametric test when sample size was small. Welch correction was applied when necessary. *P* values <0.05 were considered statistically significant.

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

Results

Low mutation rate but reduced mRNA expression of *FBXW7* in human intrahepatic cholangiocarcinoma

First, we determined the *FBXW7* mutation frequency in a collection of human iCCA (n = 120) and corresponding non-tumorous livers. Unexpectedly and contrary to previous findings,[17] *FBXW7* was mutated only in 1 iCCA (0.8%). The mutation occurred in *FBXW7* exon 8 and consisted of the previously reported R465H change[26,27] (Fig. 1A). As *FBXW7* gene downregulation can result from promoter hypermethylation and/or LOH at the *FBXW7* locus,[28,29] the promoter and locus status of *FBXW7* were determined. While no *FBXW7* promoter hypermethylation was detected (data not shown), LOH occurred in 20 of 120 iCCA (16.7%) with wild-type *FBXW7* (Fig. 1B, C). Subsequently, mRNA levels of *FBXW7* were investigated by quantitative reverse transcription PCR (qRT-PCR) using the iCCA samples from the collection whose RNA was available (n = 82). Strikingly, the vast majority of iCCA (71/82; 86.6%) exhibited lower *FBXW7* mRNA levels when compared with corresponding non-tumorous livers (Fig. 1D), in agreement with previous reports[19,30] and the FBXW7 data from The Cancer Genome Atlas (TCGA, Fig. S1A). In the TCGA dataset presented on cBioportal,[31,32] containing mutation data from 35 patients, 1 patient (2.86%) harbored the R393Q mutation (Fig. S1B). Interestingly, this mutation and that found in our study (R465H) have been identified as significant hotspots and indicate oncogenic events.[26,27] Also, the mRNA level of *FBXW7* correlates with its copy number values (Fig. S1C). No association between *FBXW7* mRNA levels and clinicopathologic features of the patients, including age, gender, etiology, presence of cirrhosis, tumor size, and tumor differentiation, was detected (data not shown). Also, since survival data from our patient cohort were not available, the relation between *FBXW7* expression and prognosis could not be determined.

Altogether, the present data indicate that downregulation of wild-type *FBXW7* gene is almost ubiquitous in human iCCA.

Inactivation of Fbxw7 synergizes with activated AKT to induce hepatocyte-derived iCCA formation in mice

Subsequently, we sought to address the role of FBXW7 along cholangiocarcinogenesis *in vivo*. Thus, we generated a mouse model harboring a dominant negative form of *Fbxw7* (*Fbxw7*ΔF), which was injected into the mice hydrodynamically. Inactivation of Fbxw7 alone did not result in any tumor development or appearance of liver histologic abnormalities up to 36 weeks post-injection (Fig. S2), implying that loss of Fbxw7 does not suffice to drive carcinogenesis in the mouse liver. Subsequently, to increase the malignant potential, another oncogenic insult was added to Fbxw7 inactivation, namely the induction of

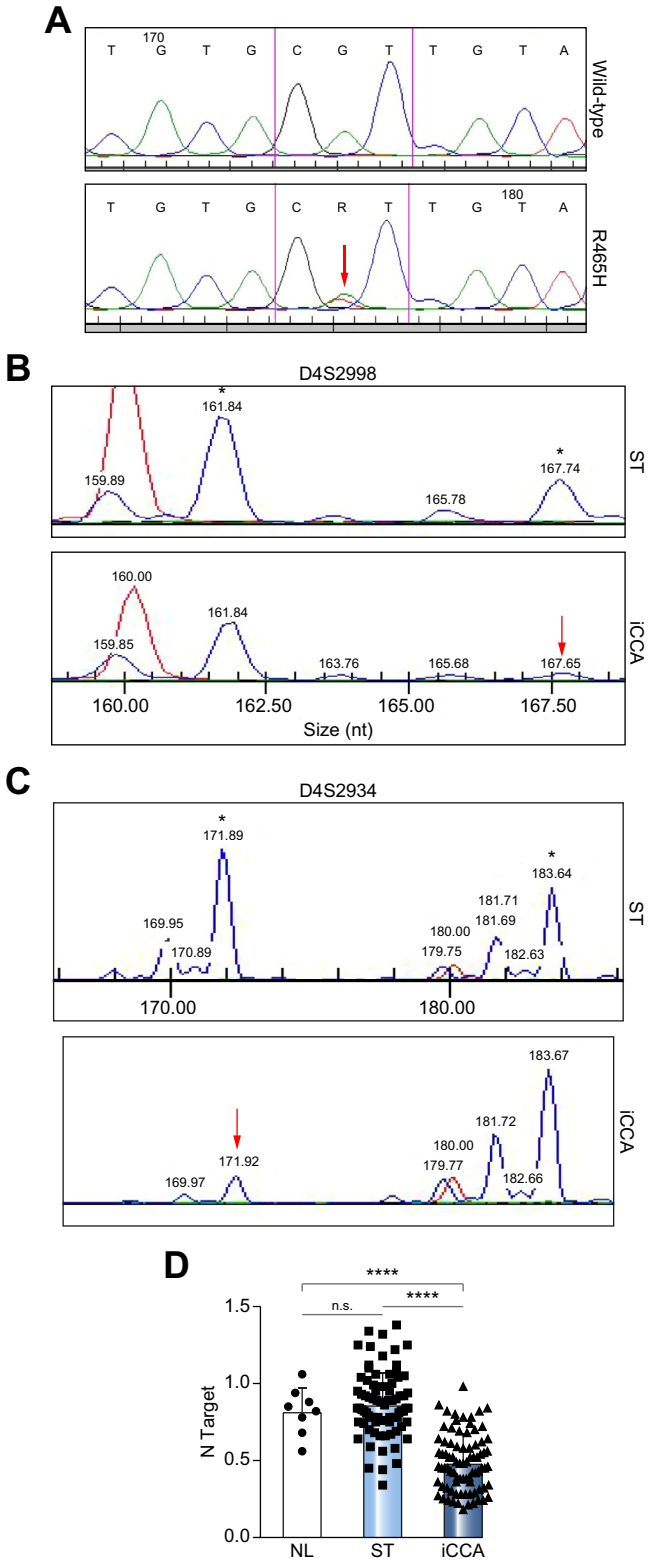

Fig. 1. Downregulation of the *FBXW7* gene in human intrahepatic cholangiocarcinoma specimens. (A) Chromatogram showing the wild-type sequence (upper panel) and the R465H mutant form (lower panel) of *FBXW7* exon 8. The red arrow indicates the mutated nucleotide. (B, C) Examples of LOH at the *D4S2998* (B) or *D4S2934* (C) locus encompassing the *FBXW7* gene. The 2 alleles are indicated by asterisks (*), and red arrows indicate the presence of LOH at 1 allele. (D) qRT-PCR analysis of *FBXW7* mRNA levels in NL (n = 8), iCCA (n = 82), and corresponding ST (n = 82). Data was analyzed by unpaired *t*-test. n.s., not significant; ****$p < 0.0001$ when compared to NL and ST. iCCA, intrahepatic cholangiocarcinoma; LOH, loss of heterozygosity; NL, normal liver; qRT-PCR, quantitative reverse transcription PCR; ST, non-tumorous surrounding tissue.

