# Unraveling the actin cytoskeleton in the malignant transformation of cholangiocyte biology

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## ARTICLE INFO

## ABSTRACT

Correct actin cytoskeleton organization is vital in the liver organ homeostasis and disease control. Rearrangements of the actin cytoskeleton may play a vital role in the bile duct cells cholangiocytes. An abnormal actin network leads to aberrant cell morphology, deregulated signaling networks and ultimately triggering the development of cholangiocarcinoma (CCA) and paving the route for cancer cell dissemination (metastasis). In this review, we will outline alterations of the actin cytoskeleton and the potential role of this dynamic network in initiating CCA, as well as regulating the course of this malignancy. Actin rearrangements not only occur because of signaling pathways, but also regulate and modify cellular signaling. This emphasizes the importance of the actin cytoskeleton itself as cause for aberrant signaling and in promoting tumorigenic phenotypes. We will highlight the impact of aberrant signaling networks on the actin cytoskeleton and its rearrangement as potential cause for CCA. Often, these exact mechanisms in CCA are limited understood and still must be elucidated. Indeed, focusing future research on how actin affects and regulates other signaling pathways may provide more insights into the mechanisms of CCA development, progression, and metastasis. Moreover, manipulation of the actin cytoskeleton organization highlights the potential for a novel therapeutic area.

## Introduction

Cholangiocarcinoma (CCA) is a dismal disease caused by the malignant transformation of cholangiocytes, the epithelial cell layer lining the bile ducts [1,2]. The function of cholangiocytes is to transport and actively sense bile, a process that is highly dependent on the organization of intracellular actin filament networks. The actin cytoskeleton plays a major role in establishing and maintaining key cellular processes in cholangiocytes, such as membrane tension, structure and cell shape, polarity of membrane proteins, regulation of transporter and ion channels, and vesicular trafficking [3]. An abnormal actin network in cholangiocytes leads to aberrant cell morphology, deregulated signaling networks and can ultimately trigger the malignant development of CCA, paving the route for cancer cell dissemination (metastasis) [4]. Numerous cancer studies have described the impact of signaling pathways on the actin cytoskeleton, including CCA [5]. However, recent studies have highlighted that actin rearrangements not only occur because of the altered signaling pathways, but also regulate and modify cellular signaling [6–8]. This emphasizes the importance of the actin cytoskeleton as a direct cause for aberrant signaling and in promoting tumorigenic phenotypes.

In this review, we will outline alterations of the actin cytoskeleton and the potential role of this dynamic network in initiating and regulating the development of CCA. We will describe the changes of actin function in (a) sensing the extracellular milieu by the primary cilium, (b) the interface of extracellular matrix and cell membrane, (c) cell plasticity and motility and (d) nuclear localization and chromatin integrity. In the second part of the review, we will explain how actin can impair signaling pathways during CCA development, specifically its role in (a) biliary inflammation and (b) increased liver stiffness during liver fibrosis-to-cirrhosis. Finally, we will highlight therapeutic opportunities for targeting the actin cytoskeleton in CCA treatment.

## Rearrangement of the actin cytoskeleton in CCA

The actin cytoskeleton is organized in a hierarchical structure based on the expression of different actin isoforms [4] (Fig. 1). Actin can function either in its monomeric form, as globular actin proteins (G-actin), or polymerize into longer actin filaments (F-actin). This polymerization process is tightly regulated by actin-binding proteins

(ABPs). Different ABPs regulate the assembly/disassembly of F-actin through a complex network formation by actin branching or cross-linking of bundle filaments into actin stress fibers. If actin filaments interact with motor proteins, such as myosin, the actin cytoskeleton can mediate mechanical strength and contractility to the cell, which is essential for example in cellular migration and invasion. These specific processes are further regulated by Rho GTPases changing the activity status of ABPs and actin by post-translational phosphorylation. A panel of inhibitors is known to manipulate the actin organization. The most important inhibitors are summarized in Table 1.

In CCA, the actin cytoskeleton network is impaired at several different levels (Table 2). Aberrant signaling in CCA leads to deregulation of the actin cytoskeleton, cell morphology changes and subsequently to altered phenotypes such as, increased invasion [9,10], aberrant transcription, and loss of the primary cilium [11,12]. In the following section, we will discuss the role(s) and mechanism(s) of actin itself, and actin cytoskeleton components in oncogenic phenotypes by focusing on the actin cytoskeleton's function in ciliogenesis, adhesion, epithelial-to-mesenchymal transition (EMT), cell motility, and chromatin integrity.

