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Hepatic and Biliary Cancer

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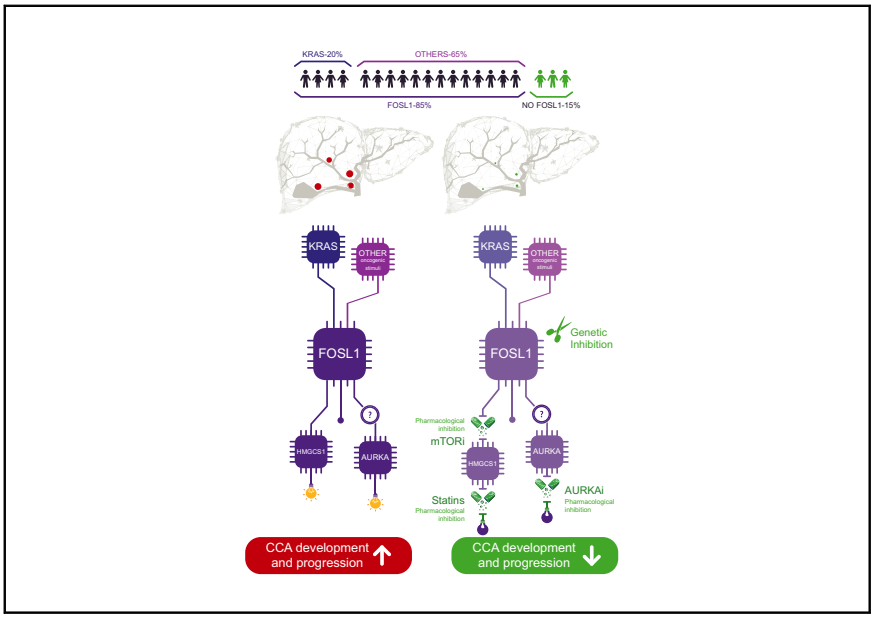
FOSL1 promotes cholangiocarcinoma via transcriptional effectors that could be therapeutically targeted

Graphical abstract



Highlights

- The transcription factor FOSL1 is upregulated in human and mouse CCA, and is independently associated with patient survival.
- Genetic FOSL1 inhibition impairs cell proliferation and cell cycle progression *in vitro*, and tumor initiation and maintenance *in vivo*.
- The mevalonate pathway gene *HMGCS1* is upregulated in human and mouse CCA, and its expression is controlled by direct FOSL1 promoter binding.
- Genetic *HMGCS1* abrogation or pharmacological blockade with mTOR inhibitors phenocopies loss of FOSL1.

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Lay summary

Understanding the molecular mechanisms involved in cholangiocarcinoma (bile duct cancer) development and progression stands as a critical step for the development of novel therapies. Through an inter-species approach, this study provides evidence of the clinical and functional role of the transcription factor FOSL1 in cholangiocarcinoma. Moreover, we report that downstream effectors of FOSL1 are susceptible to pharmacological inhibition, thus providing new opportunities for therapeutic intervention.

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FOSL1 promotes cholangiocarcinoma via transcriptional effectors that could be therapeutically targeted

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Background & Aims: Cholangiocarcinoma (CCA) is a neoplasia of the biliary tract driven by genetic, epigenetic and transcriptional mechanisms. Herein, we investigated the role of the transcription factor FOSL1, as well as its downstream transcriptional effectors, in the development and progression of CCA.

Methods: FOSL1 was investigated in human CCA clinical samples. Genetic inhibition of *FOSL1* in human and mouse CCA cell lines was performed in *in vitro* and *in vivo* models using constitutive and inducible short-hairpin RNAs. Conditional FOSL1 ablation was done using a genetically engineered mouse (GEM) model of CCA (mutant *KRAS* and *Trp53* knockout). Follow-up RNA and chromatin immunoprecipitation (ChIP) sequencing analyses were carried out and downstream targets were validated using genetic and pharmacological inhibition.

Results: An inter-species analysis of FOSL1 in CCA was conducted. First, FOSL1 was found to be highly upregulated in human and mouse CCA, and associated with poor patient survival. Pharmacological inhibition of different signalling pathways in CCA cells converged on the regulation of FOSL1 expression. Functional experiments showed that FOSL1 is required for cell proliferation and cell cycle progression *in vitro*, and for tumour growth and tumour maintenance in both orthotopic and subcutaneous xenograft models. Likewise, FOSL1 genetic abrogation in a GEM model of CCA extended mouse survival by decreasing the oncogenic potential of transformed cholangiocytes. RNA and ChIP sequencing studies identified direct and indirect transcriptional effectors such as HMGCS1 and AURKA, whose genetic and pharmacological inhibition phenocopied FOSL1 loss.

Conclusions: Our data illustrate the functional and clinical relevance of *FOSL1* in CCA and unveil potential targets amenable to pharmacological inhibition that could enable the implementation of novel therapeutic strategies.

Lay summary: Understanding the molecular mechanisms involved in cholangiocarcinoma (bile duct cancer) development and progression stands as a critical step for the development of novel therapies. Through an inter-species approach, this study provides evidence of the clinical and functional role of the transcription factor FOSL1 in cholangiocarcinoma. Moreover, we report that downstream effectors of FOSL1 are susceptible to pharmacological inhibition, thus providing new opportunities for therapeutic intervention.

Introduction

Human cholangiocarcinoma (CCA) includes a heterogeneous group of cancers of the biliary tract with dismal prognosis. The incidence of CCA is increasing globally, and currently represents ~3% of all gastro-intestinal tumours.[1] The asymptomatic nature of these tumours in early stages and their chemo-resistance lead to poor outcomes. Numerous studies have catalogued the genomic and molecular landscape of CCA in search of actionable molecular targets that hold promise for tailored treatments.[2–10] These studies unveiled *KRAS, BRAF, IDH1/2, FGFR2* or *EGFR* as

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prevalent and central oncogenic drivers in CCA. While *KRAS* is mutated across intrahepatic and extrahepatic CCA (iCCA and eCCA) subtypes,[8,11,12] *FGFR2* and *IDH1/2* genomic alterations are rarely detected in eCCA.[2–6,11,13–15] Targeted therapies to most of these oncogenes are currently undergoing evaluation in the clinic. Despite promising results with *IDH1/2* and *FGFR2* inhibitors, patients display modest responses, most likely due to intrinsic or adaptive resistance mechanisms that limit their antitumour effect.[16–20] Thus, a better knowledge of the underlying molecular mechanisms orchestrated by oncogenic drivers in CCA, especially those lacking therapeutic options such as *KRAS*, may facilitate the identification of novel interventional strategies.

Genomic alterations in CCA are associated with defined gene expression profiles. For instance, human CCAs harbouring *KRAS* mutations (8-47% of cases) feature a gene expression signature that is associated with poor prognosis.[7,10] These observations suggest that transcriptional nodes may play a functionally relevant role in CCA. A few transcription regulators, including *NOTCH* genes,[21,22] *YAP1*,[23] *FOXM1*,[24] *SOX9*,[24] *SOX17*[25] or *TCF7*[26] have been reported to function in CCA development and/or progression. Nonetheless, beyond NOTCH receptors, no targeted therapies are yet available against such transcriptional regulators. Given the dismal prognosis of patients with CCA, the identification of relevant transcription factors in the context of dominant oncogenes, as well as the characterization of their transcriptional network, remains a relevant task as this may unveil new tumour vulnerabilities amenable to pharmacological inhibition.

We previously described a cross-tumour signature upregulated in CCA, as well as in lung and pancreas cancer, whose high expression was a marker of poor survival.[27] In addition to other genes involved in CCA, such as *AREG* and *LAMC2*,[28,29] the cross-tumour signature included the transcription factor *FOSL1*, a member of the AP1 complex that hetero-dimerizes with members of the JUN family for proficient transcriptional activity.[30] *FOSL1* mRNA and protein expression is upregulated in a wide variety of tumour types, where it plays an oncogenic role by modulating various cellular processes such as proliferation, differentiation, invasion, epithelial-mesenchymal transition and/or drug resistance.[31,32] In the context of dominant oncogenes, *FOSL1* represents a vulnerability in lung and pancreatic cancer driven by oncogenic *KRAS*.[27,33–35] Nonetheless, data regarding FOSL1's role in cholangiocarcinogenesis is far from conclusive. On the one hand, early studies reported that *Fosl1* expression in transgenic mice triggers ductular proliferation and infiltration of inflammatory cells, leading to biliary fibrosis and suggesting a pro-oncogenic function.[36] On the other hand, a recent integrative analysis of human CCA data sets predicted *FOSL1* downregulation in neoplastic tissue, postulating a tumour suppressor role.[37] In light of these findings, additional studies to resolve the function of *FOSL1* in CCA are required.

Using a multimodal inter-species approach combining clinical data, human and mouse CCA cell lines, and genetically engineered mouse (GEM) models, we found that *FOSL1* is a relevant transcription factor in CCA that is in part positively regulated by *KRAS*. Moreover, molecular analysis of *FOSL1* downstream effectors using RNA and chromatin immunoprecipitation (ChIP) sequencing analysis, as well as genetic and pharmacological inhibitory strategies, unveiled direct and indirect transcriptional targets that represent actionable elements for the implementation of innovative therapeutic strategies.