AKT, frequently occurring in human iCCA,[33] through hydrodynamic co-injection of activated/myristylated (myr-)*AKT* and *Fbxw7*ΔF (Fig. 2A). Previously, we showed that myr-AKT overexpression induces lipid-rich hepatocellular preneoplastic lesions in the mouse liver, ultimately leading to the development of HCC and, more rarely, iCCA[34] (Fig. S3). In *AKT/Fbxw7*ΔF livers, similar clusters of clear-cell, lipid-rich preneoplastic hepatocytes with enlarged nuclei, were detected 3 weeks post-injection. These hepatocellular lesions, morphologically indistinguishable from those of myr-*AKT* mice, were progressively replaced by iCCA lesions, starting from 6 weeks post-injection (Fig. 2A; Fig. S4). Ten weeks post-injection, large iCCAs were detected in *AKT/Fbxw7*ΔF livers. Most iCCA exhibited large areas of necrosis in the tumor center (Fig. S4), suggesting vigorous proliferation, which was confirmed by Ki67 staining (Fig. 2A). By 12–15 weeks post-injection, livers were almost completely occupied by colliding iCCA and mice were moribund (Fig. 2A; Fig. S4). Liver weight and liver/body ratio gradually increased along cholangiocarcinogenesis (Fig. S5). Since *AKT* and *Fbxw7*ΔF were both HA-tagged, immunohistochemistry showed positive HA-tag immunoreactivity in both cytoplasm (for *AKT*) and nucleus (for *Fbxw7*ΔF) of tumor cells (Fig. 2B).

To determine whether the observed iCCA lesions derive from hepatocytes or cholangiocytes, lineage tracing technology was applied. By breeding $AlbCre^{ERT2}$ mice with $R26R^{EYFP}$ mice, $AlbCre^{ERT2}$;$R26R^{EYFP}$ heterozygotes were generated. Subsequently, tamoxifen was injected into $AlbCre^{ERT2}$;$R26R^{EYFP}$ mice 2 weeks before hydrodynamic injection to trigger specific EYFP expression in hepatocytes (Fig. S6A). Noticeably, immunofluorescence (IF) showed that iCCAs induced by *AKT/Fbxw7*ΔF co-expression originated from hepatocytes, as they exhibited positive staining for both EYFP and HA-tag (Fig. S6B).

Activation of multiple oncogenic pathways in *AKT/Fbxw7*ΔF lesions

Next, we investigated the signaling pathways activated in *AKT/Fbxw7*ΔF mouse lesions. Specifically, we focused on the oncogenic cascades that are physiologically inhibited by Fbxw7, including Hippo/Yap, Notch, and c-Myc.[11,13,14] Western blotting and immunohistochemistry showed the activation/induction of Yap, Notch2, and c-Myc proteins (Fig. 2C; Figs. S7 and S8). Yap was expressed at high level (Fig. 2C) and translocated in the nucleus (a sign of its activation; Fig. S7) of tumor cells. Consequently, Yap downstream targets, namely *Ctgf* and *Cyr61*, were significantly upregulated in the iCCA lesions when compared to the non-tumorous counterparts (Fig. 2D). Among the other Hippo pathway members, the Yap negative regulators Lats1/2 were downregulated (Fig. S8). Similarly, levels of Notch2 and its ligand Jag1 were remarkably increased in *AKT/Fbxw7*ΔF neoplastic lesions (Fig. 2C). Accordingly, the canonical downstream targets of Notch2 (*Hey1* and *Nrarp*) were induced at transcriptional level in *AKT/Fbxw7*ΔF iCCAs (Fig. 2D). As for the downstream targets of c-Myc, genes involved in cell cycle and survival, such as *Cdk4*, *Ccnd2*, *Skp2* and *Tert*, as well as energy metabolism, including *Ldha*, *Ldhc*, and *Odc1*, were uniformly upregulated in tumors (Fig. 2E). Importantly, elevated c-Myc expression was specific for *AKT/Fbxw7*ΔF iCCA lesions, as it was undetectable in un-injected normal liver, *AKT*- or *Fbxw7*-ΔF-only injected liver tissues (Fig. S9).

Since *AKT/Fbxw7*ΔF induced tumors derive from hepatocytes, we further confirmed our findings using the AML-12 mice normal hepatocyte cell line.[35] After transfection of myr-*AKT*,