*Sensing of the extracellular milieu: the primary cilium*

The primary cilium of cholangiocytes is an essential signaling hub to transmit extracellular osmotic, chemical, and mechanical changes inside the cell [13]. Although, the primary structure of the cilium is formed by microtubules protruding out of the centrosome, the actin network plays an essential role in cilium formation, length, and receptor localization. We will particularly focus on the chemosensory pathway (Hedgehog (Hh) signaling) and the mechano-sensitive receptors (polycystin (PC-1

**Table 1**
Commonly used actin inhibitors and their effects on actin organization.

| Inhibitor group | Effect on actin organization | REF |
|---|---|---|
| Cytochalasins (*e.g.,* A and D) | binds to actin filaments<br>prevents G-actin polymerization on barbed end<br>blocks assembly and disassembly of actin filaments | [125, 126] |
| Latrunculin (*e.g.,* A and B) | prevents actin polymerization by binding to G-actin<br>enhances actin depolymerization | [126] |
| Jasplakinolide | induces actin polymerization and F-actin formation by actin nucleation<br>actin stabilization, competitive binding to Phalloidin | [127, 128] |
| Phalloidin | actin filament stabilization<br>competitive binding with Jasplakinolide | [128] |
| Chondramide (*e.g.,* A and C) | actin polymerization and bundling | [129] |
| Swinholide (*e.g.,* A) | binds to barbed end of monomeric G-actin and polymerized actin | [130] |
| Miuraenamide (*e.g.,* A) | induces actin nucleation and polymerization | [131] |
| SMIFH2 | inhibits formin-mediated F-actin formation, but not *de novo* F-actin formation<br>inhibition of non-myosin IIA | [132] |
| Fasudil | inhibitor of myosin light chain (MLC) phosphorylation | [56] |
| Blebbistatin | inhibits ATPase activity of myosin II | [133] |

G-actin: globular actin; F-actin: filamentous actin; REF: references.

and PC-2)), and their impact on the actin cytoskeleton in CCA (Fig. 2).

Hedgehog (Hh) signaling is tightly regulated by the localization of the receptor smoothened (SMO), a G-protein-coupled receptor (GPCR)-
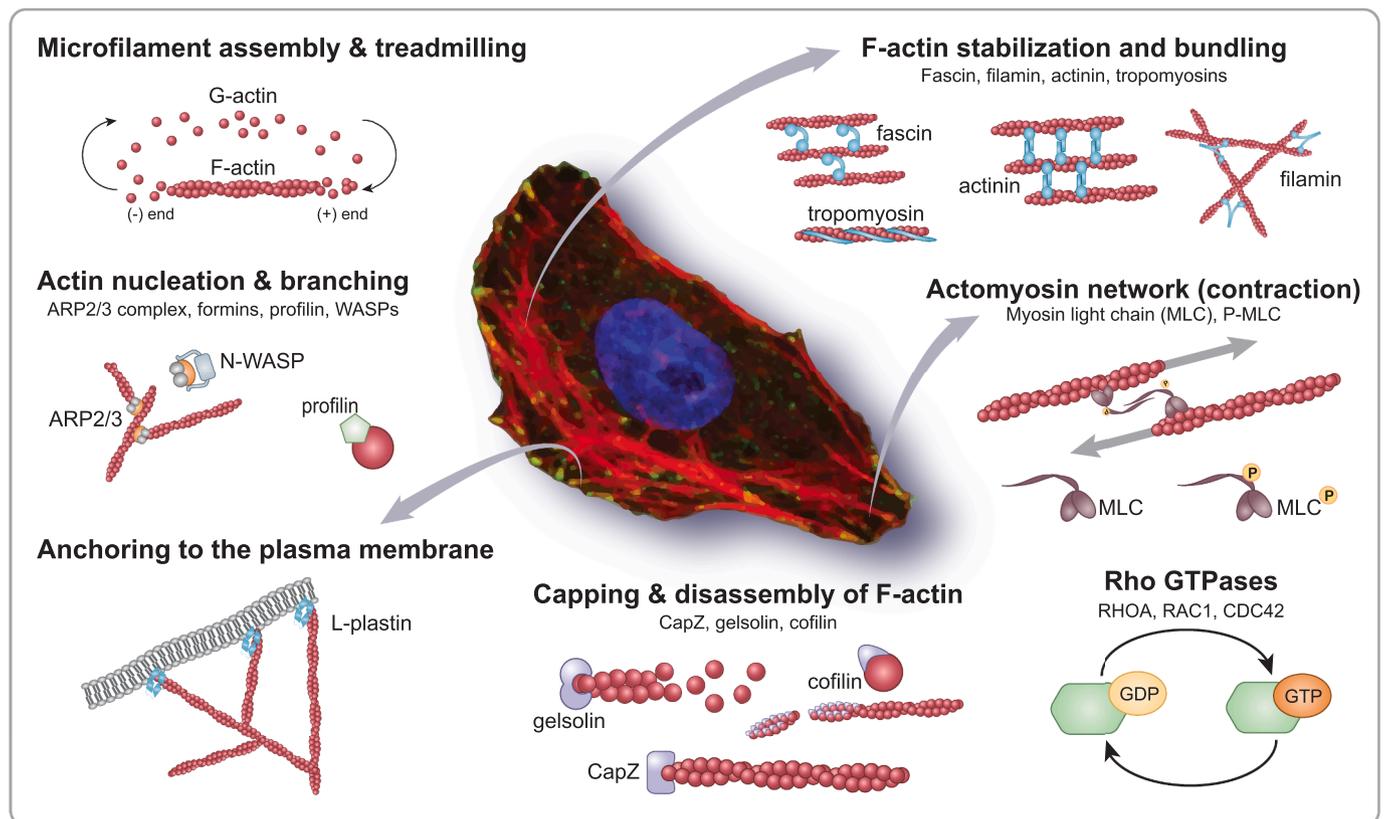


