Title
Fascin1 empowers YAP mechanotransduction and promotes cholangiocarcinoma development

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Mechanical forces control cell behavior, including cancer progression. Cells sense forces through actomyosin to activate YAP. However, the regulators of F-actin dynamics playing relevant roles during mechanotransduction in vitro and in vivo remain poorly characterized. Here we identify the Fascin1 F-actin bundling protein as a factor that sustains YAP activation in response to ECM mechanical cues. This is conserved in the mouse liver, where Fascin1 regulates YAP-dependent phenotypes, and in human cholangiocarcinoma cell lines. Moreover, this is relevant for liver tumorigenesis, because Fascin1 is required in the AKT/NICD cholangiocarcinogenesis model and it is sufficient, together with AKT, to induce cholangio-cellular lesions in mice, recapitulating genetic YAP requirements. In support of these findings, Fascin1 expression in human intrahepatic cholangiocarcinomas strongly correlates with poor patient prognosis. We propose that Fascin1 represents a pro-oncogenic mechanism that can be exploited during intrahepatic cholangiocarcinoma development to overcome a mechanical tumor-suppressive environment.
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**Results**

**Ena/VASP and Fascin1 sustain YAP/TAZ activity.** Evidence for CAPZ regulating the balance between branched and bundled F-actin mainly comes from studies on cell migration, and whether this is relevant for mechanotransduction remains unexplored. We started our investigation by testing the functional relevance of proteins promoting the bundled F-actin formation, such as Formins, Ena/VASP, and Fascin1. We used YAP/TAZ as a downstream read-out of ECM mechanotransduction, and MCF10A mechano-sensitive mammalian epithelial cells since they are an established cell model for investigating the Hippo pathway and for YAP mechanotransduction.\(^1\)\(^{-}\)\(^3\)\(^{-}\)\(^4\) The role of Formins downstream of integrin/RHO signaling and in mechanotransduction is well known\(^1\), and inhibition of Formins with the SMIFH2 small molecule in cells cultured on plastic (i.e., on a stiff substratum) resulted in the nuclear exclusion of YAP/TAZ (Fig. 1a) in line with previous data\(^1\). The role of Ena/VASP proteins is less understood. We transfected cells with F4P-GFP-Mito expressing plasmid, sequestering endogenous Ena/VASP proteins at the mitochondrial surface and thus blocking their function\(^3\). Expression of F4P-GFP-Mito, but not its A4P-GFP-Mito control, was sufficient to inhibit cell spreading, as shown by phalloidin staining, and caused a consequent inhibition of YAP/TAZ, as gauged by nuclear/cyttoplasmic localization (Fig. 1b).