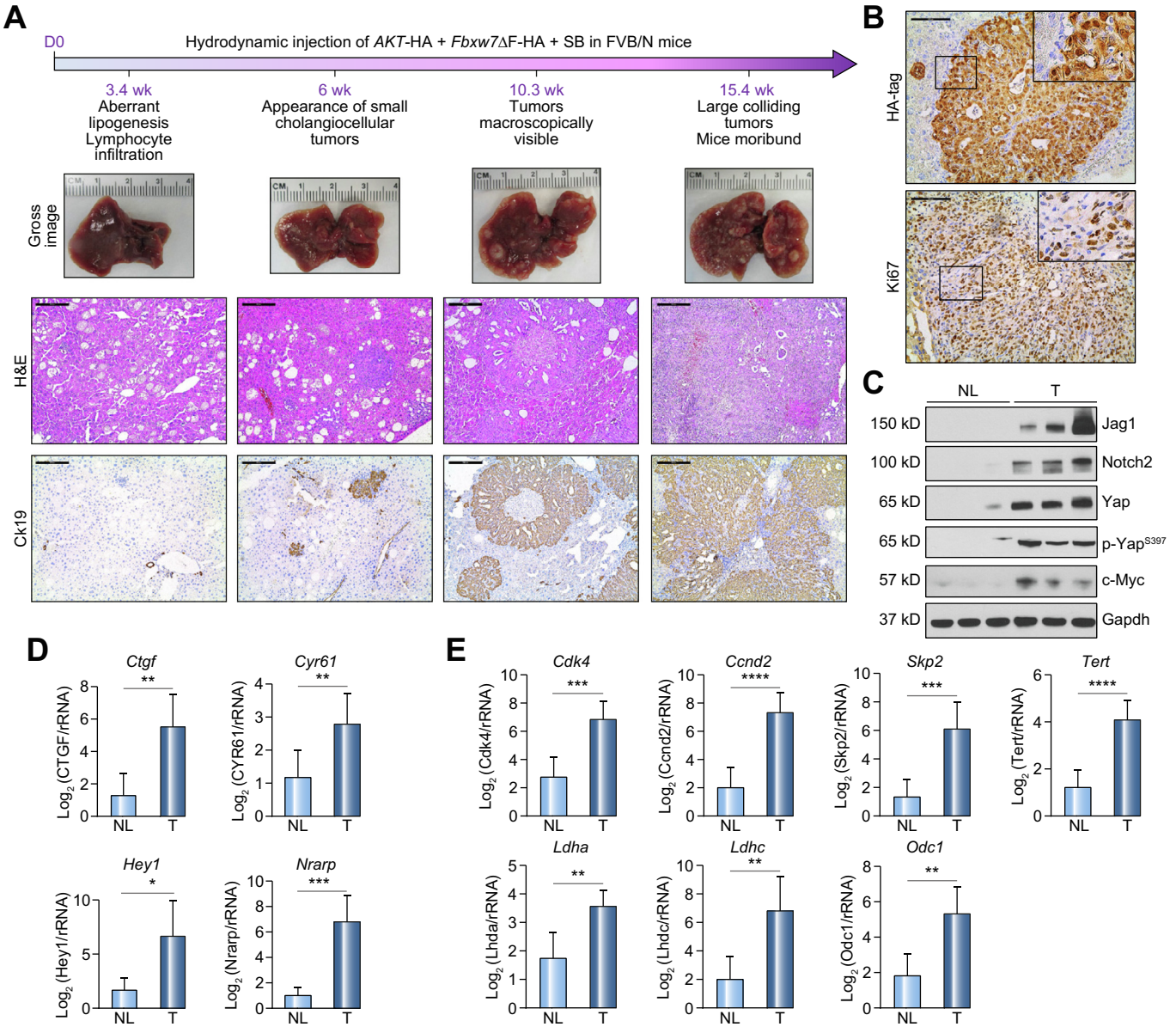

Fig. 2. Co-expression of *AKT* and a dominant negative form of *Fbxw7* (*Fbxw7*ΔF) leads to intrahepatic cholangiocarcinoma development in mice. (A) Timeline of tumor development in *AKT/Fbxw7*ΔF mice (upper panel); macroscopy of *AKT/Fbxw7*ΔF livers (second panel); histopathologic features of the lesions (third panel); Ck19 immunostaining (fourth panel). (B) HA-tag immunohistochemistry showing cytoplasmic and nuclear staining for AKT and Fbxw7ΔF. The lesions are highly proliferative, as indicated by Ki67 nuclear immunoreactivity. (C) Western blotting of pathways activated in *AKT/Fbxw7*ΔF cholangiocellular lesions. Gapdh was used as a loading control. (D) mRNA levels of canonical Yap (*Ctgf*, *Cyr61*; upper panel) and Notch (*Hey1*, *Nrarp*; lower panel) target genes. (E) mRNA levels of c-Myc targets (*Cdk4*, *Ccnd2*, *Skp2*, *Tert*, *Ldha*, *Ldhc*, and *Odc1*). Data was analyzed by unpaired *t*-test with Welch's correction. Scale bar: 200 μm for 10x; 100 μm for 20x. n.s., not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. NL, normal liver; T, tumor.

*Fbxw7*ΔF alone or together for 48 h, the protein levels of Yap, Notch2, and c-Myc were elevated in *Fbxw7*ΔF and *AKT/Fbxw7*ΔF transfected cells (Fig. S10A). Interestingly, Sox9, a biliary epithelium biomarker, was also induced in *Fbxw7*ΔF and *AKT/Fbxw7*ΔF transfected AML-12 cells. *AKT/Fbxw7*ΔF co-expression also triggered c-MYC upregulation in RBE, KMCH, and SNU1196 human CCA cell lines (Fig. S10B).

Depletion of Yap delays but does not abolish *AKT/Fbxw7*ΔF tumorigenesis

YAP activation occurs in over 90% of human CCA specimens[36] as well as in the *AKT/Fbxw7*ΔF mouse model (this study). In addition, YAP was hypothesized as a target protein of FBXW7 in HCC[13] and pancreatic cancer.[37] We therefore investigated the role of Yap in *AKT/Fbxw7*ΔF driven cholangiocarcinogenesis. Thus, hydrodynamic injection of *AKT/Fbxw7*ΔF/pCMV (as control) and *AKT/Fbxw7*ΔF/*Cre* plasmids was employed in *Yap*$^{flox/flox}$ mice (Fig. 3A). Ablation of the endogenous *Yap* significantly delayed tumor development in *AKT/Fbxw7*ΔF mice (Fig. 3B and 3C). By 7.7 weeks post-injection, while the control group mice developed iCCAs, no frankly malignant lesions occurred in *AKT/Fbxw7*ΔF/*Cre* livers, whereas small clusters of infiltrating lymphocytes (Fig. 3C) and scattered clusters of clear-cell, lipid-rich hepatocytes were detected (Fig. S11). Twenty weeks post-injection, several tumors were observed in *AKT/Fbxw7*ΔF/*Cre* livers, with a diameter of 1 mm–5 mm. They consisted of