**Fig. 1. Overview of basic actin cytoskeleton components and their impact on the formation of actin networks.** Actin microfilament assembly is based on the treadmilling of globular actin (G-actin) to filamentous actin (F-actin). Actin nucleation and branching is regulated by actin-related protein 2/3 (ARP2/3) complex, formin, profilins and Wiskott-Aldrich syndrome proteins (WASPs). F-actin can be stabilized or bundled by fascin, filamin, actinin or tropomyosins and anchored to the membrane by L-plastin. Cellular contractions are mediated by myosin light chains (MLC) in the actomyosin network and post-translational phosphorylation by Rho GTPases (RHOA, RAC1, CDC42). Proteins promoting capping and disassembly of F-actin are CapZ, gelsolin and cofilin.

**Table 2**
List of altered genes in actin cytoskeleton regulation during cholangiocarcinogenesis generated based on the KEGG signaling pathway "Regulation of actin cytoskeleton" (hsa04810). If not indicated otherwise, expression levels are in relation to benign/normal.

| Gene name | Gene/ RNA/ protein | Origin | Expression level | Stage in cholangiocarcinogenesis | Effect, regulation mechanism and clinical impact | REF |
|---|---|---|---|---|---|---|
| ACTB | gene | tissue | up | CCA | Genomic amplification | [120] |
| | protein | tissue | up | iCCA | – | [134] |
| ACTN1 | protein | tissue | up | CCA | Biomarker | [135] |
| ACTN4 | RNA (circular) | tissue | up | iCCA | Tumor growth and metastasis Poor prognosis | [136] |
| | protein | tissue | up | CCA | Biomarker | [135] |
| APC | gene | tissue | mutation | iCCA | In less than 5% of cases | [137] |
| ARAF | gene | tissue | mutation | iCCA | Constitutive active mutation N217I Increased viability | [138] |
| ARP3 | protein | plasma | up | liver fluke | – | [139] |
| BAIAP2 | protein | EVs (Patients, cell models) | up | combined HCC/CCA | – | [140] |
| BRAF | gene | tissue | mutation | iCCA | Characterized in proliferation subclass | [141] |
| CDC42 | protein | tissue | down | congenital biliary atresia | Disruption of cell junctions and polarity | [142] |
| | RNA | tissue | down | CCA | Poor survival | [143] |
| CRKL | protein | tissue | up | combined HCC/CCA | Genomic amplification | [144] |
| CXCL12 | RNA | tissue, cell model | up | liver metastasis in CCA | Increased migration/invasion poor prognosis and survival | [145] |
| CXCR4 | protein | cell models | up | CCA | Increased migration/invasion activation of Akt signaling | [10] |
| | protein | tissue, cell models | up | iCCA | *In vitro* and *in vivo* tumorigenicity Poor overall survival | [146] |
| EGFR | gene | tissue | mutation | eCCA | Genomic amplification | [66] |
| | RNA | tissue | up | CCA | Worse survival | [55] |
| ERK1 | protein | tissue | up | CCA | – | [147] |
| ERK2 | protein | tissue | up | CCA | – | [147] |
| EZR | protein | tissue, cell models | down | CCA | Increased migration Increased tumor size with satellite nodules | [148] |
| | protein | tissue | down | iCCA | Worse survival | [149] |
| | protein | tissue (mouse) | down | cholestasis | Increased proliferation and morphology defects Fibrosis | [77] |
| F2 | RNA | tissue | down | CCA | DNA hypermethylation | [150] |
| FAK | protein | tissue | up | iCCA | Activates Akt signaling | [151] |
| FGF10 | protein | tissue (mouse) | up | IPNB | Induction of cholangiocarcinogenesis | [152] |
| FGFR2 | gene | tissue (mouse) | mutation | CCA | Constitutive active gene fusions | [153] |
| FGFR4 | protein | tissue, cell models | up | CCA | Glycosylated form leads to increased migration | [154] |
| FLNA | protein | tissue | up | pCCA | – | [155] |
| | protein | tissue | up | PSC-derived CCA | – | [156] |
| ITGA6 | RNA, protein | tissue, cell models | up | CCA | Increase migration, invasion, proliferation Inhibition by repressed miR-29–3p family | [44] |
| ITGB1 | RNA, protein | tissue, cell models | up | CCA | Increase migration, invasion, proliferation Inhibition by repressed miR-29–3p family | [44] |
| | RNA, protein | tissue | up | iCCA, pCCA, dCCA | Increase proliferation, migration, invasion Worse prognostic factor | [45] |
| | RNA | cell models | up | CCA | Increased adhesion and proliferation, Inhibition by lovastatin | [47] |
| ITGB3 | RNA | cell models | up | CCA | Increased adhesion and proliferation, Inhibition by lovastatin | [47] |
| ITGB4 | protein | tissue | up | iCCA | Bile duct invasion | [40] |
| ITGB6 | protein | tissue | up | iCCA | Bile duct invasion Worse survival | [40] |
| | protein | tissue | up | hilar CCA | Worse prognosis, drug resistance | [41, 42] |
| | protein | tissue, cell models | up | CCA | Increased proliferation, migration, invasion lymph node metastasis | [43] |
| KNG1 | protein | bile | up | CCA compared to benign/ PSC | – | [157] |
| | protein | serum | up | CCA compared to PSC | Upregulation of fucosylated protein form | [158] |
| KRAS | gene | tissue | mutation | iCCA | Recurrent mutations in G12A/C/D leads to deregulation of actin cytoskeleton pathway | [67] |
| LIMK1 | RNA | tissue | up | CCA | – | [159] |
| MAPK3 | protein | cell models | up | CCA | Inhibition by sorafenib leads to reduced MAPK3 phosphorylation and reduced proliferation | [160] |
| MEK1 | protein | tissue | up | CCA | – | [147] |
| | protein | cell models | up | CCA | CXCL12 activates MEK1 phosphorylation, actin polymerization and invasion | [10] |
| MEK2 | protein | cell models | up | CCA | CXCL12 activates MEK2 phosphorylation, actin polymerization and invasion | [10] |
| MYL9 | RNA | tissue | up | CCA | – | [161] |
| NRAS | gene | tissue | mutation | iCCA | – | [162] |
| PDGFA | RNA | tissue | up | CCA | – | [163] |