Besides Ena/VASP proteins, another key component regulated by CAPZ is Fascin2. Fascin is a highly conserved protein encoded by three orthologous genes in mammals, which promotes in vitro the formation of rigid and contractile bundles\(^3\), and which is found in filopodia and in F-actin bundles around the nucleus\(^3\)\(^{-}\)\(^4\). Fascin1 is the isoform with the broadest expression in mice, whereas Fascin2 and Fascin3 expression is limited to the retina and testis, respectively\(^4\). Recent data suggest a role for Fascin1 as a regulator of the Hippo pathway in WM793 melanoma cells and in A549 non-small cell lung cancer cells\(^3\)\(^{-}\)\(^4\). However, the functional relevance for this regulation has not been addressed neither in vivo nor in the context of mechanotransduction. MCF10A cells express undetectable levels of Fascin2 and Fascin3 mRNA, as measured by qPCR (Supplementary Fig. 1a). We therefore knocked-down Fascin1 by RNA interference, which caused the reduction of radial F-actin bundles (Fig. 1c and Supplementary Fig. 1b, c), and a concomitant translocation of YAP/TAZ towards the cytoplasm (Fig. 1D). This was independently confirmed by treating cells with the G2 small-molecule inhibitor of Fascin (Fig. 1e)\(^4\), and in human cholangiocarcinoma cell lines (see Fig. 4 below). Accordingly, inhibition of Fascin1 reduced YAP/TAZ transcriptional activity measured by the established 8XGTIIC-lux luciferase reporter assay in MDA-MB-231 breast cancer cells (Fig. 1f), which display high level of YAP/TAZ activity\(^1\)\(^{-}\)\(^4\), and whose metastatic ability depends on Fascin\(^4\). Similar results were obtained by monitoring endogenous YAP/TAZ target genes by qPCR in mouse E0771 breast cancer cells stably expressing Fascin1 shRNAs (Fig. 1g), which we used to validate shRNAs to be used in vivo (see Fig. 4 below). Collectively, this data indicates that the pool of bundled F-actin promoted by Ena/VASP and Fascin1 sustains YAP/TAZ activity when cells are on a stiff substratum.
CAPZ and Arp2/3 antagonize Fascin1-dependent actin during mechanotransduction. We and others previously described CAPZ as a YAP/TAZ inhibitor in the context of ECM mechanotransduction, but whether this relates with the ability of CAPZ to promote branched F-actin networks and to oppose Fascin1 (see introduction and Supplementary Fig. 2a) remains unknown. To test this hypothesis, we inhibited the Arp2/3 complex, i.e., the master regulator of branched actin, by treating cells plated on a soft ECM—i.e., the condition in which CAPZ is relevant— with the CK-869 small molecule. As shown in Fig. 2a (quantified in...
Fig. 1 Bundled F-actin promoted by Formins, Ena/VASP, and Fascin1 sustains YAP activity on stiff ECM substrata. a Representative immunofluorescence (IF) of MCF10A cells treated with 60 µM SMIFH2 Formin inhibitor or with the same amount of vehicle (DMSO) for 24 h and stained for YAP/TAZ, F-actin (phalloidin), and DAPI as nuclear counterstain. On the right, quantification of YAP/TAZ subcellular localization, expressed as the percentage of cells in which the nucleus had a stronger staining than the surrounding cytoplasm (Nuclear) or equal/lower than the cytoplasm (Cytoplasm). n = 3 (>60 cells per condition in total). p = 0.0133 by unpaired Welch’s t-test. Scale bar = 10 µm. b Representative IF of MCF10A cells transfected with a plasmid encoding for the Ena/VASP inhibitor FAP-GFP-Mito, or for its mutated control A4P-GFP-Mito, and stained for YAP/TAZ, F-actin (phalloidin) and DAPI. On the bottom/left, quantification of YAP/TAZ localization. n = 3 (>60 cells per condition in total). p = 0.0357 by unpaired Welch’s t-test. Scale bar = 10 µm. c, d Representative IF of MCF10A cells transfected with FSCN1 siRNA (siFSCN1) or control siRNA (siCo.). In c, cells were stained for Fascin1, F-actin (phalloidin), and DAPI. In d, cells were stained for YAP/TAZ and DAPI. On the right, quantification of YAP/TAZ localization. Similar results were obtained with siFSCN1 B. n = 3 (>60 cells per condition in total). p = 0.0283 by unpaired Welch’s t-test. Scale bars = 5 µm. f BXGTIIC-luciferase reporter assay for YAP/TAZ in MDA-MB-231 cells transfected with two independent siRNAs targeting FSCN1 (siFSCN1 A and siFSCN1 B) or control siRNA (siCo.), or treated with different doses of G2 (50 µM or 100 µM), latrunculinA (LatA 0.5 µM) as positive control for inhibition of YAP/TAZ, or DMSO as vehicle. Mean expression in controls was set to 100 and all other samples are relative to this. Data are mean and s.d. g qPCR for YAP/TAZ target genes (CTGF and ANKRDP) in mouse E0771 cells stably expressing control short hairpin RNA (shCo.) or two different short-hairpin RNAs targeting FSCN1 (shFSCN1 A and shFSCN1 B). Data are relative to GAPDH expression. Mean expression levels in control cells were set to 1, all other samples are relative to this. Data are mean and s.d.