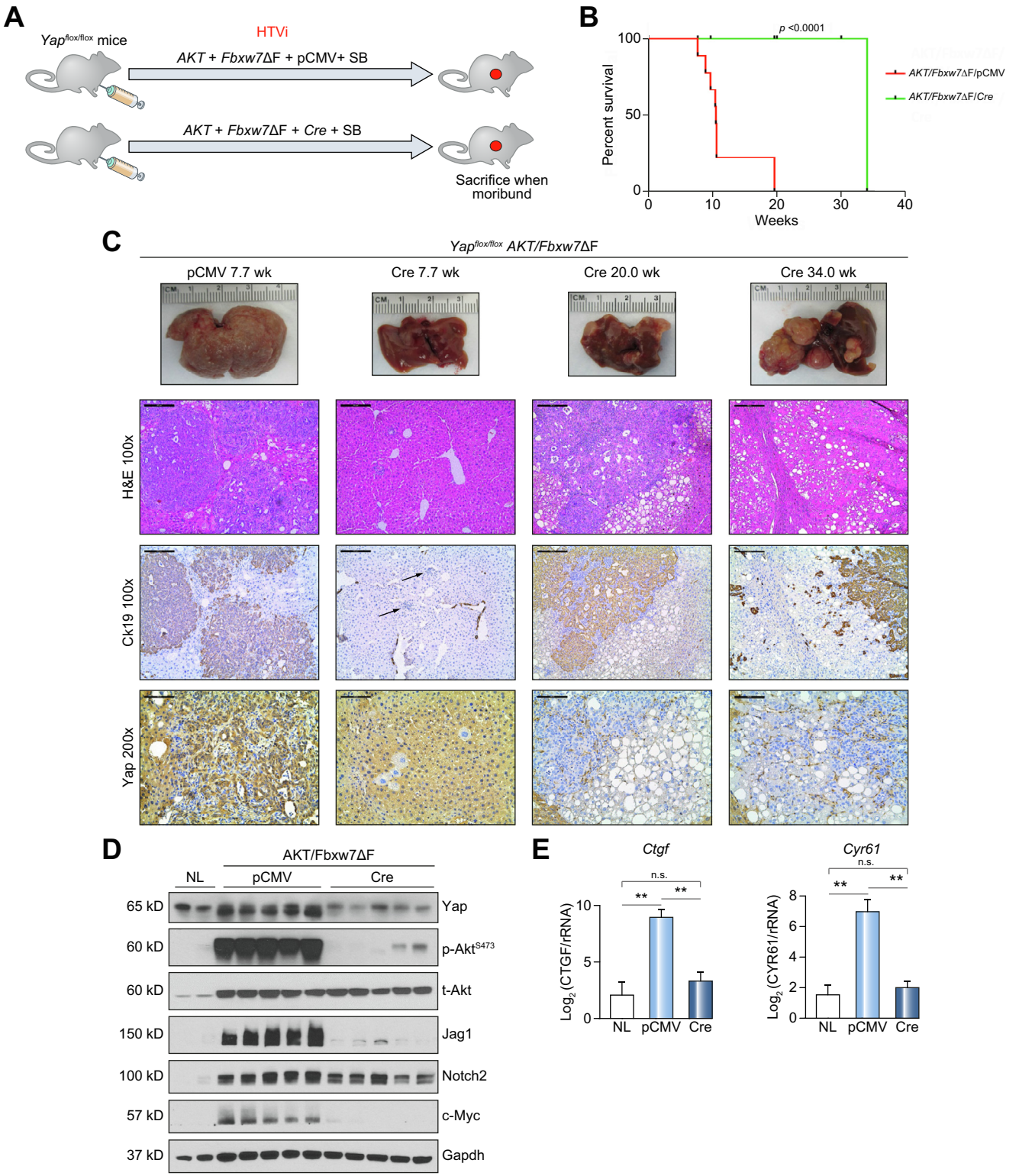

Fig. 3. Deletion of Yap delays, without suppressing, cholangiocarcinogenesis in *AKT/Fbxw7*ΔF mice. (A) Study design. *Yap*$^{flox/flox}$ conditional knockout mice were subjected to HTVi of either *AKT/Fbxw7*ΔF/pCMV (control, n = 9) or *AKT/Fbxw7*ΔF/*Cre* (n = 10) plasmids. (B) Survival curve showing the delay of intrahepatic cholangiocarcinoma development following deletion of *Yap*. (C) Delay of tumor development in *AKT/Fbxw7*ΔF mice, as revealed by macroscopic examination of the livers (upper panel), histopathology of the lesions, Ck19 immunoreactivity (as a marker of biliary tumors) and Yap immunoreactivity. (D) Western blotting of *AKT/Fbxw7*ΔF mouse livers from control and *Yap* deleted (Cre) mice. Gapdh was used as a loading control. (E) mRNA expression of *Ctgf* and *Cyr61*. Data was analyzed by Mann-Whitney test. Scale bar: 200 μm for 10x; 100 μm for 20x. HTVi, hydrodynamic tail vein injection; NL, normal liver; SB, sleeping beauty transposase.

clear-cell hepatocellular lesions, containing small areas of cholangiocellular differentiation inside, and they were of mixed-type morphology at this time point (Fig. S11). Noticeably, by 34 weeks post-injection, most of the hepatocellular large tumors were replaced by pure cholangiocellular lesions (Fig. S11). Both CCA and HCC lesions were highly proliferative

(Fig. 3C and Fig. S12). To determine whether these tumor cells were escapers (namely tumor cells retaining Yap expression), we performed immunohistochemistry with the anti-Yap antibody. Hepatocytes with no immuno-positivity for Yap expression were detectable as early as 7.7 weeks post-injection in *AKT/Fbxw7*ΔF/Cre livers. Importantly, all *AKT/Fbxw7*ΔF/*Cre* tumor cells were negative for Yap immunoreactivity, indicating effective *Yap* deletion (Fig. 3C and Fig. S12). Robust Yap immunolabeling was instead detected in tumor stromal cells, which displayed Vimentin immunoreactivity (Fig. S12), and in surrounding non-tumorous hepatocytes. The observation was corroborated by western blotting showing low Yap expression (Fig. 3D), and by qRT-PCR demonstrating decreased levels of *Ctgf* and *Cry61* genes in *AKT/Fbxw7*ΔF/*Cre* tumors (Fig. 3E). Noticeably, phosphorylated/activated (p-)Akt was remarkably decreased following *Yap* ablation, together with profound downregulation of *Jag1*, a Yap target.[38] Interestingly, Notch2 levels were only mildly reduced, while c-Myc expression was decreased, in *AKT/Fbxw7*ΔF/*Cre* samples.

Altogether, these data indicate that *Yap* ablation significantly slows, but does not suppress, *AKT/Fbxw7*ΔF induced tumorigenesis in mice.

Notch2 activation is partially required for *AKT/Fbxw7*ΔF induced cholangiocarcinogenesis

We previously discovered that Notch2, instead of Notch1, is the main contributor to hepatocyte-derived iCCA formation.[39] As the present data indicate elevated expression of Notch2 and its ligand Jag1 in *AKT/Fbxw7*ΔF iCCA lesions, we investigated whether Notch2 signaling is required for *AKT/Fbxw7*ΔF induced cholangiocarcinogenesis. Thus, we hydrodynamically injected *AKT/Fbxw7*ΔF/pCMV and *AKT/Fbxw7*ΔF/*Cre* plasmids in *Notch2*$^{flox/flox}$ conditional knockout mice (Fig. 4A). Although deletion of *Notch2* delayed the fatal tumor development by 4–5 weeks (Fig. 4B), both cohorts, namely mice injected with *AKT/Fbxw7*ΔF/pCMV and *AKT/Fbxw7*ΔF/*Cre*, developed large tumors with positive Ck19 staining (Fig. 4B and 4C). As described in the AKT/Yap iCCA model,[39] ablation of *Notch2* significantly disrupted the biliary properties of tumor lesions, as indicated by the predominance of large clear-cell hepatocellular tumors over cholangiocellular lesions, and lower Ck19 and higher Hnf-4α immunoreactivity (Fig. 4C and 4D; Fig. S13). Interestingly, a trend of replacement of HCC lesions with cholangiocellular lesions was also observed in later stages of tumor development in this model (Fig. S13). Western blotting confirmed the efficient deletion of *Notch2*, which was paralleled by decreased *Jag1* and *Sox9* expression (Fig. 4E). Yap, the oncogenic effector of the Hippo pathway, was downregulated following *Notch2* ablation (Fig. 4E), confirming the positive signal loop between Yap and Notch pathways.[38] Intriguingly, c-Myc expression was not affected by *Notch2* ablation.