Materials and methods

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

Results

FOSL1 is upregulated in cholangiocarcinoma and correlates with poor survival

To address the role of FOSL1 in CCA, we first performed immunohistochemistry (IHC) analyses in a cohort of 209 patients with surgically resected iCCA (median resection age: 67; sex: 93 female, 116 male; stage: 176 early, 24 late, 9 unknown). Only 13 patients (6.22%) received neoadjuvant therapy prior to surgery (Table S1). A uniform nuclear FOSL1 staining was observed in the tumorous tissue (>90% of cells stained positive) of most patients (n = 177, 84.689%) while no expression was found in non-tumorous liver from adjacent normal tissue and in normal bile ducts (Fig. 1A and Fig. S1A). In addition, FOSL1 positive staining was observed in 18 of 21 preinvasive lesions, including 11 intraductal papillary biliary neoplasms (IPBN) and 7 biliary epithelial neoplasias (not shown) (Fig. S1B).

Patients with CCA and FOSL1 expression had shorter overall survival (p = 0.0371) (Fig. 1B). Given the adverse effect of *KRAS* and *BRAF* mutations on overall CCA survival,[7,10] patients were stratified according to their mutational status. Patients carrying CCA tumours with combined FOSL1 expression and mutations in *KRAS* or *BRAF* had the lowest survival outcome (p = 0.0017) (Fig. 1C). Furthermore, direct comparison of FOSL1-expressing CCAs with and without *KRAS* or *BRAF* mutations revealed a significant survival difference (p = 0.019) (Fig. 1C). Multivariate analysis showed that FOSL1 expression is independently associated with survival (LogTest p value: 0.006; FOSL1 p value: 0.046; TNM p value: 0.023; KRAS/BRAF mut p value: 0.002; sex p value: 0.366; age p value: 0.777).

Next, we investigated FOSL1 expression in a panel of CCA cell lines with distinct mutational background as well as in normal primary cultures and SV-40 immortalized human cholangiocytes. FOSL1 upregulation was generally observed in CCA cells compared to non-tumourous cholangiocytes, as revealed by different bands representative of various human FOSL1 isoforms (Fig. 1D), suggesting regulation by cell-autonomous mechanisms.

Then, we studied FOSL1 expression in a GEM model of iCCA driven by liver-specific *Kras* mutations and *Trp53* deletion (*Albumin*$^{Cre/+}$; *Kras*$^{LSL\text{-}G12D/+}$; *Trp53*$^{f/f}$, hereafter referred to as AKP).[38] This model recapitulates histologic and molecular features of multistage progression of human iCCA, including the biliary typical lesion IPBN. IHC analysis of adjacent normal tissue showed a lack of FOSL1 expression in normal hepatocytes and cholangiocytes (Fig. 1E). Conversely, CCA lesions, featuring a prominent stroma and Ck19 expression, exhibited pronounced nuclear FOSL1 expression in tumour cells (Fig. 1E). FOSL1 expression was also found in mouse IPBN (Fig. S1C). Differentiated CCA displayed heterogenous FOSL1 expression (n = 6) while a more uniform and intense pattern was found in poorly differentiated CCA (n = 6) (Fig. S1D and E).

In agreement with the human data, FOSL1 expression was preferentially observed in cancer cell lines derived from AKP mice compared to normal mouse cholangiocytes (Fig. 1F), although only 1 band was observed in mouse lysates. FOSL1

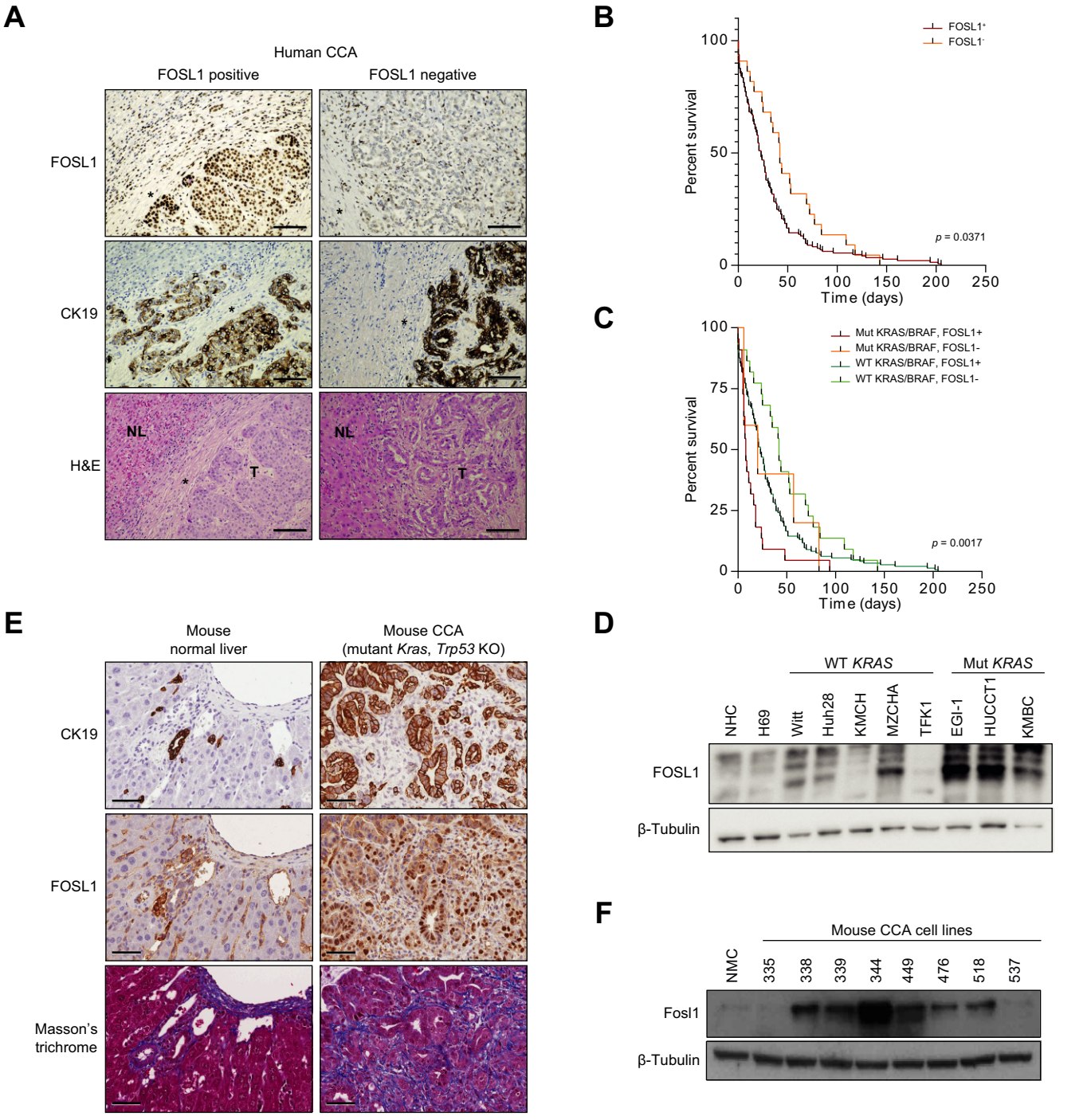

Fig. 1. FOSL1 expression in cholangiocarcinoma. (A) Representative images of human CCA stained with indicated antibodies or H&E. Scale bars are 100 μm. NL: Normal liver; T: Tumour; Asterisk: Fibrotic tissue. (B) Survival analysis of patients with iCCA stratified by FOSL1 expression (Log-rank test). (C) Survival analysis of patients with iCCA based on FOSL1 expression and *KRAS* or *BRAF* mutations (Log-rank test). (D) FOSL1 immunoblot in non-tumourous cells and human CCA cell lines. (E) Representative images of mouse normal liver and CCA from *Albumin*$^{Cre/+}$; *Kras*$^{LSL-G12D/+}$; *Trp53*$^{f/f}$ (AKP) mice stained with indicated antibodies or Masson's trichrome. Scale bars are 60 μm. (F) Fosl1 immunoblot in NMC and iCCA cell lines derived from AKP mice. CCA, cholangiocarcinoma; iCCA, intrahepatic cholangiocarcinoma; KO, knockout; NHC, normal human cholangiocyte; NMC, normal mouse cholangiocyte.

protein expression in mouse CCA cell lines was consistent but variable among tumor cell lines with regard to normal mouse cholangiocytes. This is in accordance with the heterogeneous FOSL1 expression reported in cell lines derived from other epithelial tumors driven by oncogenic Kras and p53 loss.[27] The variability in FOSL1 expression may be explained in part by potential copy gain of the mutant *Kras* allele[39,40] or by a different chromosomic instability pattern elicited upon heterozygous or homozygous inactivation of p53 in mouse CCA cells.[38] Collectively, the human and mouse observations suggest that FOSL1

expression is intimately associated with CCA and has implications for patient prognosis.