The results suggest that on a soft ECM the competition between branched and bundled F-actin networks, we then tested whether Arp2/3 and CAPZ modulate YAP/TAZ by regulating Fascin1. In line with this hypothesis, treatment of cells with CK-869 or depletion of CAPZ led to the accumulation of Fascin1-positive structures at cell edges (Fig. 2b and Supplementary Fig. 2b), where CAPZ is usually found (Supplementary Fig. 2c). These structures in part co-localized with Vinculin, a marker for mature focal adhesions (Supplementary Fig. 2d). Moreover, we observed enhanced Fascin1 immunoreactivity in CAPZ-depleted cells plated on a soft ECM (Fig. 2c). Most importantly, Fascin1 activity was required for nuclear YAP/TAZ localization induced by Arp2/3 inhibition or by CAPZ knockdown (Fig. 2d, e). Collectively, these results suggest that on a soft ECM the competition between CAPZ-Arp2/3 and Fascin1 is relevant for the regulation of YAP/TAZ nuclear localization (see scheme in Supplementary Fig. 2a).

To reinforce this idea, overexpressing active S39A Fascin1 in cells on a soft ECM promoted partial YAP/TAZ nuclear accumulation (Fig. 2f, see Supplementary Fig. 2e for controls). This did not occur in cells treated with latrunculinA (Supplementary Fig. 2f), indicating an F-actin-dependent function of Fascin1. Finally, we built on the notion that YAP/TAZ are antagonized Fascin1-dependent YAP activity in the liver. To challenge the relevance of our observations in vivo, we sought to trans impose our observations to the mouse liver, a model system to study YAP/TAZ biology and an organ where cells lay in a soft environment. Moreover, the hepatocyte-specific inactivation of Capzb triggers YAP mechanotransduction as well as proliferation and dedifferentiation of hepatocytes into atypical ductal cells (ADC) of cholangiocellular identity.

We initially tested whether Fascin1 is downstream of CAPZ also in vivo. For this purpose we inhibited Fascin1 in liver cells by administering the G2 Fascin inhibitor via i.p. injection to CAPZ LKO mice and scored hepatocyte dedifferentiation as a read-out of YAP function. As shown in Fig. 3a and Supplementary Fig. 3, G2 treatment restricted the expansion of the cholangiocellular marker CK19 in CAPZ LKOs. The effect was partial, likely due to the relatively low affinity of G2 for Fascin1. To test whether Fascin1 was sufficient to activate YAP in vivo, we subsequently overexpressed Fascin1 in the liver of adult wild-type mice using hydrodynamic tail vein (HTV) injection and scored established YAP-induced phenotypes. Expression of Fascin1 was sufficient to increase hepatocyte proliferation, as shown by EdU incorporation (Fig. 3b), and to induce the formation of ADCs, as gauged by staining for the A6 cholangiocellular marker (Fig. 3c). Importantly, these phenotypes were prevented when Yap1/Wwtr1 (TAZ) were knocked out in Fascin1-expressing hepatocytes (Fig. 3c), indicating an effect mediated by activation of YAP/TAZ. These results suggest that CAPZ maintains hepatocyte cell fate by inhibiting Fascin1-dependent YAP activation.

Fascin1 has a pro-oncogenic function in intrahepatic cholangiocarcinomas. In the liver, YAP activation not only induces differentiation of hepatocytes into ADCs, but also promotes the development of hepatocellular carcinomas (HCC), cholangiomas and intrahepatic cholangiocarcinomas (iCCA) which prompt us to explore the role of Fascin1 in liver tumorigenesis. Overexpression in the mouse liver of myristoylated AKT, an established driver of liver carcinogenesis, did not result in any histopathological alteration (Fig. 3d–f). AKT-expressing cells were not detected, implying their elimination, likely due to insufficient fitness in the C57BL/6 N background. In striking contrast, expression of Fascin1 anc AKT was sufficient to induce the appearance of macroscopic nodules on the liver surface (Fig. 3d). The AKT/FSCN1 lesions display enhanced proliferation as gauged by EdU incorporation, they exhibited cholangiocellular features based on the immunoreactivity for the CK19 and A6 markers, and histopathologic analyses indicated them as neoductular proliferation or cholangiomas (Fig. 3e, f). A similar phenotype was observed following expression of AKT and activated TAZ (Fig. 3d–f) or YAP84. Furthermore, we detected the presence of hepatocellular adenomas or hepatocellular foci, or of mixed lesions, in AKT/FSCN1 and AKT/TAZ livers (Fig. 3e).
Finally, we did not observe malignant progression features in the C57BL/6N background, indicating delayed or reduced progression compared to FVB/N mice. Thus, Fascin1 cooperates with activated AKT to induce liver neoplasia, including cholangiomas.