Overall, the present data imply a relevant but dispensable role of Notch2 in *AKT/Fbxw7*ΔF dependent cholangiocarcinogenesis.

c-Myc is necessary for cholangiocarcinogenesis in *AKT/Fbxw7*ΔF mice

Subsequently, we investigated the role of c-Myc, another important target of FBXW7,[11] in *AKT/Fbxw7*ΔF mice. Thus, *MadMyc*, a dominant negative form of c-Myc,[40] and the *AKT/Fbxw7*ΔF plasmids, were simultaneously hydrodynamically delivered into the mouse liver and tumor development was monitored. Hydrodynamic injection of *AKT/Fbxw7*ΔF/pT3 construct was used as control (Fig. 5A). Strikingly, inhibition of c-Myc completely suppressed iCCA formation in *AKT/Fbxw7*ΔF mice (Fig. 5B and 5C). Indeed, mice were healthy with no gross liver tumors by 31 weeks post-injection. Consistently, histopathological evaluation revealed the absence of premalignant and malignant lesions in *AKT/Fbxw7*ΔF/*MadMyc* livers (Fig. 5C). Importantly, sporadic HA-positive cells were detected in *AKT/Fbxw7*ΔF/*MadMyc* livers (Fig. S14A), and Western blotting revealed the ectopically expressed HA-tagged AKT, Fbxw7ΔF and MadMyc, leading to the inhibition of c-Myc expression (Fig. S14B). In striking contrast, numerous highly proliferative iCCAs emerged in the liver parenchyma of *AKT/Fbxw7*ΔF/pT3 mice, with strong immunoreactivity for the biliary marker Ck19 and the proliferation marker Ki67 (Fig. 5C). All *AKT/Fbxw7*ΔF/pT3 mice had to be euthanized by 10–12 weeks post-injection (Fig. 5B).

c-MYC is a pivotal player downstream of FBXW7 in human iCCA

Next, we investigated the potential targets of FBXW7 in human iCCA. We transfected RBE, KMCH, and SNU1196 CCA cell lines with lentivirus particles encoding *EGFP* (as control), *FBXW7* (wild-type), and *FBXW7*ΔF, and the expression of known canonical FBXW7 targets was analyzed. Protein levels of CCNA, CCND1, CCNE, GSK3β, HSF1, MCL-1, mTOR, NOTCH1, NOTCH2, PCNA, RICTOR and YAP did not show any consistent change following FBXW7 modulation (Fig. 6). Additional proteins involved in tumor development, including p-AKT, p-ERK, p21, etc. were also analyzed, and their levels were not consistently affected by the same approach in the 3 cell lines (Fig. S15). In striking contrast, c-MYC was consistently downregulated following FBXW7 wild-type overexpression and strongly induced by FBXW7 inhibition (via *FBXW7*ΔF transfection) in the 3 cell lines (Fig. 6). Accordingly, transcriptional activity of c-MYC exhibited the same pattern of modulation following transient transfection of control, *FBXW7,* and *FBXW7ΔF* plasmids in RBE cells and KMCH cells (Fig. S16).

Finally, we investigated the relationship between FBXW7 and c-MYC in the collection of human iCCA specimens (n = 82). No significant correlation was detected between *FBXW7* and *c-MYC* mRNA levels (Fig. 7A), whereas a strong, inverse correlation was found between *FBXW7* mRNA levels and those of c-MYC transcriptional activity (Fig. 7B). Subsequently, we determined the protein levels of FBXW7 and c-MYC in the same sample collection using immunohistochemistry (Fig. 8). Upregulation of FBXW7 and c-MYC in iCCA when compared with non-tumorous surrounding livers occurred in 14 and 44 of 82 specimens (17.1% and 53.6%, respectively). FBXW7 and c-MYC were concomitantly upregulated in only 4 of 82 samples (4.9%). The remaining 10 iCCA specimens showing elevated levels of FBXW7 displayed c-MYC downregulation. On the other hand, 39 of 44 (88.6%) iCCA specimens showing high levels of c-MYC exhibited absent/weak immunoreactivity for FBXW7.

Altogether, these findings support a central role played by c-MYC both in mouse and human cholangiocarcinogenesis driven by FBXW7 inactivation.

Discussion

Here, we demonstrated that inactivation of Fbxw7 synergizes with activated AKT to induce hepatocyte-derived iCCA formation in mice. By conducting loss of function experiments using