(*continued on next page*)

**Table 2** (*continued*)

| Gene name | Gene/ RNA/ protein | Origin | Expression level | Stage in cholangiocarcinogenesis | Effect, regulation mechanism and clinical impact | REF |
|---|---|---|---|---|---|---|
| | RNA, protein | tissue | up | biliary atresia | Biliary defects and hedgehog pathway activation in zebrafish larvae, DNA hypomethylation | [164] |
| | protein | tissue, cell models | up | CCA | – | [165] |
| PDGFB | protein | tissue, cell models | up | CCA | – | [165] |
| PDGFC | protein | tissue | up | CCA | – | [165] |
| PDGFD | protein | tissue, cell models | up | eCCA | Invasive tumors present higher expression | [166] |
| | protein | tissue | up | CCA | – | [165] |
| PDGFRA | RNA | tissue | up | CCA | – | [163] |
| | protein | tissue | up | CCA | – | [165] |
| PDGFRB | protein | cell models | up | CCA | Activation by myofibroblast derived PDGFB, Inhibition by imatinib induces apoptosis | [167] |
| PIK3CA | gene | tissue | mutation | BTC | – | [65] |
| PIK3CB | protein | plasma | up | CCA (non-/liver-fluke) | – | [168] |
| PIK3R1 | RNA | cell models | up | CCA | Mediates drug resistance Inhibition by repressed miR-29b | [169] |
| PPP1CB | RNA | cells (rat) | down | cholestasis | Inhibition by miR-218–5p in rat | [170] |
| PPP1R12A | RNA | tissue | up | CCA | Increased proliferation and decreased apoptosis Inhibition by repressed miR-455–5p | [171] |
| PXN | protein | cell models | up | iCCA | *Clonorchis sinensis* excretory-secretory products stimulate PXN expression and invasion | [172] |
| | RNA, protein | cell models, xenograft | down | CCA | clobenopropit treatment (H4 histamine receptor agonist) stimulates PXN expression and EMT, migration and invasion | [173] |
| RAC1 | protein | cell models | up | CCA | Induced by mechanical stretching | [56] |
| | RNA | tissue | up | CCA | Worse survival | [55] |
| RAF1 | gene | tissue | mutation | eCCA | Mutation in a single patient | [174] |
| RHOA | protein | cell models | up | CCA | Induced by mechanical stretching | [56] |
| ROCK2 | RNA | cell models | up | CCA | Increased migration and invasion Inhibition by repressed miR-200b/c | [175] |
| SOS1 | RNA | tissue | down | CCA | Worse survival | [143] |
| SRC | protein | cell models | mutation | cholangiocytes with mutated CFTR ΔF508 | Rearrangement of F-actin and increased inflammation | [176] |
| TIAM1 | RNA, protein | tissue, cell models | up | CCA | – | [177] |
| VAV1 | RNA | tissue | up | CCA | Positive correlation with CXCR4 expression | [178] |
| VCL | protein | cell models | up | iCCA | *Clonorchis sinensis* excretory-secretory products stimulate VCL expression and invasion | [172] |
| | RNA, protein | cell models | up | iCCA | VCL in combination with inactive LKB1 induces decreased adhesion, increased migration and metastasis | [179] |
| WAVE3 | RNA | tissue, cell models | up | iCCA | Worse overall survival Increased EMT, migration, invasion and proliferation | [180] |