FOSL1 is regulated by multiple signaling pathways in CCA

The results in human CCA suggested that different oncogenic signals regulate FOSL1 expression. We carried out pharmacological inhibition of several signaling pathways involved in CCA[41] to dissect their potential involvement in FOSL1 regulation. In eCCA (EGI-1) and iCCA (HUCCT1) cell lines, various signaling pathways including MEK1/2, MEK5, JNK1/2, PI3K and IKK regulated FOSL1 expression (Fig. 2A). Of note, FOSL1 downregulation upon inhibition of MEK1/2, MEK5 and PI3K was also seen in a mouse CCA cell line (339) directly derived from the AKP GEM model (Fig. 2B).

Based on the clinical data suggesting an association between *FOSL1* and *KRAS*, gain- and loss-of-function experiments were carried out to test their potential relationship. Overexpression of exogenous mutant *KRAS* in immortalized cholangiocytes led to upregulation of FOSL1 (Fig. 2C). Conversely, *KRAS* knockdown in eCCA and iCCA cell lines decreased FOSL1 expression (Fig. 2D). To test if *FOSL1* regulation by *KRAS* extended to the mouse setting, we deployed mouse cholangiocytes isolated from *Kras*$^{LSL\text{-}G12D/+}$ (K) and *Kras*$^{LSL\text{-}G12D/+}$; *Trp53*$^{f/f}$ (KP) mice, following previously described protocols.[42,43] In these cholangiocytes, *Kras* is regulated through its endogenous promoter and its activation, along with *Trp53* deletion, is achieved by exogenous administration of adenoviral Cre recombinase (adCre) (Fig. S2A). Upon adCre administration, *Fosl1* mRNA and protein expression increased in K and KP cholangiocytes compared to wild-type counterparts (Fig. 2E), indicating that *Fosl1* expression is under the control of the *Kras* oncogene. FOSL1 protein was also slightly upregulated in *Trp53* knockout cholangiocytes compared to wild-type ones, suggesting that *Trp53* also regulates FOSL1 expression (Fig. S2B). Interestingly, expression of mutant *Kras* alone or in the presence of concomitant *Trp53* deletion increased the proliferative and clonogenic potential of mouse cholangiocytes (Fig. S2C). These results indicate that *KRAS* mutations contribute to *FOSL1* regulation and highlight the oncogenic role of *Kras* in cholangiocarcinogenesis.

CCA cell lines are sensitive to *FOSL1* silencing *in vitro*

To determine the functional relevance of *FOSL1* in human CCA, its expression was depleted in eCCA and iCCA cell lines by tetracycline-inducible short-hairpin RNAs (shRNAs). Genetic

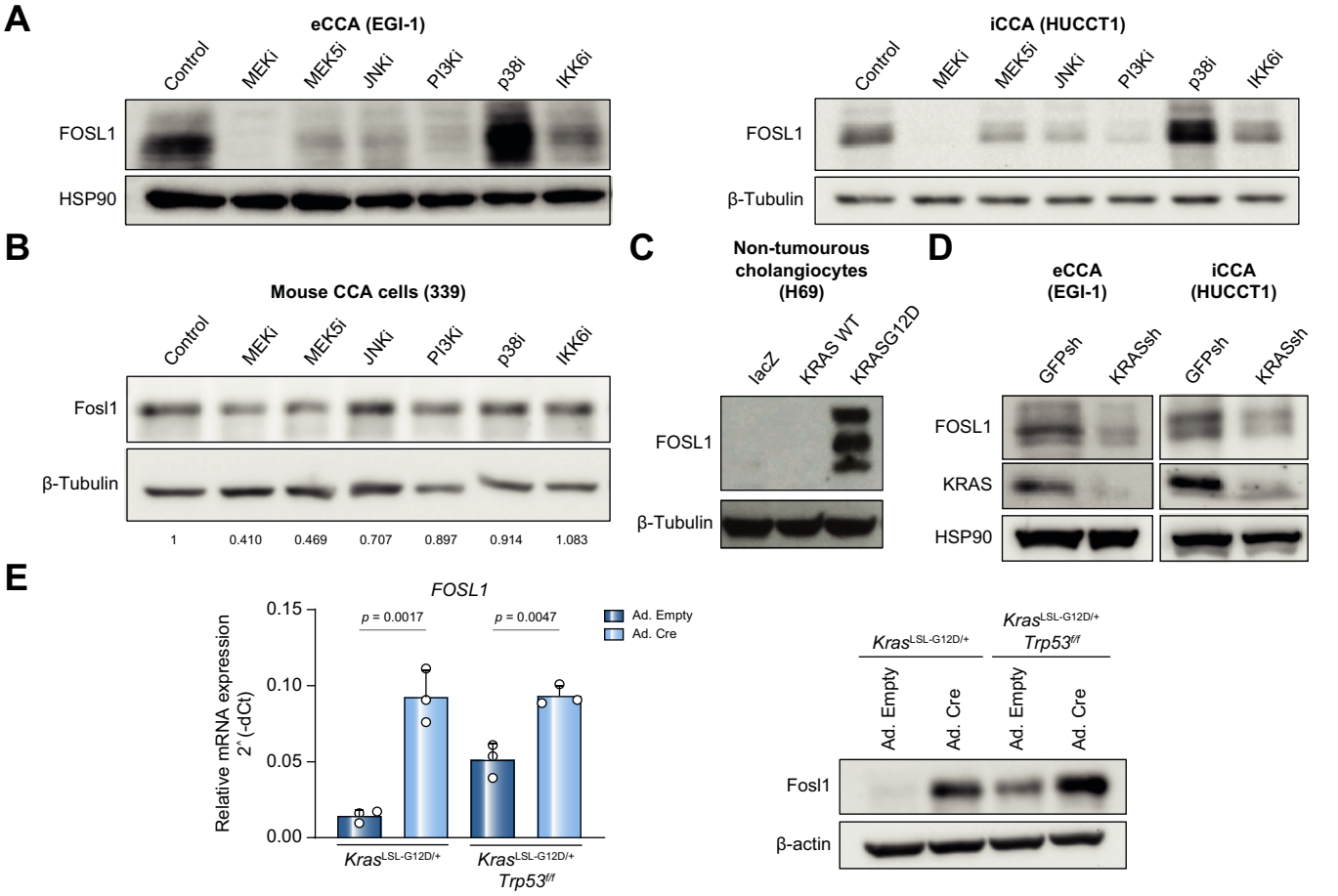

Fig. 2. FOSL1 expression is regulated by various signalling pathways. (A) FOSL1 immunoblot in eCCA and iCCA cell lines incubated with MEKi (0.5 μmol/L, MEK5i (10 μmol/L), JNKi (10 μmol/L), PI3Ki (0.1 μmol/L), p38i (10 μmol/L) and IKKi (1 μmol/L) for 20 h. (B) Fosl1 immunoblot and corresponding quantification in mouse CCA cells (339) treated as in A. (C) FOSL1 immunoblot in WT- and oncogenic *KRAS* (G12D)-overexpressing H69 cholangiocytes compared to lacZ-expressing (control) cells. (D) KRAS and FOSL1 immunoblot in eCCA and iCCA cell lines after KRAS shRNA-mediated knockdown compared to control cells (E) Fosl1 mRNA and protein expression in cholangiocytes from *Kras*$^{LSL\text{-}G12D/+}$ (K) and *Kras*$^{LSL\text{-}G12D/+}$; *Trp53*$^{f/f}$ (KP) mice treated with adCre or adEmpty (t-test). All data are mean ± SD. AdCre, adenoviral Cre recombinase; eCCA, extrahepatic cholangiocarcinoma; iCCA, intrahepatic cholangiocarcinoma; shRNA, short-hairpin RNA; WT, wild-type.