To explore the functional requirement of Fascin1 in the context of cholangiocarcinoma, we next investigated the levels of Fascin1 in experimental cholangiocarcinomas induced in mice by different oncogene combinations. For this purpose we stained livers of FVB/N mice harboring cholangiocarcinomas induced by HTV injection of AKT together with activated N-Ras-V12D (AKT/N-Ras), with activated Notch Intracellular Domain (AKT/NICD), or with activated YAP-S127A (AKT/YAP).
all models tested, a robust Fascin1 immunoreactivity was detected in endothelial cells, and used as an internal control of the staining. However, only the AKT/NICD combination displayed consistent, strong, and diffuse cytoplasmic Fascin1 expression in tumor cells when compared to the surrounding normal tissue (Fig. 4a, b, see higher magnifications in Supplementary Fig. 4a). The absence of Fascin1 overexpression in other oncogenic combinations likely reflects different underlying molecular mechanisms. Tumors induced by NICD alone were also negative for Fascin1 expression in tumor cells, suggesting the need for combined NICD and AKT signaling (Fig. 4a).

The development of cholangiocarcinomas by AKT/NICD relies on endogenous YAP in cancer cells57,60, thus representing the ideal experimental set-up to test the requirement for Fascin1. We
Fascin1 staining was restricted to vascular structures with the G2 Fascin inhibitor or with vehicle (5% DMSO), stained for the cholangiocellular marker CK19 and DAPI as nuclear counterstain. Analyses were carried out 15 days after tamoxifen injection. On the right, quantification of CK19-positive area in sections of the portal area for the indicated conditions. Mean and single data (mice, n = 3). Scale bar = 100 µm.

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 ARTICLE

Fig. 3 Fascin1 regulates hepatocyte cell fate through YAP/TAZ and promotes cholangiocarcinoma development. a Representative immunofluorescence stainings of liver sections from adult tamoxifen-injected Albumin-CreERT2; Capzl2/−/ mice (CAPZ LKO) mice injected i.p. with the G2 Fascin inhibitor or with vehicle (5% DMSO), stained for the cholangiocellular marker CK19 and DAPI as nuclear counterstain. Analyses were carried out 15 days after tamoxifen injection. On the right, quantification of CK19-positive area in sections of the portal area for the indicated conditions. Mean and single data (mice, n = 3). Scale bar = 100 µm. b Representative immunofluorescence stainings of liver sections from mice transduced by hydrodynamic tail vein (HTV) injection with transposon plasmids encoding for GFP or for GFP + Fascin1. Liver sections were stained for EdU. Analyses were carried out 15 days after HTV injection. On the right, quantification of EdU-positive and GFP-positive hepatocytes. Mean and single points (mice, n = 3). Scale bar = 5 µm. c Representative immunofluorescence stainings of liver sections from mice transduced by HTV injection with transposon plasmids encoding for GFP or GFP + Fascin1. YAP/TAZ double KO was obtained by adding the CRE recombinase to the HTV mix, and by injecting Yapi1fl/fl; Wwp1fl(TAZ)fl/fl; ROSA26-LSL-loxZ mice. Analyses were carried out 15 days after HTV injection and induction of CRE activity by tamoxifen. YAP/TAZ recombination was controlled by β-galactosidase staining. Mean and single points (mice, n = 2). Scale bar = 10 µm. d Representative pictures and quantification of the macroscopic lesions detected at the liver surface in mice transduced by HTV injection with transposon plasmids expressing myristoylated-HA-AKT alone, together with Fascin1 (AKT/FSCN1), or together with activated TAZ 4SA (AKT/TAZ). Livers were analyzed 7 months after HTV. Mean and single points (mice, n = 3). e Representative immunochemistry for the cholangiocellular marker CK19 on liver sections from mice transduced by HTV injection as in d. The black arrow indicates an example of hepatocellular adenoma. Squares and insets: higher magnification on CK19-positive areas, including cholangiomas and ductular reactions. Livers were analyzed 7 months after HTV. f Representative immunofluorescence images of liver sections from mice transduced by HTV injection as in d. Liver sections were stained for HA to localize AKT-expressing cells, the cholangiocellular marker A6, and DAPI. The white arrow indicates a normal A6-positive bile duct. Above the pictures: quantification of EdU-positive and HA-positive cells. Cells expressing only AKT were undetectable, likely due to the high resistance to oncogenic transformation of the C57BL/6 N strain. EdU incorporation in this condition refers to non-transduced hepatocytes. Livers were analyzed 7 months after HTV. Average and single points (mice, n = 3). Scale bar = 80 µm.