iCCA: intrahepatic CCA; pCCA: perihilar CCA; eCCA: extrahepatic CCA; dCCA: distal CCA; IPNB: intraductal papillary neoplasm of the bile duct; BTC: biliary tract cancer, PSC: primary sclerosing cholangitis; EVs: extracellular vesicles; EMT: epithelial mesenchymal transition; REF: references.

like receptor, affecting chemotaxis, proliferation and apoptosis in CCA [11,14]. The canonical Hh signaling pathway gets activated by binding of the agonist Sonic hedgehog (Shh) to the ciliary located protein Patched 1 (PTCH1). PTCH1 is degraded in the lysosome, released, and enables the translocation of SMO into the cilium. The translocation of SMO to the ciliary tip increases intracellular G-protein signaling and leads to activation of the glioma-associated transcription factors (GLI1, GLI2A), that in turn, translocates into the nucleus and initiates transcription of Hh target genes. Although, the primary cilium is lost in CCA and thus, SMO is unable to translocate to the cilium, CCA cells remain chemosensitive to SMO agonist purmorphamine, indicating a still active Hh signaling [11,15]. The stimulation of human and rat CCA cells by purmophamine induces chemotaxis by actin rearrangements, lamellipodia and filopodia formation *in vitro*, and increased metastasis formation *in vivo* [5,11]. Similar observations have been shown in mouse embryonic fibroblasts (MEF) with two different *Smo* mutants either 1) lacking the ciliary localization domain (CLD) ($Smo^{\Delta CLD}$) or 2) harboring a C151Y point mutation in the N-terminal cysteine-rich domain ($Smo^{C151Y}$). These domains are both essential for proper ciliary translocation [16,17]. Expression of *Smo* mutants in MEFs on a $Smo^{-/-}$ background induces a stronger chemotaxis phenotype than observed in $Smo^{WT}$. In fibroblasts as well as CCA cells, SMO-dependent chemotaxis is

inhibited by $G\alpha_{i/o}$-protein inhibitor pertussin toxin (PTX). G-proteins are the downstream effectors of GPCRs, and form heterotrimeric complexes named after their alpha subunits ($G\alpha_{i/o}$, $G\alpha_q$, $G\alpha_s$ and $G\alpha_{12/13}$). $G_{i/o}$-proteins induce actin polymerization, mediating migration and chemotaxis to SMO agonists [16]. However, it remains unclear, if this mechanism is mediated directly or indirectly via activation of signaling cascades, including adenylyl cyclase (AC)/cAMP, PI3K/Akt, and RhoA/ROCK signaling [18]. These pathways are all deregulated in CCA and known to affect the actin cytoskeleton (**see section 1C**).