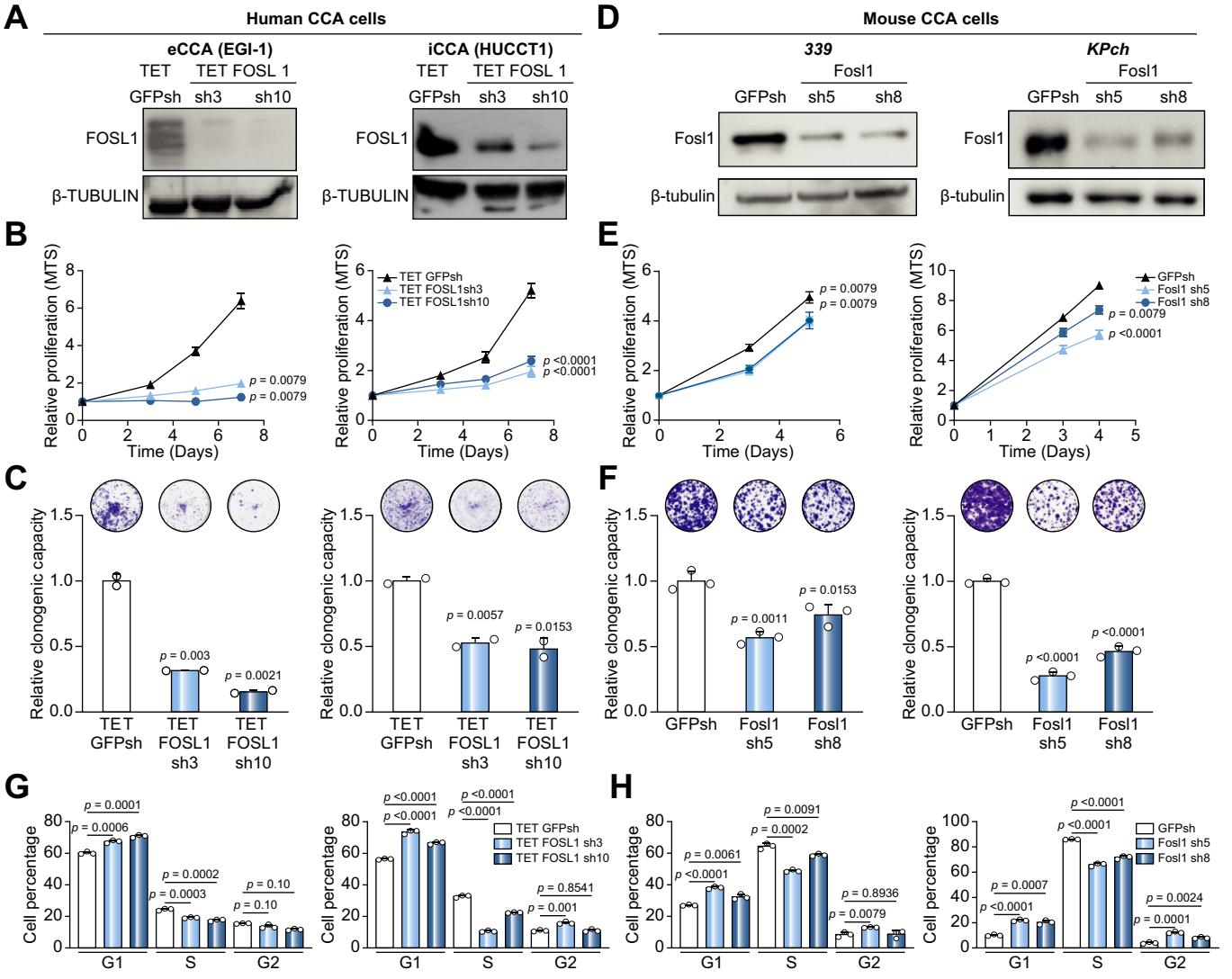

Fig. 3. CCA cell lines are sensitive to FOSL1 inhibition *in vitro*. (A) FOSL1 immunoblot in human eCCA and iCCA cell lines expressing FOSL1 shRNAs. (B) Cell proliferation assay by MTS in the same cells as in A (Mann Whitney or *t* test). (C) Representative images and quantification of colony forming ability in the same cells as in A (*t* test). (D) FOSL1 immunoblot in mouse CCA (339 and KPch) cell lines. (E) Cell proliferation assay by MTS in the same cells as in D (Mann Whitney or *t* test). (F) Representative images and quantification of colony forming ability in the same cells as in D (*t* test). (G-H) Cell cycle analysis 24 h after seeding of human (G) and mouse (H) cell lines. Error bars correspond to mean ± S.D (*t* test or Mann-Whitney). The assays are representative of at least 3 independent experiments. eCCA, extrahepatic cholangiocarcinoma; iCCA, intrahepatic cholangiocarcinoma; shRNA, short-hairpin RNA.

inhibition of *FOSL1* was achieved by 4 days of incubation with the tetracycline analogue doxycycline (Fig. 3A), when the different functional assays were seeded. First, *FOSL1* inhibition decreased cell proliferation of CCA cells (Fig. 3B). Additionally, *FOSL1* knockdown impaired the colony forming ability of CCA cell lines (Fig. 3C). *Fosl1* function was also investigated in mouse CCA using 339 cells and an additional cell line, KPch, isolated from tumours generated by subcutaneous injection of adCre-treated KP mouse cholangiocytes. Both cell lines expressed Ck19, a marker of cholangiocyte lineage (Fig. S3A). In line with the human data, *Fosl1* depletion by 2 constitutive shRNAs led to a reduction in the proliferative and colony formation potential of both mouse CCA cell lines (Fig. 3D-F).

At the cellular level, *FOSL1* knockdown robustly impaired the cell cycle in both human and mouse CCA cells by inducing a G1 phase arrest and, consequently, a reduction in the percentage of cells progressing to S phase (Fig. 3G and H). By contrast, no consistent differences regarding apoptosis were observed (Fig. S3B and C). The observed G1 phase arrest was related to an increase in the percentage of senescent cells. This result suggests that FOSL1 expression favours senescence escape and proliferation in CCA cell lines (Fig. S3D).

CCA growth and maintenance is compromised by *FOSL1* inhibition *in vivo*

Next, we asked whether *FOSL1* would be required for CCA growth *in vivo*. To do this, human eCCA and iCCA cell lines expressing the same inducible shRNAs targeting *FOSL1* were used. Immune-deficient mice were inoculated with these cells before being given 2 mg/ml of doxycycline from day 0 post-injection until the end of the experiment. In both cell-derived xenograft (CDX) models, *FOSL1*-silenced cell lines yielded