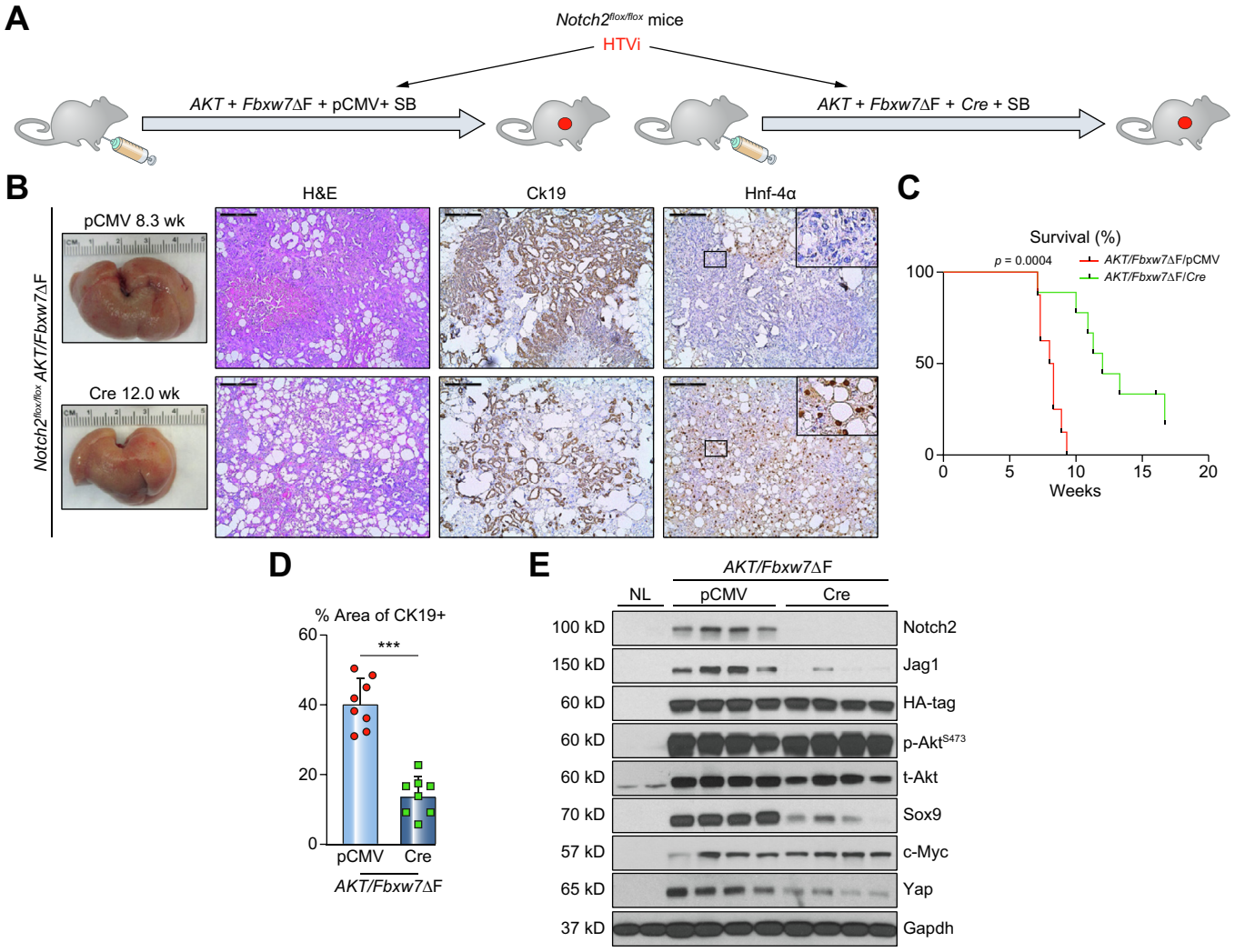

Fig. 4. Deletion of *Notch2* slows, without impairing, cholangiocarcinogenesis in *AKT/Fbxw7*ΔF mice. (A) Study design: *Notch2*$^{lox/flox}$ conditional knockout mice were subjected to HTVi of either *AKT/Fbxw7*ΔF/pCMV (control, n = 8) or *AKT/Fbxw7*ΔF/Cre (n = 10) plasmids. (B) Delay of tumor development in *AKT/Fbxw7*ΔF mice, as revealed by macroscopic examination of the livers and histopathology of the lesions, is accompanied by reduction of the Ck19 biliary marker and increase of the Hnf-4α hepatocellular marker. (C) Survival curve showing the delay of intrahepatic cholangiocarcinoma development following deletion of Notch2. (D) Analysis of Ck19-positive areas in control (pCMV) and *Notch2*-depleted (Cre) livers. Data was analyzed by Mann-Whitney test. (E) Western blotting of *AKT/Fbxw7*ΔF livers from control and *Notch2* deleted mice. Scale bar: 200 μm. ***$p < 0.001$. HTVi, hydrodynamic tail vein injection; NL, normal liver.

Yap$^{flox/flox}$ and *Notch2*$^{flox/flox}$ conditional knockout mice, we discovered that inhibition of each of the 2 oncogenic pathways delays, but does not suppress, *AKT/Fbxw7*ΔF-induced iCCA development. In particular, histopathological analysis revealed that hepatocellular lesions appear first in all models. Subsequently, cholangiocellular lesions emerge, co-existing with the hepatocellular ones. During tumor progression, the cholangiocellular lesions replace either completely (in the *AKT/Fbxw7*ΔF model) or partly (in *AKT/Fbxw7*ΔF depleted of Yap or Notch2) the hepatocellular lesions. These findings suggest that additional alterations are needed for cholangiocellular lesions to develop in these models, but, once these alterations are acquired, cholangiocellular lesions seem to have a growth advantage over hepatocellular lesions.

By employing a comprehensive analysis of *in vitro* and *in vivo* approaches as well as validation in human iCCA specimens, we identified c-Myc as a crucial mediator of cholangiocarcinogenesis following downregulation of FBXW7. Although Yap and Notch are considered to be canonical Fbxw7 targets,[13,14] our *in vitro* studies demonstrates that neither YAP nor NOTCH2 levels (nor related signaling cascades) are consistently influenced by FBXW7-mediated degradation in human iCCA cells. Similarly, several other FBXW7 targets, including mTOR, CCND1, and CCNE, were heterogeneously modulated following FBXW7 manipulation. The discrepancy between our findings and the literature requires further elucidation. Presumably, the recognition substrates for FBXW7 differ depending on the context and the tissue type as well as the genetic background/alterations present. This might also explain the reason why, besides c-MYC, most FBXW7 target proteins were heterogeneously affected by FBXW7 modulation in distinct CCA cell lines. Similarly, we found that c-MYC activation was AKT-independent both *in vitro* and *in vivo* models of iCCA, against the assumption that c-Myc is an AKT target.[35] Once again, tissue- and context-dependent mechanisms might be responsible for these unexpected results.

c-MYC is a known substrate for FBXW7.[11,41,42] Alterations of FBXW7 leading to c-MYC accumulation have been detected in