Mechanobiological changes in the bile flow are sensed by the ciliary protein complex PC-1/PC-2, containing the transmembrane receptor protein PC-1 and the calcium channel PC-2 [19]. Increasing intracellular calcium levels caused by changes in the biliary flow inhibit adenylyl cyclase 6 (AC-6), which is essential for the conversion of ATP into cAMP. Subsequently, lower cAMP levels reduce the catalytic activity of the cAMP-dependent kinase A (PKA) [19]. PC-1 and PC-2 are both genes highly mutated in autosomal dominant polycystic kidney disease (ADPKD) in up to 90% of patients, leading to polycystic liver disease (PLD). PLD is characterized by abnormal tubular structures and increased cyst formations in the biliary, renal, and pancreatic ducts [20]. Transcriptomic profiling of rat polycystic kidney (PCK) and human ADPKD compared to normal cells has revealed an overexpression of
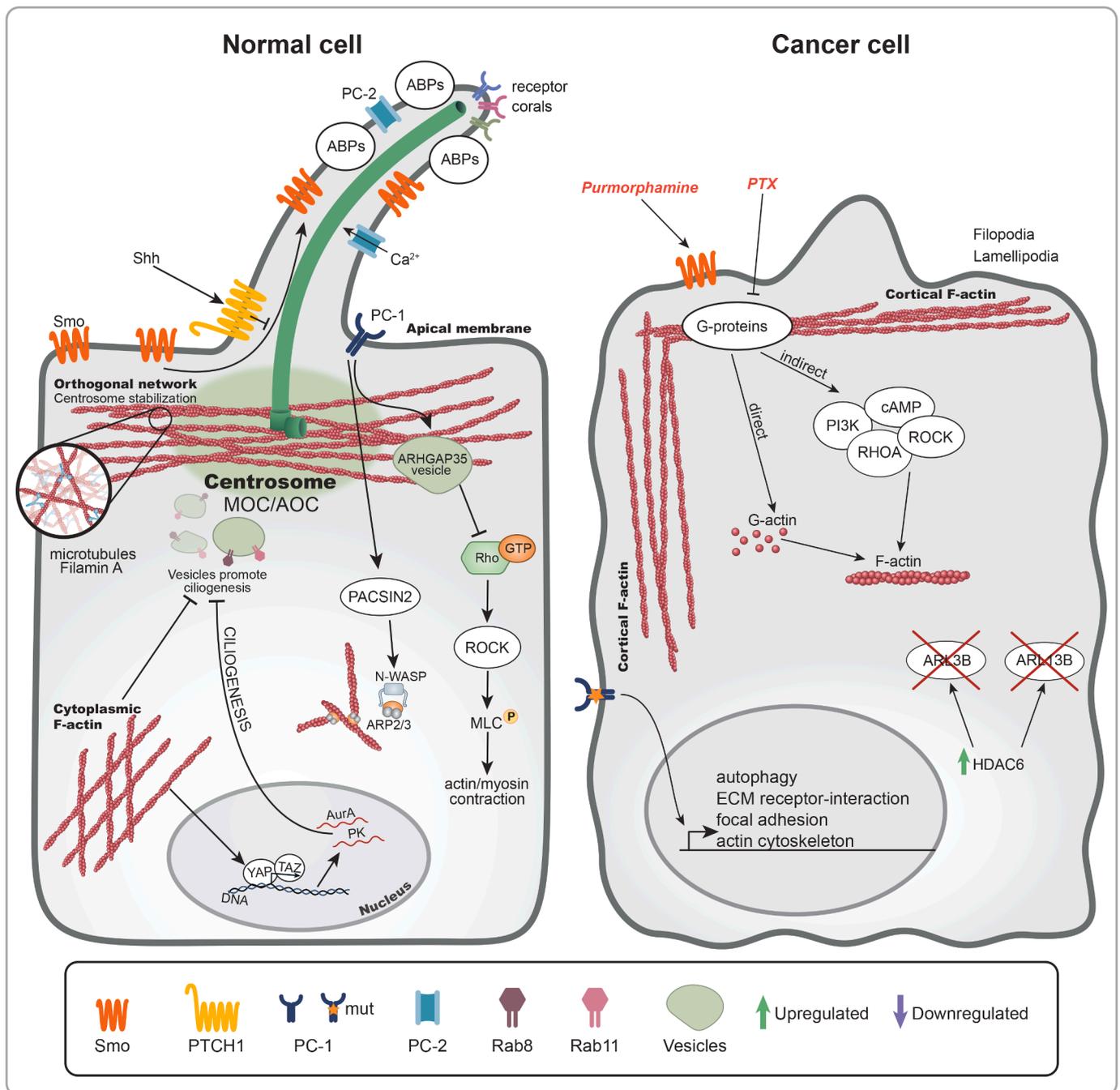
**Fig. 2. Overview of ciliary signaling pathways impacting actin cytoskeleton in the malignant transformation of cholangiocytes.** Ciliogenesis is promoted by degradation of cytoplasmic F-actin and transport of ciliary proteins to the apical membrane by rabin (Rab8, Rab11) positive vesicles. The centrosome is stabilized at the apical membrane by orthogonal actin networks crosslinked by filamin A and functions as microtubule and actin organizing center (MOC/AOC). Ciliary loss in CCA mediates a switch from canonical hedgehog signaling (Hh) in normal cholangiocytes to non-canonical Hh signaling in CCA cells. Activation of non-canonical Hh signaling increases PI3K, cAMP and RhoA/ROCK signaling, F-actin formation and subsequently migratory phenotypes. This process can be inhibited by the inhibitors Purmorphamine and PTX labelled in red. Polycystin-1 (PC-1) is regulating actin directly by PACSIN2 and indirectly by Rho GTPase Activating Protein 35 (ARH-GAP35) vesicles inhibiting RhoGTPases/ROCK signaling and phosphorylation of myosin light chains (P-MLC). In ADPKD, mutated PC-1 increases autophagy and expression of histone deacetylase 6 (HDAC6) leading to ubiquitination and degradation of the ciliary proteins ADP ribosylation factor-like GTPase 3b (ARL3B) and ARL13B. ABPs: actin binding proteins; Smo: Smoothened;PTCH1: Patched 1; Shh: Sonic hedgehog.