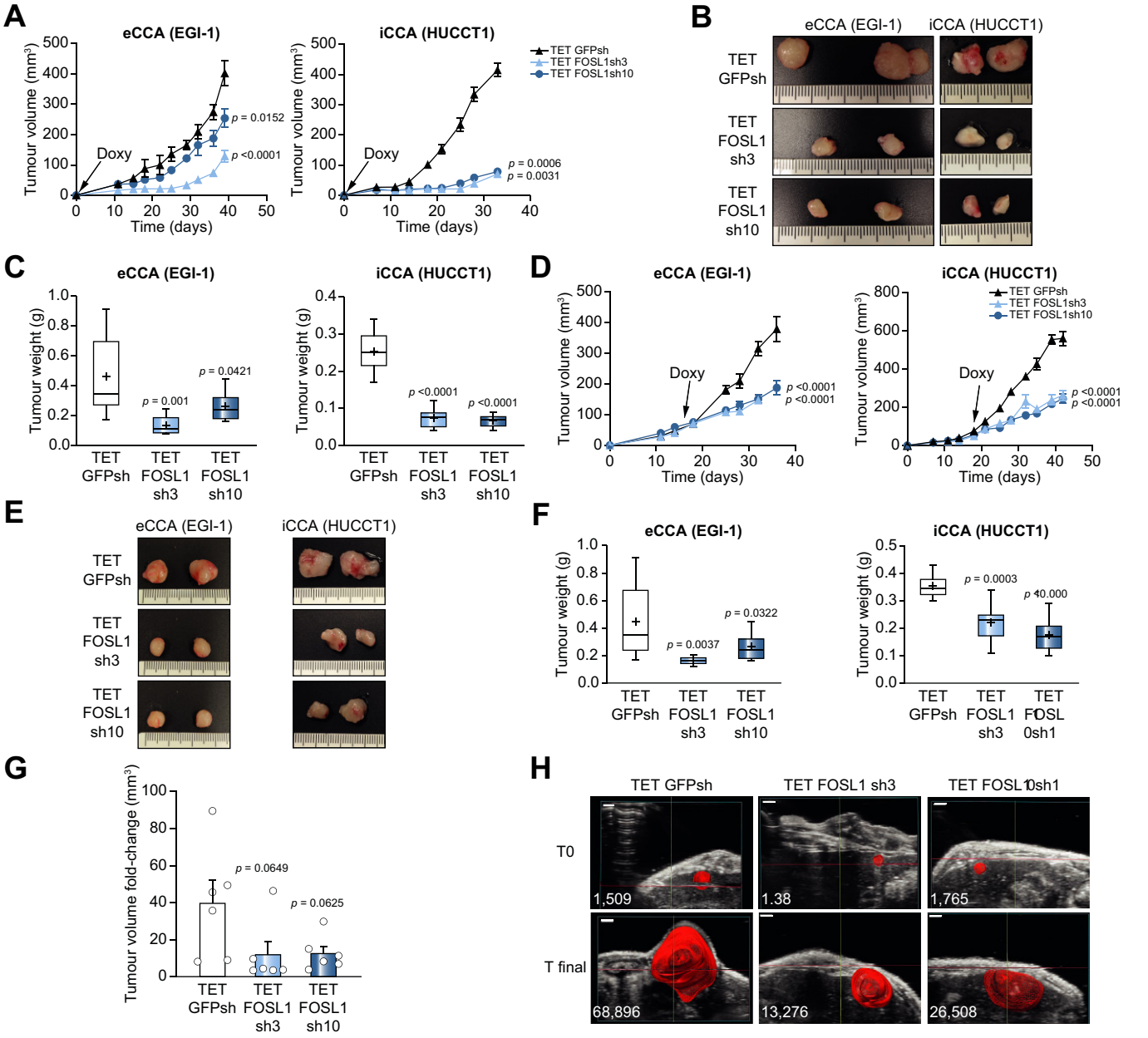

Fig. 4. *In vivo* FOSL1 inhibition impairs tumour formation and growth in CCA cell lines. (A) Growth kinetics of tumours from eCCA (EGI-1, n = 6-8) and iCCA (HUCCT1, n = 8) expressing 2 shRNAs against FOSL1 or a control shRNA. Doxy: 2 mg/ml doxycycline in drinking water containing 5% sucrose (Dunnett's or Dunn's multiple comparison test). (B) Representative images of the tumours in A at sacrifice. (C) Box and whiskers plot showing tumour weight at sacrifice (EGI-1, n = 6-8; HUCCT1, n = 8) (Dunnett's multiple comparison test). (D-F) Growth kinetics of tumours from eCCA (EGI-1, n = 8) and iCCA (HUCCT1, n = 8) expressing 2 shRNAs against FOSL1 or a control. Doxy: 2 mg/ml doxycycline in drinking water containing 5% sucrose (Dunnett's multiple comparison test). (E) Representative images of the tumours in D at sacrifice. (F) Box and whiskers plot showing tumour weight at sacrifice (EGI-1 n = 8 and HUCCT1 n = 8) (Holm-Sidak's or Dunnett's multiple comparison test). (G) Tumour volume at day 40 after doxycycline treatment start (n = 6) (Dunn's multiple comparison test). (H) Representative ultrasound images at the beginning of doxycycline treatment and at the end of the experiment (day 40). Scale bar is 1 mm. All data are mean ± SEM. Mean is shown as '+'. CCA, cholangiocarcinoma; eCCA, extrahepatic cholangiocarcinoma; iCCA, intrahepatic cholangiocarcinoma; shRNA, short-hairpin RNA.

tumours of significant smaller volume and weight than those derived from control cells (Fig. 4A-C). Similar results were obtained in CDXs derived from mouse CCA cells (KPch) (Fig. S4A-C).

To test the functional relevance of *FOSL1* as a potential molecular target, we investigated its role in established mutant *KRAS* CCA tumours. ShRNA activation was done when tumours reached an average tumour volume of 80-100 mm^3. IHC analysis of CDXs 5 days after doxycycline administration showed successful FOSL1 inhibition (Fig. S4D). *FOSL1* depletion led to tumour volume reduction compared to control shRNA in the 2 cell lines investigated (Fig. 4D and E). Likewise, tumour weight of *FOSL1*-inhibited cells was smaller at the end of the experiment (Fig. 4F).

To rule out differences emerging from the site of implantation, we developed an orthotopic model by engrafting small pieces of a human CCA CDX into the liver of immune-deficient mice (n = 6 per group) as we previously described.[44] To do this, we capitalized on the human EGI-1 cells already engineered

to express TET-inducible shRNAs. Tumours were monitored by ultrasound and doxycycline treatment was initiated (to activate shRNA expression) when an average volume of 1.5–2.0 mm^3 was reached. Relative analysis of tumour volume at day 0 and at the end of the experiment revealed an average reduction of 60% in the group of mice implanted with *FOSL1*-depleted cells compared to the control group (Fig. 4G and H). Collectively, these results suggest that FOSL1 plays a relevant functional role in CCA progression, where it may represent a molecular target.

Fosl1 abrogation extends overall survival and decreases tumour burden in a GEM model

To study *FOSL1* function in a physiologically relevant system that recapitulates cholangiocarcinogenesis, we took advantage of the AKP model. First, we bred AKP mice to $Fosl1^{f/f}$ mice to obtain $Albumin^{Cre/+}$; $Kras^{LSL\text{-}G12D/+}$; $Trp53^{f/f}$; $Fosl1^{f/f}$ (AKPF) mice (Fig. 5A). Then, survival analyses were undertaken in AKP (n = 19) and AKPF (n = 23) mice. We observed that AKPF mice have a higher overall survival compared to AKP mice expressing *Fosl1* (p <0.0001) (Fig. 5B). Of note, the survival differences remained significant even when mice were stratified by sex (p = 0.0077, male; p = 0.0074, female) (Fig. 5C and D).

In the AKP model, expression of mutant *Kras* and *Trp53* in adult mouse cholangiocytes induces CCA.[45] To test if *Fosl1* had any role on CCA originated from adult mouse cholangiocytes transformed by simultaneous *Kras* mutation and *Trp53* ablation, normal cholangiocytes were isolated from $Kras^{LSL\text{-}G12D/+}$; $Trp53^{f/f}$ (KP) and $Kras^{LSL\text{-}G12D/+}$; $Trp53^{f/f}$; $Fosl1^{f/f}$ (KPF) mice. These

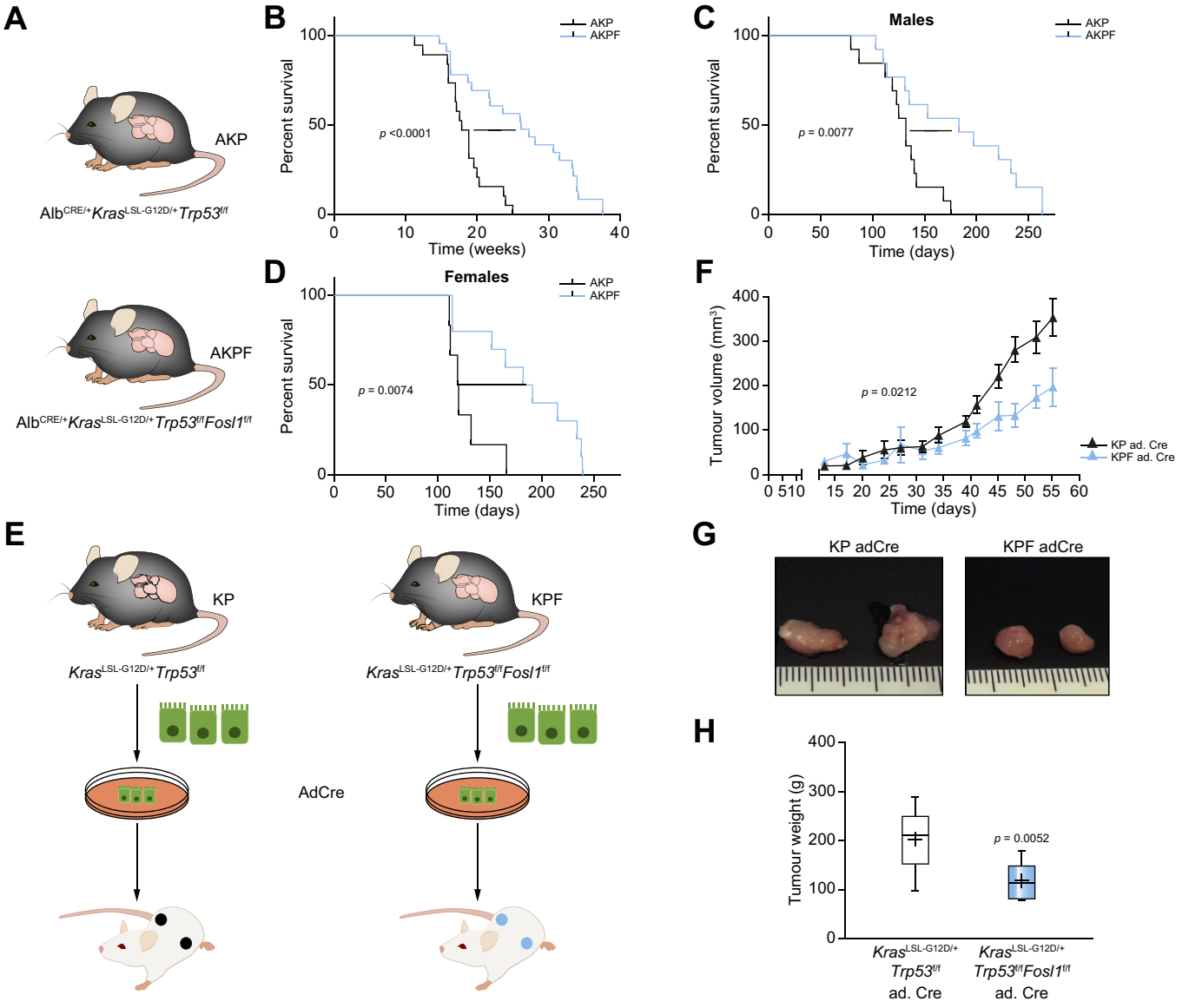

Fig. 5. FOSL1 depletion reduces tumour growth *in vivo*. (A) Schematic representation of transgenic mice used for survival studies. (B) Kaplan-Meier plot of AKP (n = 19) and AKPF (n = 23) mice (log-rank test). (C) Kaplan-Meier plot of AKP (n = 12) and AKPF (n = 13) male mice (log-rank test). (D) Survival analysis of AKP (n = 7) and AKPF (n = 10) female mice (log-rank test). (E) Schematic representation of transgenic mice used for cholangiocyte isolation and transformation. (F) Tumour volume of xenografts derived from adCre-treated KP and KPF cholangiocytes. Data are mean ± SEM (n = 8-10, *t* test). (G) Representative images of tumours at sacrifice. (H) Box and whiskers plot of tumour weight at sacrifice. Mean is shown as '+' (n = 8, *t* test). AdCre, adenoviral Cre recombinase.

cholangiocytes were treated with adCre *in vitro* to flox the corresponding alleles (Fig. 5E and Fig. S5A-B) and subsequently injected into the lower flank of immune-deficient mice. Both KP- and KPF-derived CDXs expressed Ck19 (Fig. S5D). Analyses of tumour volume from KP and KPF cholangiocytes revealed that tumours arising from *Fosl1*-depleted cells had a smaller volume compared to *Fosl1*-proficient ones (Fig. 5F and G). Likewise, weight was also reduced in tumours lacking *Fosl1* expression (Fig 5H). The data from the GEM and the primary cholangiocyte models provide evidence that *Fosl1* has a relevant role in mouse cholangiocarcinogenesis by affecting overall survival, a finding explained in part by the down-modulation of the oncogenic potential of transformed cholangiocytes.