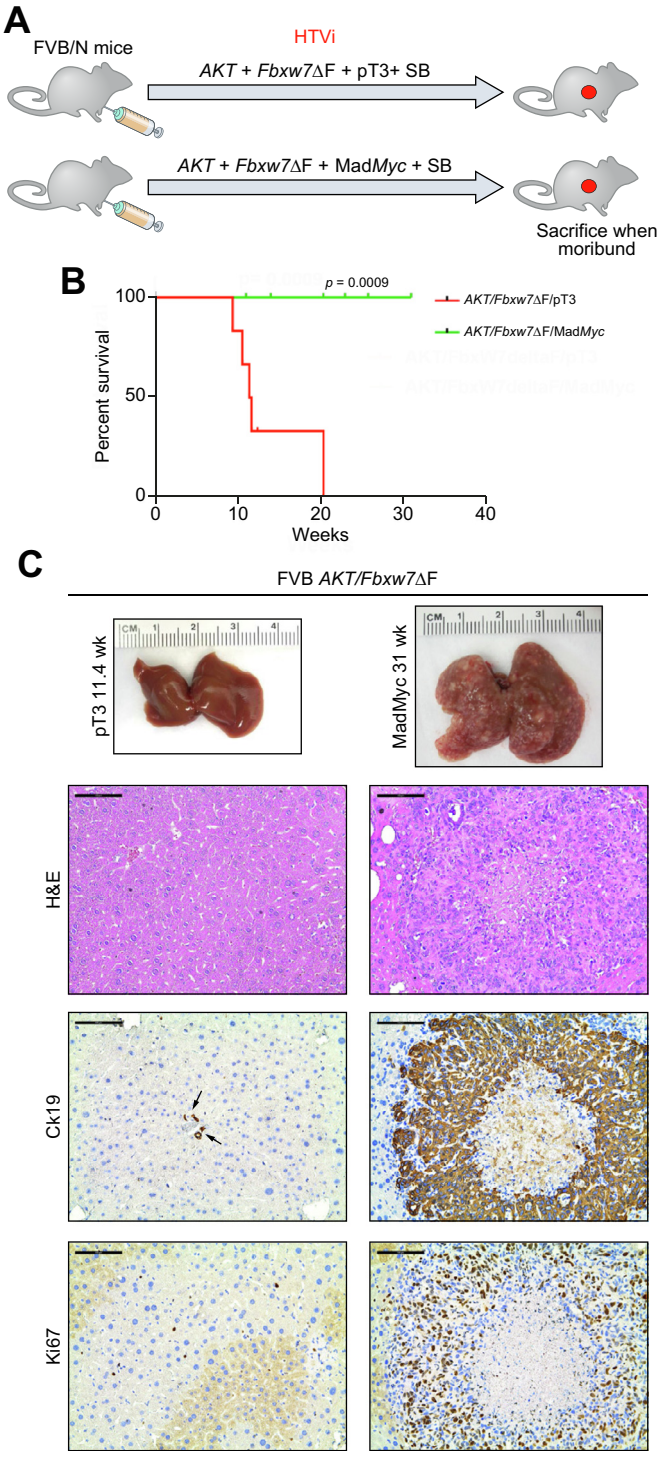

Fig. 5. Inactivation of c-Myc completely abolishes cholangiocarcinogenesis in *AKT/Fbxw7*ΔF mice. (A) Study design: FVB/N mice were subjected to HTVi of either *AKT*/*Fbxw7*ΔF/pT3 (control, n = 6) or *AKT*/*Fbxw7*ΔF/*MadMyc* (n = 8) plasmids. *MadMyc* is a dominant negative form of c-Myc. (B) Survival curve of *AKT*/*Fbxw7*ΔF/pT3 and *AKT*/*Fbxw7*ΔF/*MadMyc* mice. (C) Inhibition of tumor development in *AKT*/*Fbxw7*ΔF/*MadMyc* mice, as revealed by macroscopic examination of the livers and histopathology of the lesions, is accompanied by immunoreactivity for the Ck19 biliary marker limited to normal biliary epithelial cells (indicated by arrows) and low/absent immunoreactivity for the proliferation marker Ki67. Scale bar: 100 μm. HTVi, hydrodynamic tail vein injection.

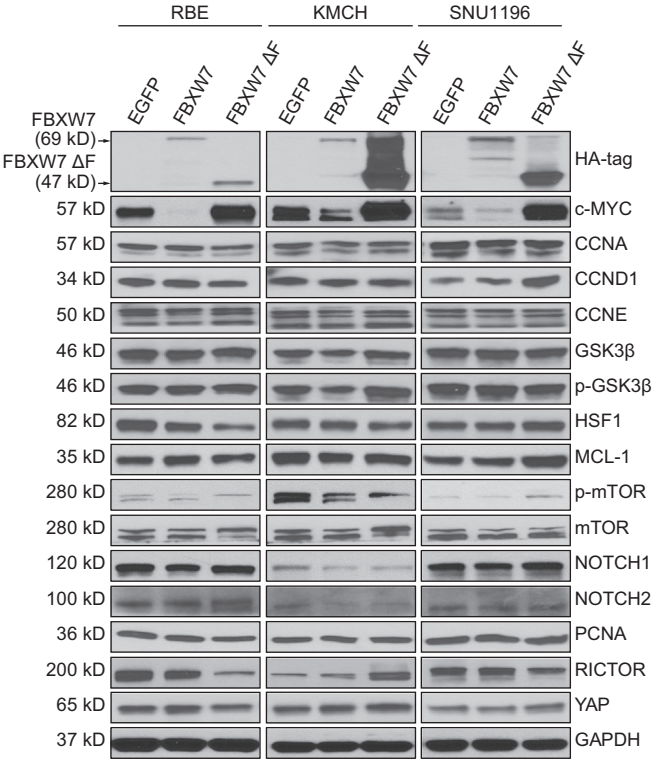

Fig. 6. Western blot analysis showing c-MYC as a consistent target of FBXW7 in cholangiocarcinoma cell lines. GAPDH was used as a loading control.

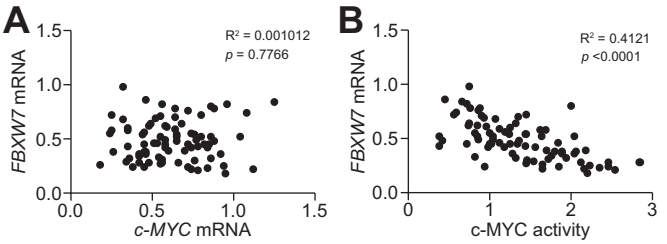

Fig. 7. Expression levels of the *FBXW7* gene are inversely correlated with c-MYC activity in human intrahepatic cholangiocarcinoma. (A) Absence of significant correlation between *FBXW7* and *c-MYC* mRNA levels, as assessed by linear regression analysis. (B) A significant, negative correlation was found between mRNA levels of *FBXW7* and c-MYC activity using the same statistical approach.