pathways involved in autophagy, extracellular matrix (ECM) receptor interaction, focal adhesion and genes involved in actin cytoskeleton regulation [21]. Indeed, mutations in PC-1 in renal cells cause major rearrangements of the actin cytoskeleton, as shown by phalloidin staining of F-actin [22]. Organized F-actin on the basal membrane of normal cells is redistributed into disorganized, short and thick F-actin bundles in the cellular cortex of cystic cells [22]. This rearrangement is

caused by two indirect regulatory mechanisms of actin mediated by PC-1. First, PC-1 induces F-actin polymerization by activation of nucleation factors (neural Wiskott-Aldrich syndrome protein (N-WASP) and actin-related protein 2/3 (ARP2/3)) through protein-protein interaction with the C-terminus of Pacsin-2 [23]. Second, PC-1 regulates actin-myosin interaction through Rho GTPase Activating Protein 35 (ARHGAP35). Wildtype PC-1 recruits ARHGAP-35 to the centrosome by

vesicular trafficking promoting GTP hydrolysis of RHOA, thereby inhibiting the activation of ROCK kinase signaling. PC-1 mutations result in loss of this ARHGAP-35-dependent RHOA inhibition, ROCK activation, and increased phosphorylation of myosin light chains (P-MLC), causing contraction of the actin cytoskeleton [22,24]. Additionally, autophagy (an actin-regulated process) plays an essential role in the aberrant cilium-formation in ADPKD and CCA [25]. In ADPKD, the overexpression of histon deacetylase 6 (HDAC6) leads to ubiquitination and degradation of the ciliary proteins ADP ribosylation factor-like GTPase 3b (ARL3B) and ARBL13B [20]. Interestingly, HDAC6 is overexpressed in CCA and has been shown to impair cilia formation [26,27]. Yet, if the role of HDAC6 in cilia formation is through autophagy, it remains unknown how actin is involved in this process in CCA [25].

Microtubules are the basic structure of the cilium. However, several studies are highlighting the importance of actin in the process of regulating cilia formation, the cilium length and cellular signaling [12]. For cilia formation, ciliary proteins and the centrosome are transported to the apical membrane by the actin cytoskeleton. Ciliary proteins can be transported by actin filaments directly by actin-binding motif proteins [28] or indirectly in rabin (Rab8/Rab11)-positive vesicles [29]. Following degradation of cortical F-actin [12], the centrosome re-localizes and is stabilized by an orthogonal actin network, which is formed by the actin-branching protein Filamin A (FLNA) during ciliogenesis [29]. Here, the centrosome functions as the basal body both in microtubule and actin organization centers (MOC and AOC), regulating the nucleation and network formation of either microtubule- or actin-filaments [30]. It is only recent that studies have identified actin and ABPs within the primary cilium and started to elucidate their function within renal cells [29,31]. Using techniques like cryo-electron tomography, filamentous actin structures have been visualized in the primary cilium of MDCK-II cells [31]. This is further supported by proximity-based proteomics of the ciliary protein 5-hydroxytryptamine receptor 6 (HTR6) [29], which revealed the localization of actin and ABPs at the ciliary base and within the cilium. Proteomics has helped to identify high abundance of actin proteins (ACTA1, ACTB, ACTG1), F-actin fragmentation proteins (GSN) and ABPs (ACTN1, ACTN4, TPM) within the cilium [29]. A potential function of actin and ABPs in the cilium can be the correct organization of receptors, such as GPCR somatostatin receptor type 3 (SSTR3) and the establishment of membrane polarity [32]. The clustering of receptors in 'receptor corals' or free diffusion is essential in fine-tuning the downstream signaling [32]. These studies were performed in renal cells, showing the localization of actin and ABPs in the primary cilium. We are still lacking similar studies to be performed in cholangiocytes.

*Cellular and extracellular matrix interface*

Besides the cilium, cholangiocytes sense their cellular environment and the ECM by plasma membrane proteins. Accumulation of ECM is at the origin of a desmoplastic reaction that typically characterizes CCA tumors [33,34]. The role of ECM in CCA has been experimentally demonstrated to cooperate to carcinogenesis [35] and tumor progression [34]. Among ECM proteins, periostin (PN), laminin gamma 2 (LAMC2), osteopontin (SPP-1), secreted protein acidic and rich in cysteine (SPARC), thrombospondin-1 (THBS1), collagen type-1, 3 and 4 (COL1, COL3, COL4), are correlated with poor prognosis in human CCA [33,34,36–38]. One of the major membrane protein families involved in ECM sensing and cellular adhesion in the tumor microenvironment (TME) are integrins, transmembrane receptors expressed at the cell surface, which allow for the 'integration' of signals coming from the external milieu to the interior actin cytoskeleton (Fig. 3) [39]. Integrins are heterodimeric receptors composed by two chains (18 alpha and 8 beta subunits), providing at least 24 receptor combinations [39]. Several integrins are overexpressed in human intrahepatic [40] or hilar [41,42] CCA compared to non-tumor tissues. Upregulation of integrin β6
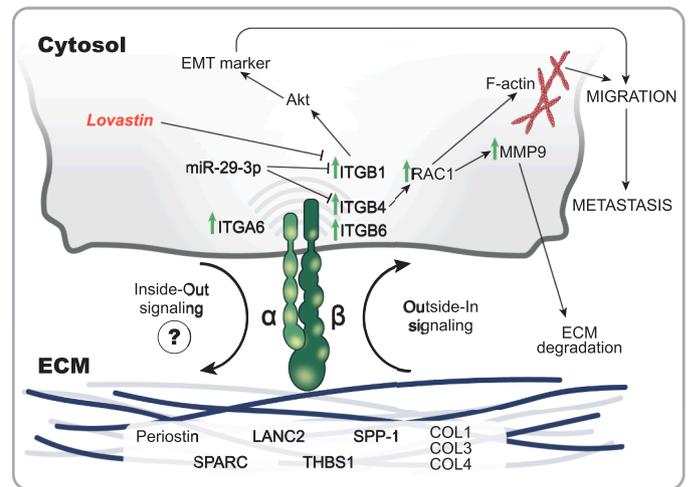