Direct and indirect regulation of pro-oncogenic transcriptional effectors by *FOSL1*

Next, we explored the transcriptome changes controlled by *FOSL1*. RNA sequencing was performed in EGI-1 cells where *FOSL1* was knocked-out by 2 inducible shRNAs and compared with control cells (B >0). This analysis yielded a list of 56 downregulated and 73 upregulated genes (Table S2). We focused on downregulated genes for subsequent analysis as they could represent potential molecular targets for eventual genetic and pharmacological inhibitory strategies (Fig. 6A). This list included several cyclin kinases involved in G1 to S transition that could explain the G1 arrest elicited by *FOSL1* inhibition, such as CDK2, CDK6 and CDK7 (Fig. S6A). Gene Ontology (GO) analysis of the downregulated genes revealed an enrichment of biological processes involving cholesterol and steroid biosynthesis, and mitochondrial electron transport and ATP synthesis (p <0.005) (Fig. 6B). Next, protein-protein interaction (PPI) examination of the downregulated gene set using STRING identified 3 main independent clusters of genes (Fig. 6C). One cluster featured genes involved in mitotic progression, including *AURKA,* previously described as a downstream target of *FOSL1* in lung and pancreatic cancer.[27] The second cluster involved various genes implicated in the mevalonate (MVA) pathway, cholesterol biosynthesis and regulation of steroids and lipids (*HMGCS1*, *ELOVL5*, *IDI1*, *INSIG1*, *MSMO1* and *SCD*). A third cluster was formed by mitochondrial genes with NADH dehydrogenase and reductase activity (*MT-ND4L, MT-ND5* and *MT-ND3*) as well as by the membrane gene *PTGS1*, which synthesizes the formation of thromboxanes and prostaglandins. STRING analysis also revealed similarly enriched biological pathways to those found by GO (Fig. S6B). Reverse transcription quantitative PCR (qPCR) analysis of several genes in each cluster validated the RNA-seq data (Fig. S6C).

To unveil FOSL1 direct targets, complementary ChIP-seq analyses were performed. We used EGI-1 cells transduced with a Flag-tagged *FOSL1* expression construct (Fig. S6D). A heat map centred on the FOSL1 peaks illustrated strong enrichment in chromatin immune-precipitated conditions with regard to input control (Fig. S6E). Global analysis of the FOSL1-bound regions revealed that the top-ranking sequence motifs predicted by MEME and DREME corresponded to members of the AP1 transcriptional complex (Fig. 6D). Binding of FOSL1 to various RNA species was detected (Fig. 6E and Table S3). Among coding protein genes, the *HMGCS1* promoter sequence stood out as one of the most enriched regions (-log10pval = 119.43; fold change [FC] = 13.9) (Fig. 6F). Promoter regions of additional genes within the cholesterol cluster, such as *ELOVL1* (-log10pval = 33.78; FC = 7.46), *INSIG1* (-log10pval = 27.45; FC = 9.16) and *MSMO1* (-log10pval = 12; FC = 4.8) were also bound by FOSL1 (Fig. S6F). Notably, no enrichment of FOSL1 binding to the *AURKA* promoter was found, suggesting transcriptional regulation by indirect mechanisms. To validate the ChIP-seq results, ChIP-qPCR analyses were carried out on independent samples. We focused on *HMGCS1*, an indispensable enzyme of the MVA pathway responsible for the generation of farnesyl-pyrophosphate and geranyl-geranyl-pyrophosphate forms as well as MVA and cholesterol, which localizes upstream of the remaining genes in the shared PPI cluster (Fig. S6G). A 9-fold enrichment signal was observed for the *HMGCS1* gene compared to control input (Fig. 6G). Altogether, these results demonstrate that FOSL1 controls *HMGCS1* expression in CCA cells through direct promoter binding.

Genetic and pharmacological inhibition of *FOSL1* targets impairs CCA growth

The RNA- and ChIP-seq analyses indicated that *HMGCS1* is a downstream FOSL1 target. Supporting these data, human specimens were analysed and HMGCS1 was shown to be highly expressed in nearly half of all CCA cases (46%). Likewise, AURKA was also highly expressed in over two-thirds of all cases (70%). No HMGCS1 or AURKA staining was detected in non-tumorous biliary ducts (Fig. 7A, Fig. S7A and Table S2). More notably, a strong association between those CCA samples with high expression levels of FOSL1 and those ones with high levels of either HMGCS1 (p <0.001) or AURKA (p <0.001) was found by Fisher's exact test. Furthermore, we took advantage of a human data set that allows direct comparison of CCA to normal biliary epithelial cells[46] and found upregulation of *HMGCS1* mRNA in cancer specimens (Fig. 7B). HMGCS1 downregulation was observed upon *FOSL1* knockdown both in *in vitro* and in CDX models (Fig S7B,C). Notably, HMGCS1 was also decreased in mouse 339 and KPch CCA cell lines when *FOSL1* was inhibited (Fig. S7D). In addition, we found HMGCS1 expression in CCA lesions of the AKP GEM model, coinciding with tumour areas where FOSL1 levels were also upregulated (Fig. 7C).

The *HMGCS1* expression findings prompted us to assess *HMGCS1* function in CCA. To do this, 2 shRNAs were deployed to silence *HMGCS1* in eCCA and iCCA cell lines (Fig. 7D). Genetic inhibition of *HMGCS1* reduced cell proliferation in human CCA cell lines compared to control cells (Fig. 7E). Likewise, a decrease in the number of colonies formed by *HMGCS1*-depleted cells was observed (Fig. 7E). Thus, *HMGCS1* inhibition partially phenocopies *FOSL1* loss in CCA.

Next, we followed a pharmacological approach to inhibit HMGCS1. We deployed inhibitors that target HMGCS1 either upstream or downstream. First, given that the mTOR pathway can regulate *HMGCS1* expression,[47] we used the mTOR inhibitor (mTORi) AZD8055. In agreement with the published data, decreased HMGCS1 was seen in all human and mouse CCA cells treated with AZD8055 (Fig. S7E). Colony forming assays with various doses of the mTORi showed that a 20 nM dose was sufficient to reduce the clonogenic efficiency by 60–85% in human and mouse cell lines (Fig. 7F). Second, we used pitavastatin, an inhibitor that prevents the production of MVA from HMG-CoA by targeting HMGCR, an enzyme that functions right downstream of HMGCS1 (Fig. S6F). Colony forming assays revealed that pitavastatin administration in the low micromolar range