Discussion

The identification of the YAP mechanotransduction system provided a powerful model to study the effects of tissue mechanical properties on cell behavior. Yet, despite a wealth of knowledge on the most upstream players of this pathway (i.e., at the level of focal adhesions)1,7,67 and some very recent hints on the mechanisms proximal to YAP regulation68–71, the relevant intermediate players and the F-actin structures involved remain largely unknown. Moreover, the identity of the pivotal inducers of YAP mechanotransduction in vivo, and their eventual role in cancer progression, remain even less charted territories.

Here, by the known function of CAPZ in shifting the balance between actin bundled vs. branched structures during cell migration29,31,1 we identify Ena/VASP and Fascin1 proteins as required for YAP activity in response to ECM stiffness. On a soft ECM CAPZ and Arp2/3 complexes, promoters of branched F-actin, inhibit YAP activity by limiting the formation of Fascin1-dependent actin. Indeed expression of activated Fascin1 is sufficient to drive nuclear YAP on a soft ECM and in confluent monolayers. The concept of competition between different actin networks in cells has been previously proposed in yeast (where Profilin regulate the balance between Arp2/3 and Formin activity)31,72, in epithelial cells of liver origin (where actomyosin bundles prevent the formation of Arp2/3 dependent sub-membranous actin)59, and in axon growth cones (where Arp2/3 activity can restrict myosin-mediated contractility)73. Our data suggests that such competition also occurs during ECM mechanotransduction, and becomes relevant when cells are in conditions of decreased ECM stiffness. In these conditions, not only is RHO signaling reduced, leading to reduced activity of ROCK/MLCK and of Formins1, but at the same time actin structures induced by CAPZ and Arp2/3 outbalance actin bundles promoted by Ena/VASP, Fascin1 and potentially other F-actin bundling proteins. We speculate this might be particularly relevant at intermediate stiffness levels, ultimately tipping the balance in favor of YAP/TAZ activity.
The role of Ena/VASP and Fascin1, key factors driving filopodia formation, might indicate a specific function of filopodia in the phenotypes we observed. Filopodia are mechanosensitive structures, they support the formation of nascent adhesions that can subsequently develop into mature focal adhesions in response to ECM forces, and they provide migrating cells the ability to probe the mechanics of the microenvironment. However, we did not observe major induction of filopodia upon Fascin1 overexpression, Arp2/3 inhibition or CAPZ inactivation, at least under our imaging conditions.
conditions. Moreover, Fascin1 staining in cholangiocarcinomas appears cytoplasmic diffuse and it is not limited to the cell periphery/protrusions. Thus, these players might thus regulate other cytoplasmic bundled actin structures relevant for mechanotransduction, as suggested by delayed radial bundles upon loss of Fascin1.