T-cell acute lymphoblastic leukemia (T-ALL),[42] melanoma,[15] skin carcinogenesis,[43] prostate cancer,[44] and HCC.[45] In CCA, one group reported that overexpression of FBXW7 in QBC-939 and MZ-CHA1 CCA cell lines decreased the expression of c-MYC.[11] The study also showed that FBXW7 overexpression inhibits xenograft CCA tumor growth in nude mice.[11] In our study, c-MYC levels were profoundly downregulated by FBXW7 overexpression in 3 human CCA cell lines. Conversely, when transfecting a dominant negative form of FBXW7, c-MYC expression was strongly upregulated in the same cells. Based on our analysis of various genes induced by c-Myc in mouse iCCA tissues, c-Myc most likely regulates multiple processes during cholangiocarcinogenesis, including tumor cell proliferation, survival and metabolism. Most importantly, we found that impairing c-Myc activity triggers inhibition of cholangiocarcinogenesis induced by inactivation of FBXW7 along with AKT activation *in vivo*. The results were validated *in vitro* using AML-12 normal mouse hepatocytes (Fig. S10A), indicating that c-Myc is required for iCCA initiation. To investigate the role of c-Myc in

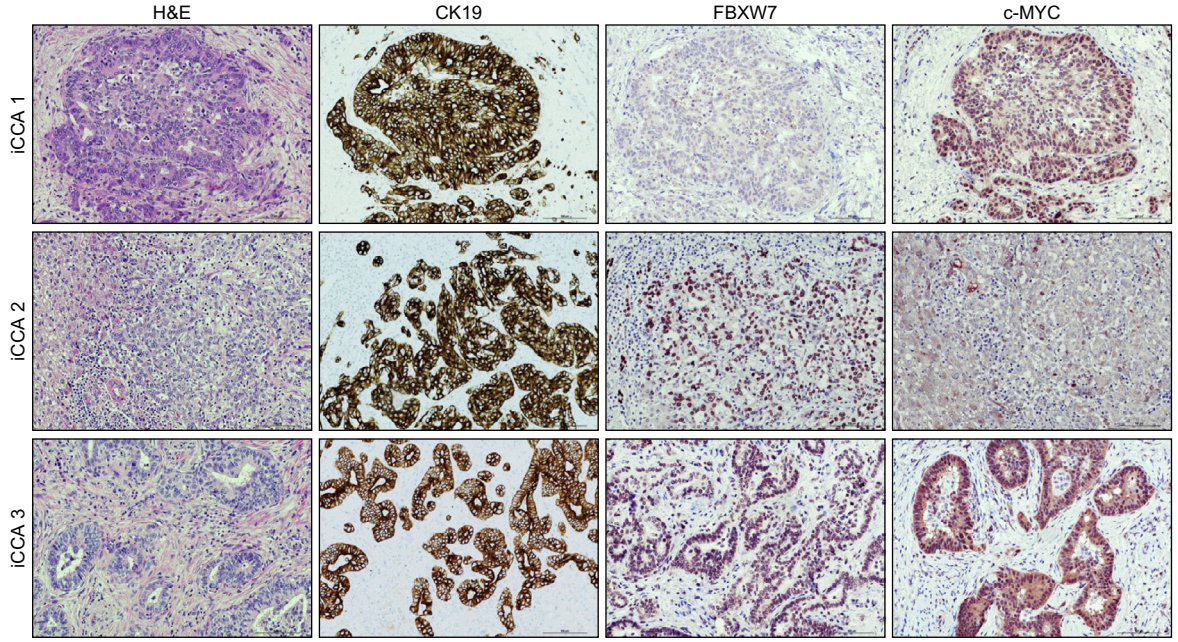

Fig. 8. Immunohistochemical patterns of FBXW7 and c-Myc in human intrahepatic cholangiocarcinoma specimens. Three representative cases (iCCA 1-3) are shown. Specifically, iCCA 1 (upper panel) recapitulates the most frequent pattern observed in the human iCCA collection, consisting of tumors with low levels of FBXW7 and strong immunoreactivity for c-MYC. iCCA 2 (middle panel) shows instead the example of an iCCA with high immunoreactivity for FBXW7 and weak/punctate staining of c-MYC protein. Finally, iCCA 3 (lower panel), consisting of a tumor displaying concomitant, elevated immunolabeling for both FBXW7 and c-MYC. CK19 staining was used as a biliary differentiation marker of the tumors. Scale bar: 100 μm. iCCA, intrahepatic cholangiocarcinoma.

iCCA progression, we infected the 3 CCA cell lines with tetracycline (Tet) inducible Mad*Myc* lentivirus. Upon Doxycycline treatment, Mad*Myc* expression was induced and inhibited CCA cell proliferation and colony formation (Fig. S17). Thus, c-MYC is a pivotal player in iCCA initiation and progression.

Another important finding of our study is that, although FBXW7 was almost ubiquitously downregulated in iCCA specimens, mutations in its coding region as well as LOH at the gene locus were rare, and no hypermethylation at the FBXW7 locus was detected. Thus, other molecular mechanisms are involved in the downregulation of FBXW7 in human iCCA. Previous studies have suggested that long non-coding RNAs and microRNAs as well as various oncogenes, such as Pin1, EZH2, CNS6, and Usp28 can downregulate FBXW7 in cancer.[10,46] Clearly, additional studies, which are beyond the scope of the present work, are required to identify the pivotal molecular mechanisms responsible for FBXW7 downregulation in human iCCAs. Nonetheless, additional events might limit FBXW7 activity in cholangiocellular tumors independent of *FBXW7* transcription. Indeed, we recently identified alternative splicing forms of FBXW7 in 27/82 (32.9%) iCCA, which were not detected in corresponding non-tumorous livers (Fig. S18). The presence of these splicing forms has been shown to result in very low translational efficiency of the FBXW7 protein in prostate, bladder, and kidney tumors.[47] Although this issue should be more comprehensively addressed, the present findings imply the existence of several, distinct mechanisms by which FBXW7 expression and/or activity are suppressed in human iCCA.

Finally, our data might possess relevant therapeutic implications. As loss of FBXW7 is extremely frequent in iCCA, targeting c-MYC might be highly detrimental for the growth of many of these tumors. Unfortunately, c-MYC is not easily druggable and no effective drugs against c-MYC are commercially available. Nonetheless, bromodomain and extra-terminal (BET) inhibitors recently provided encouraging results for the treatment of c-MYC driven tumors.[48,49] Thus, BET inhibitors (and similar small inhibitors) should be considered as potentially important drugs for innovative therapies against human iCCA.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

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

Authors' contributions

Jingxiao Wang, Haichuan Wang, Ning Ding, Meng Xu, Xinyan Chen, Xinhua Song, Michele Peters, Silvia Ribback, Kirsten Utpatel, and Antonio Cigliano acquired experimental data. Li Che, Frank Dombrowski, Matthias Evert, and Antonio Cossu provided administrative, technical, or material support. John Gordan assisted in study design and interpretation. Jingxiao Wang and

Haichuan Wang analyzed the data and drafted the manuscript. Yong Zeng, Xin Chen and Diego F. Calvisi were involved in study design, drafting of the manuscript, study supervision and obtaining funding.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2019.05.027.

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Author names in bold designate shared co-first authorship

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