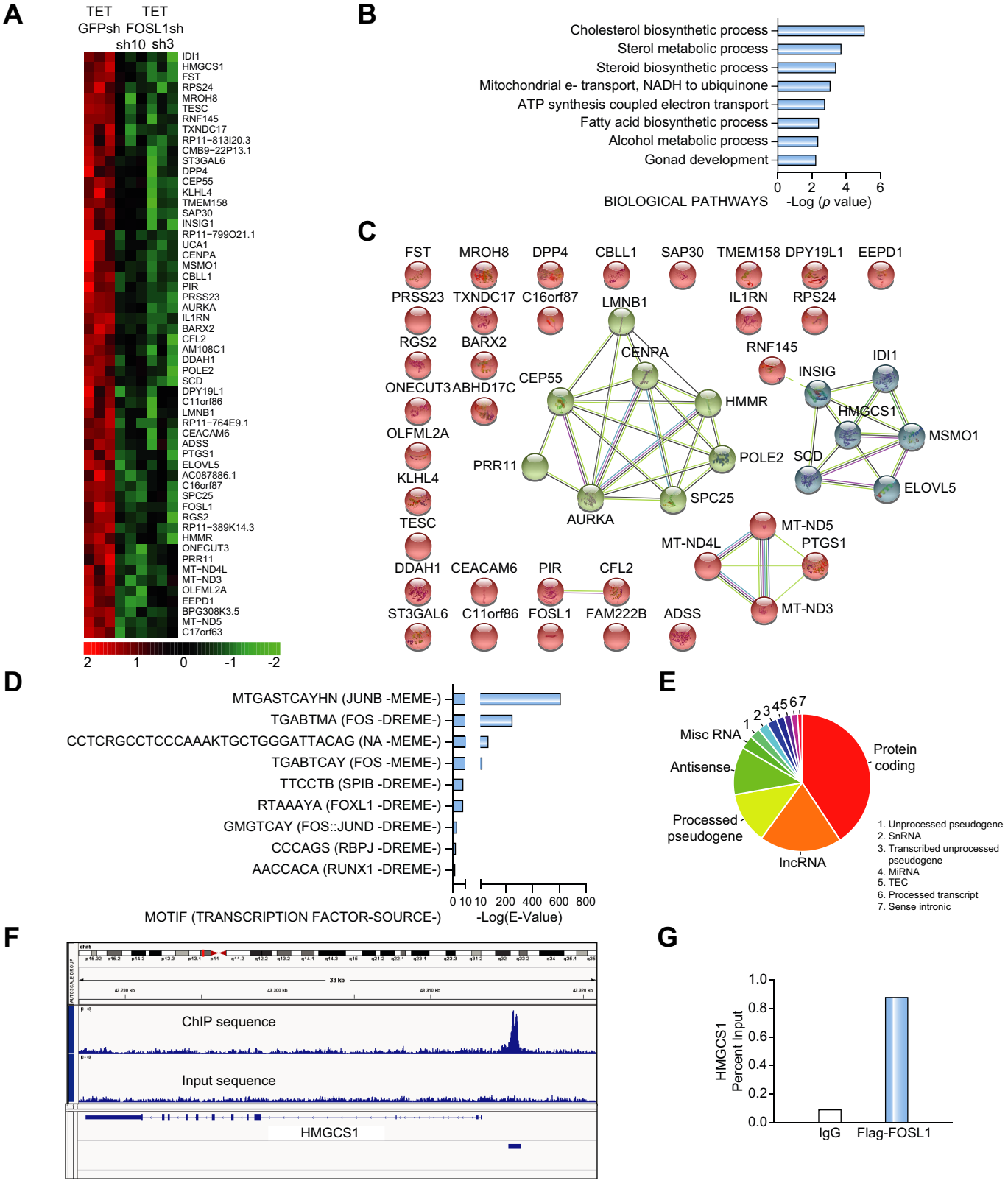

Fig. 6. Identification of transcriptional FOSL1 effectors. (A) Heat map of down-regulated genes in EGI-1 eCCA cells expressing 2 FOSL1 shRNAs compared to control group (shRNA GFP). (B) Gene Ontology analysis of the downregulated gene set. (C) Protein-protein interaction network of the downregulated gene set using STRING. (D) Transcription factor motif analysis of FOSL1-bound regions by MEME-DREME. (E) Pie chart of RNA species bound by FOSL1. (F) *HMGCS1* promoter sequence enrichment as per ChIP-seq data. (G) ChIP-qPCR analysis of FOSL1 binding to *HMGCS1* promoter region. Signal is relative to percent input. ChIP, chromatin immunoprecipitation; eCCA, extrahepatic cholangiocarcinoma; lncRNA, long non-coding RNA; MiRNA, microRNA; qPCR, quantitative PCR; shRNA, short-hairpin RNA; snRNA, small nuclear RNA; TEC, to be experimentally confirmed.

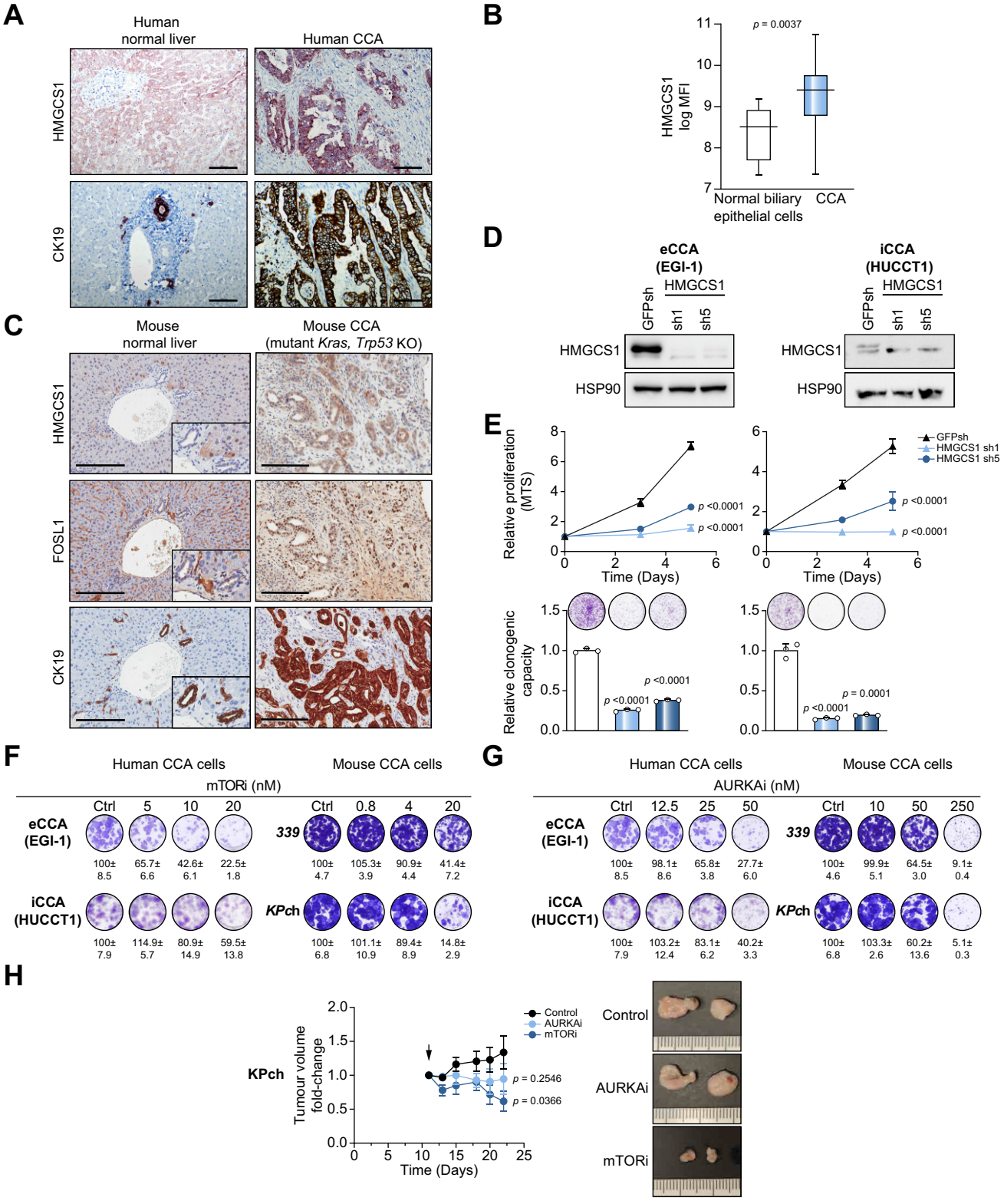

Fig. 7. Genetic and pharmacological inhibition of FOSL1 targets impairs CCA growth. (A) Representative images of normal human liver and CCA stained with indicated antibodies. Scale bars are 100 μm. (B) *HMGCS1* expression in human NBEC and CCA samples (Mann-Whitney test). (C) Representative images of serial sections of normal mouse liver and CCA from AKP mice stained with indicated antibodies. Insets represent normal bile ducts. Scale bars are 200 μm. (D) HMGCS1 immunoblot of human CCA cell lines expressing 2 HMGCS1 shRNAs compared to controls. (E) Cell proliferation assay using MTS (Dunnett's multiple comparison test) and representative images and quantification of clonogenic assays (Dunnett's multiple comparison test). (F-G) Colony forming assays in human (EGI-1 and HUCCT1) and mouse (339 and KPch) CCA cell lines treated with different doses of mTORi (AZD8055) or AURKAi (Alisertib). Numbers indicate relative percentage of cells with regard to control condition. All data are mean ± SD. Assays are representative of at least 3 independent experiments. (H) Growth kinetics of subcutaneous tumours from KPch mouse CCA cell lines in sv129 immunocompetent mice treated by oral gavage 5 days/week during 2 weeks with mTORi (20 mg/kg, n = 6), AURKAi (25 mg/kg, n = 8) or vehicle (n = 8). Data are mean ± SEM (*t* test). CCA, cholangiocarcinoma; shRNA, short-hairpin RNA.

(0.5–2.5 μM) also reduces the clonogenic capacity by 50% or more in human and mouse CCA cell lines (Fig. S7H).

In line with the observed AURKA upregulation in CCA, *AURKA* knockdown also decreased cell proliferation and clonogenic ability (Fig. S7F-G). Furthermore, we observed that pharmacological inhibition of AURKA, using concentrations ranging from 50 to 250 nM of the AURKAi alisertib, reduced clonogenic ability between 40 and 90% (Fig. 7G).