Importantly, this new activity of Fascin1 is relevant in the liver, a model system to study YAP and mechanotransduction. We found that Fascin1 activation is sufficient to induce mouse hepatocyte dedifferentiation into atypical ductal cells (ADC, also called oval cells or bipotent liver progenitor cells) in a YAP/TAZ-dependent manner, and that Fascin1 is required for ADC formation downstream of CAPZ inactivation, where enhanced contractility drives YAP activation25. These findings led us to explore the role of Fascin1 in liver cancer. We found that human intrahepatic cholangiocarcinomas (iCCA) exhibit high levels of Fascin1, promoting tumor cell proliferation, and whose expression correlates with more invasive iCCAs and poor prognosis. Moreover, we found a similar overexpression in cholangiocellular lesions induced by experimental overexpression in cholangiocellular lesions induced by experimental administration of drug (iCCA, STR profiled, checked for endogenous mutations against other derivatives) were cultured in DMEM/F12 supplemented with 5% Horse Serum, 2 mM Glutamine, insulin (Sigma), cheolaru toxin (Sigma), hEGF (Peprotech) and hydrocortisone (Sigma) as in Debnath et al., 2003. Human breast cancer MDA-MB-231 cells (ATCC, STR profiled) were cultured in DMEM/F12 with 10% FBS and 2 mM Glutamine. Human intrahepatic cholangiocarcinoma cell lines KU-KU-156, KU-KU-213 (STR profiled) were cultured in RPMI1640 with 10% FBS, 2 mM Glutamine, and 1 mM Sodium Pyruvate, RBE and HUCCT1 (STR profiled) were cultured in DMEM high glucose with 10% FBS, 10 mM HEPES, 2 mM Glutamine, and 1 mM Sodium Pyruvate. Murine breast cancer E0771 cells were cultured in RPMI medium supplemented with 10% FBS, 2 mM Glutamine, and 1% HEPES. All cell lines were routinely tested with universal mycoplasma ATCC detection kit 30-102 K and were negative. siRNA transfections were done with Lipofectamine RNAi MAX (Invitrogen) and plasmid DNA transfections were done with Transfect-LE-1 (MirusBio) according to the manufacturer’s instructions. Where indicated, transfections were carried out on plastic vessels and cells were subsequently replated on hydrogels. Fibronectin-coated polyacrylamide hydrogels (E = 0.5 kPa) were assembled in-house as in ref. 25.

**Methods**

**Cell lines, transfections, and microfabrications.** Human mammary epithelial MCF10A cells (ATCC, STR profiled, checked for endogenous mutations against other derivatives) were cultured in DMEM/F12 supplemented with 5% Horse Serum, 2 mM Glutamine, insulin (Sigma), cheolaru toxin (Sigma), hEGF (Peprotech) and hydrocortisone (Sigma) as in Debnath et al., 2003. Human breast cancer MDA-MB-231 cells (ATCC, STR profiled) were cultured in DMEM/F12 with 10% FBS and 2 mM Glutamine. Human intrahepatic cholangiocarcinoma cell lines KU-KU-156, KU-KU-213 (STR profiled) were cultured in RPMI1640 with 10% FBS, 2 mM Glutamine, and 1 mM Sodium Pyruvate, RBE and HUCCT1 (STR profiled) were cultured in DMEM high glucose with 10% FBS, 10 mM HEPES, 2 mM Glutamine, and 1 mM Sodium Pyruvate. Murine breast cancer E0771 cells were cultured in RPMI medium supplemented with 10% FBS, 2 mM Glutamine, and 1% HEPES. All cell lines were routinely tested with universal mycoplasma ATCC detection kit 30-102 K and were negative. siRNA transfections were done with Lipofectamine RNAi MAX (Invitrogen) and plasmid DNA transfections were done with Transfect-LE-1 (MirusBio) according to the manufacturer’s instructions. Where indicated, transfections were carried out on plastic vessels and cells were subsequently replated on hydrogels. Fibronectin-coated polyacrylamide hydrogels (E = 0.5 kPa) were assembled in-house as in ref. 25.

**Reagents.** The small-molecule inhibitors were SMIFH2 (Sigma #826, 60 microM), CKK-869 (Sigma C9124, 80 microM) LatsatinA (Sigma L5163, 0.5 microM), G2 (Xcnsio M60289 for cell treatments, Valuetech custom synthesis for mouse injections). All plasmids were sequence-verified before use and transfected as endotoxin-free maxi preps. siRNAs were selected among pre-validated Mission plK01-shRNA (Sigma) and the corresponding U6-shRNA-CPP7 cassettes were subcloned into PB-empty vector25. Sequences of siRNAs are provided in Table 1.