Lastly, we studied the effect of pharmacological abrogation of HMGCS1 and AURKA *in vivo*. To do this, we first generated subcutaneous tumours from KPch cells in sv129 mice with a competent immune system. The mTORi was chosen to abrogate HMGCS1 signalling. Single administration of mTOR and AURKA inhibitors induced tumour regression compared to control treatment, although tumour volume decrease was only significant upon mTORi treatment, more likely due to sample heterogeneity (Fig. 7H). Notably, tumour growth resumed when treatment was terminated (Fig. S7I), suggesting that sustained drug administration is necessary to yield anti-tumour responses. Pharmacological mTOR inhibition did not adversely impact mouse weight (Fig. S7J).

A second experiment was performed to compare single and dual treatments, expected to yield superior outcomes.[48] However, the effect of the combined treatment was not superior to single treatments (Fig. S7K), suggesting that a dual strategy involving mTORi and AURKAi is not justified in this CCA model.

Discussion

Our study points at *FOSL1* as a relevant transcription factor in CCA with clinical and functional implications. Furthermore, it unveils targets within the *FOSL1* transcriptional network amenable to pharmacological inhibition (Fig. 8).

At the clinical level, we observe that FOSL1 expression independently associates with poor patient outcomes irrespective of mutational status. Notably, those patients with tumours carrying *KRAS* mutations and FOSL1 expression had the worst survival outcome. A series of follow-up experiments using human and mouse *in vitro* and *in vivo* models demonstrated that *FOSL1* play a functional role in CCA, providing evidence of its potential involvement in patient outcome.

This study illustrates the involvement of major effector pathways participating in CCA, such as the RAF-MEK-ERK or the PI3K-AKT pathways,[41] in *FOSL1* regulation. Although we showed that FOSL1 expression is controlled by the *KRAS* oncogene, given the large percentage of patients with CCA expressing FOSL1, it is likely that additional oncogenes or extracellular cues derived from abnormal activation of cancer pathways also participate in *FOSL1* regulation. Supporting this data, recent work in perihilar CCA showed that the WNT pathway regulates *FOSL1* via the transcription factor *TCF7*, which played a functional role in this CCA subtype.[26]

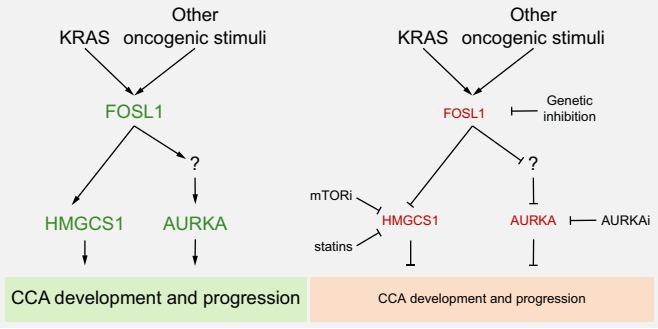

Fig. 8. Proposed model of *FOSL1* regulation and function in CCA. FOSL1 expression favours CCA development and progression via direct and indirect transcriptional effectors such as HMGCS1 and AURKA. FOSL1 genetic inhibition or single/dual pharmacological blockade of downstream targets adversely affect CCA. CCA, cholangiocarcinoma.

Our studies shed light on new molecular mechanisms controlled by *FOSL1*. In addition to regulating genes implicated in mitosis progression previously reported in lung and pancreatic cancer,[27] *FOSL1* regulates different functions such cholesterol and steroid biosynthesis or mitochondrial electron transport and ATP synthesis in CCA. Lipid metabolism-related processes were characterized by a subset of genes composed of *HMGCS1*, *ELOVL1*, *IDI1*, *INSIG1*, *MSMO1* and *SCD*, some of which are direct FOSL1 targets. Liver cancer cells undergo drastic metabolic reprogramming to meet their increased demand for energy and macromolecules,[49] and increased cholesterol synthesis through the MVA pathway may be part of such adaptative mechanisms.[50] The MVA pathway does not only lead to the production of cholesterol, but also results in important non-sterol end products including farnesyl and geranylgeranyl isoprenoids, which can be important post-translational modulators of oncogenic effectors in CCA such as KRAS or RAC1.[51,52] The fact that *HMGCS1* is necessary for proliferation of CCA cells supports the idea that the MVA pathway fuels the oncogenic phenotype. Notably, *HMGCS1* has been reported to be a 'synthetic lethal' partner of $BRAF^{V600E}$ in human melanoma and colon cancer cells ([53]). Moreover, in an attempt to unveil novel sensitizers that could be combined to maximize anti-cancer efficacy of statins, *HMGCS1* was identified as a top scoring gene of a genome-wide RNAi analysis required for cell survival in lung cancer cells.[54] Despite our current observations, the contribution of other members of the *FOSL1* signature remains unexplored and may also explain the impact of *FOSL1* inhibition in *KRAS*-mutated CCA.

Different targeted therapies have been tested with limited success in CCA. Some of them include inhibition of effectors of the RAF-MEK-ERK and PI3K-AKT pathways. Given the mounting evidence from our group and others on the role of *FOSL1* in cancer, including CCA,[27,32] considering FOSL1 as a therapeutic target would be justified. However, based on FOSL1 regulation experiments *in vitro*, inhibitors against the RAF-MEK-ERK or the PI3K-AKT modules are expected to decrease expression levels of the transcription factor and, thus, one would anticipate that targeting FOSL1 is a meaningless endeavour as inhibitors against upstream kinases are already available. However, the fact that FOSL1 expression levels are absent or barely detectable in normal liver tissues suggests that FOSL1 inhibitors may have a better therapeutic window (*i.e.* less toxicity) compared to MEK or PI3K inhibitors. Although transcription factors have been historically difficult to target, in part because their activity is ignited by protein-protein or protein-DNA interactions, inhibition of key transcription factors such as c-MYC, long-thought to be undruggable, has recently been demonstrated[55,56] providing groundwork to attempt FOSL1 inhibition.

As alternative strategies of FOSL1 inhibition, our observations suggest that disruption of the *HMGCS1* and *AURKA* networks represent potential options to treat CCA. These results are in tune with previous studies proposing statins[57,58] or AURKAi[59] as

potential therapeutic approaches for CCA. Furthermore, recent data has shown that statins reduce the risk of eCCA and may improve survival in patients with distal CCA,[60] highlighting the clinical relevance of statin treatment. Lastly, our study goes one step further by nominating mTOR inhibition as a potential therapy for CCA.

Abbreviations

AdCre, adenoviral Cre recombinase; AdEmpty, adenoviral empty (control); AURKA, aurora kinase A; CCA, cholangiocarcinoma; CDX, cell-derived xenograft; ChIP, chromatin immunoprecipitation; CK19, cytokeratin 19; eCCA, extrahepatic CCA; EFGR, epidermal growth factor receptor; ELOVL1, ELOVL fatty acid elongase 1; ERK, extracellular signal-regulated kinase; FC, fold change; FGFR2, fibroblast growth factor receptor 2; FOSL1, FOS-ligand 1; FOXM1, forkhead box protein M1; GEM, genetically engineered mouse; GO, Gene Ontology; HMGCS1, HMG coenzyme A synthetase 1; HMGCR, HMG-CoA reductase; iCCA, intrahepatic CCA; IDH1/2, isocitrate dehydrogenase; IDI1, isopentenyl-diphosphate delta isomerase 1; IHC, immunohistochemistry; INSIG1, insulin-induced gene 1; IPBN, intra-ductal papillary biliary neoplasms; MOI, multiplicity of infection; MSMO1, methylsterol monooxygenase 1; MTOR, mechanistic target of rapamycin kinase; MVA, mevalonate; PI3K, phosphoinositide 3-kinase; PPI, protein-protein interaction; RAC1, Ras-related C3 botulinum toxin substrate 1; ROI, region of interest; qPCR, quantitative PCR; SCD, stearoyl-CoA desaturase; shRNA, short-hairpin RNA; SOX9, SRY-box transcription factor 9; TCF7, transcription factor 7; YAP1, Yes-associated protein 1.

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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

S.V. conceived and designed the project and supervised the work. A.V., O.E, R.E., I.F., M.J.P., P.O., I.M., B.R-F, S.O-E., F.L., M.A.A., M.G.F, M.P-S., D.C., J.M.B and S.V. designed and planned the experiments. E.G. carried out computational analyses. A.M., M.E. and D.F.C. performed human IHC and survival analyses. M.O. and A.F.H. provided cell lines from AKP mice. A.V., O.E., R.E., M.A.A., M.G.F., M.P-S., D.F.C., J.M.B. and S.V. were responsible for the data analysis and interpretation. A.V., O.E., R.E., D.F.C., J.M.B. and S.V. wrote the manuscript and were in charge of the manuscript preparation. All the authors reviewed, edited, and approved the manuscript.

Data availability statement

The authors agree to share any additional data not directly available in the current version of the manuscript upon request.

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Supplementary data

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Author names in bold designate shared co-first authorship

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