**Human tissue samples.** Sixty-two intrahepatic cholangiocarcinomas (iCCA) and fifty hepatocellular carcinomas (HCC) as well as the corresponding surrounding non-tumorous liver tissues were used for the study. Patients’ clinicopathological features are summarized in Supplementary Tables 1 and 2. Tumors were divided in iCCA/HCC with shorter/poorer (iCCA, n = 35; HCC, n = 28) and longer/better (iCCA, n = 27; HCC, n = 28) survival, characterized by <3 and ≥3 years’ survival following partial liver resection, respectively. Liver tissues were collected at the Universities of Greifswald (Greifswald, Germany) and Regensburg (Regensburg, Germany). Institutional Review Board approval was obtained at the local Ethical Committees of the Medical Universities of Greifswald (approval code: BB 67/10) and Regensburg (17-1015-101), in compliance with the Helsinki Declaration. Written informed consent was obtained from all individuals.

**Mice and treatments.** Mice were C57BL/6 N as in ref. 25. Sex allocation was random. For G2 treatment, mice were administered 100 mg/kg of G2 by daily i.p.
Hydrodynamic tail-vein injection was used to transduce hepatocytes of 4/6-week-old mice with exogenous DNA. 50 μg of total PiggyBac (PB) and/or Sleeping Beauty (SB)-transposon plasmid DNA together with 10 μg of hyperactive PB Transposase plasmid DNA (hyPBase) or hyperactive Sleeping Beauty transposase (pCMV-T7-SB100, Addgene 34879) were diluted in sterile Ringer +4°. The day after, cells were incubated without prior fixation with PFA.

For EdU labeling, mice were injected with 12.5 mg/kg of EdU in sterile 1XPBS (A10044 Molecular Probes) 15 h before tissue sampling. Cells were incubated for 1 h with EdU in sterile 1XPBS. Liver slice or cells were fixed in PFA 4% and block-permeabilize for 30 min in 1xPBS 5% BSA for 30 min at RT.

**Histology and immunohistochemistry.** Human and mouse liver specimens were fixed overnight in 4% paraformaldehyde and embedded in paraffin. Tumors arising in mice from different oncogene combinations and used for Fascin IHC were induced in C57Bl/6n females (see references in the pertaining section of text). Sections were done at 5 μm in thickness. Liver lesions were evaluated and classified by two board-certified pathologists and liver experts (S.R. and M.E.). For immunohistochemistry, slides were deparaffinized in xylene, dehydrated through a graded alcohol series, and rinsed in PBS. Antigen unmasking was achieved by boiling in 10 mM sodium citrate buffer (pH 6.0) for 10 min, followed by a 20-min cool down at room temperature. After a blocking step with the 5% goat serum and Avidin-Biotin blocking kit (Vector Laboratories, Burlingame, CA), human tissue slides were incubated with primary antibody overnight at 4°C (see Table 2). Slides were subject to 3% hydrogen peroxide for 10 min to quench endogenous peroxidase activity and, subsequently, the biotin conjugated secondary antibody was applied at a 1:50 dilution for 30 min at room temperature. Immunoreactivity was visualized with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) using DAB chromogen. Slides were counterstained with hematoxylin.

**Luciferase assays.** Cells were plated in 24-well plates and transfected with YAP/TAZ luciferase reporter 8XGFIIC-lux plasmid (50 ng/cm2) (Addgene 34615) together with CMV-lacZ (75 ng/cm2) on or immediately after transfection. Transfected DNA content was kept equal using pKs Bluescript. Cells were harvested in luciferase lysis buffer (25 mM Tris pH 7.8, 2.5 mM EDTA, 10% glycerol, 1% NP-40). Luciferase activity was determined in a Tecan plate luminometer with freshly reconstituted assay reagent (0.5 mM D-Luciferin, 20 mM tricine, 1 mM (MgCO3)4Mg(OH)2, 2.7 mM MgSO4, 0.1 mM EDTA, 33 mM DTT, 0.27 mM CoA, 0.53 mM ATP). Each sample was transfected in two biological duplicates; each experiment was repeated independently with consistent results.

**RNA extraction and gene expression studies.** Total RNA was isolated using commercial kits with DNase treatment (Norgen). CDNA synthesis was carried out

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**Table 1 siRNA and shRNA sequences.**

| Target gene | siRNA or shRNA Sequence |
|-------------|-------------------------|
| hFSCN1 A    | UGGCAAGUUUGUGACCUCC    |
| hFSCN1 B    | CAGCGUACCCGUAAGCGC     |
| hCAPZB A    | GAAGUACCGUGAAGGAGAuck  |
| hCAPZB B    | GGAGUGAUCCUAAAAAGA     |
| AllStars negative control (Qiagen) | Not available—proprietary information |
| Scramble control shRNA | UUCUCGAACGUGACCGU |
| mFSCN1 A    | CCGGCGATCCCGTGAAGCTTAAACG |
| mFSCN1 B    | TACTACGGAGATCTTTTGG    |

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**Table 2 Antibodies.**

| Epitope | Brand and catalog | Validation |
|---------|------------------|------------|
| CK19    | DSHB TROMAII     | Positive IF staining on bile ducts. NOTE: the antibody requires inclusion of fresh liver tissues in OCT without prior fixation with PFA. |
| A6      | DSHB A6 BCM      | Positive IF staining on bile ducts. |
| YAP/TAZ | SC-10199 used in vitro | Detects a prominent band in WB of the expected size, which fades in human cells treated with YAP siRNA. Detects nuclear/cytoplasmic shuttling of YAP in multiple conditions. |
| YAP     | PROTEINTECH 13584-1-AP | Detects a prominent band in WB of the expected size, which fades in human cells treated with YAP siRNA. Detects nuclear/cytoplasmic shuttling of YAP in multiple conditions in vitro. If signal is lost in YAP-null liver tissue. |
| FSCN1   | SC-21743         | IF staining shows a specific localization in filopodia and in cytoplasm colocalized with phalloidin. The staining fades in human cells and mouse tumors expressing FSCN1 siRNAs or shRNAs. |
| FSCN1   | ab126772         | Staining is consistent with the other antibody used in the study |
| HA      | SC-7392          | Positive WB or IF staining only in cells transduced with plasmids encoding for HA-tagged proteins |
| β-gal   | ab9361           | Positive IF staining only in tissues or cells expressing a lacZ transgene. |
Table 3 Primer sequences.

| qPCR Primer | For | Rev |
|-------------|-----|-----|
| hGAPDH      | CTCCTGCACCCACCAACTGCT | GGGCCATCCACAGTCTTCTG |
| hFSCN1      | CAAGAAAGAATGGGAGCTCGG | CTTTTGATGTTAGGCGCCA |
| hFSCN2_1    | ACGAGACTCTCTTGAATTCA  | CCAGCTGCCCATCCTCTTC |
| hFSCN2_2    | GAAGAAAGATGGGAGCTCGG | CAGGTGGAAAGACGTCTGAGA |
| hFSCN3_1    | TCCAGGCCCAATAGGGAATG | CTTGTGCCGTAAGGTTGAGA |
| hFSCN3_2    | GGCGTTAAAACGAAATGCTT | ATAGTCTGCGCAAGTCTC |
| mGAPDH      | ATCCCTGCACCCACCAACTGCT | GGGCCATCCACAGTCTTCTG |
| mANKR01     | CTGTTAGGCTGAACTCGCTAT | TCTCCTTGGACGCTTCGAAT |
| mCTGF       | CTGCCATCCAGCTGAGAGACG | CATTGTTAATCCTCGGTTGAGG |
| mFSCN1      | CAAGTTTGTGACCGCCAAGA | GTAGGCGCCGTCATTGAATT |

with M-MLV Reverse Transcriptase (Thermo) and oligo-dT primers. qPCR reactions were assembled with FastStart SYBR Green Master Mix (Roche) and run on a QuantStudio6 thermal cycler (Thermo). Gene expression levels for each biological sample were quantified as the mean between three technical replicates; GAPDH expression levels were used to normalize gene expression between samples. See Table 3 for primer sequences.

Statistics and reproducibility. Sample size was determined based on previous experience. All data consist of independent experiments with independent biological replicates. All r values are pooled between independent experiments. Samples were not blinded for analyses. Data analyses were performed using GraphPad Prism software. Graphs indicate mean values and single points of all biological sections. Significance was calculated by applying unpaired Welch’s t-tests between the indicated samples unless indicated otherwise in the figure legends.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Source data for the figures are available in the Supplementary Data 1 file and any remaining information can be obtained upon reasonable request to the corresponding author.

Code availability
No custom generated codes were used.

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**Additional information**

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