**Fig. 3. Extracellular matrix sensing by integrin signaling affects actin cytoskeleton components and induces migration and metastasis formation**. Extracellular matrix components (Periostin, SPARC, LANC2, THBS1, SPP-1, COL1, COL3, COL4) are sensed by integrins composed of an alpha and beta subunit chain. Overexpression of different integrins (ITGA6, ITGB4, ITGB6, ITGB1) activates Akt signaling, RAC1 and F-actin formation resulting in ECM degradation, migration and finally metastasis formation. Several integrins can be inhibited by miR–29–3p or inhibitor Lovastatin. Integrin-mediated inside-out signaling remains unknown in CCA. Cellular and clinical phenotypes are written in capital letters and inhibitors are highlighted in red.

(ITGB6) is correlated with clinicopathological features in iCCA [40], including lymph node and distal metastases [43]. *In vitro*, ITGB6 increases RAC1 activity in human CCA cell lines, resulting in remodeling of the actin cytoskeleton with an increase of F-actin polymerization and metalloproteinase-9 (MMP9) expression [43]. These events both result in ECM degradation and cell migration. In hilar CCA, upregulation of ITGB6 is associated with poor prognosis [42]. Other integrins are upregulated in CCA, including ITGA6 [42,44], ITGB1 [44–46] and ITGB4 [40]. ITGB1 is a prognostic factor for CCA [45], and both ITGB1 and ITGB6 are negatively regulated by the miR-29-3p family and contribute to CCA cell invasion [44]. As such, PN binding to ITGB1 induces the expression of mesenchymal markers favoring EMT and migratory properties of CCA cells via an AKT-dependent signaling pathway [46]. A potential treatment option to target integrin overexpression in CCA is Lovastatin, a 3-hydroxy-3-methylglutaryl-coenzyme-CoA (HMG-CoA) reductase inhibitor, which was shown to inhibit the expression of ITGB1 and ITGB3 in CCA cell lines (RBE and Huh-28) [47]. Lovastatin treatment efficiently decreases the proliferation and migration of CCA cells by inhibition of stress-fiber formation and cellular adhesion [47]. The above discussed research on integrin signaling solely focuses on their intracellular function to integrate external signals (so called outside-in signaling), but does not describe the integrin-mediated inside-out signaling. It still remains to be elucidated if and how intracellular actin rearrangements may affect ECM repositioning potentially enhancing desmoplastic phenotypes in CCA [48].

Interaction between ECM proteins and malignant cells drives the phenotypic cellular changes, acquiring metastatic features. If cultured on ECM gel (3-D culture model), CCA cells display a drastically modified actin cytoskeleton, with an increase expression of key ABPs such as L-plastin, ezrin (villin 2), fascin and cofilin-1 [9]. L-plastin localizes to actin-rich membrane structures *in vitro*, and its inhibition reduces CCA cell invasion. In human CCA samples, L-plastin is mainly localized in the cell nuclei, in which it may play a role in the regulation of nuclear actin and transcription (**see section 1D**). In tumor cells that display a more mesenchymal-like phenotype and can invade the basement membrane (metastasize), L-plastin is found in the cytoplasm [9]. Uniquely, CCA

cells cultured in 3-D release L-plastin into the extracellular milieu, a phenotypic trait not seen when culturing mixed hepatocellular-cholangiocarcinoma (CHC) cells [49]. Thus, L-plastin has been proposed to serve as a diagnostic biomarker that can differentiate tumor types (CCA and CHC).

*Cell plasticity and motility*

CCA is characterized by an early and high metastatic burden [2]. One prerequisite for metastasis formation is the tumor cell's ability to migrate and invade into the tumor-adjacent tissue (liver parenchyma). Migration and invasion are two malignant phenotypes caused by several signaling pathways that lead to a rearrangement of the actin cytoskeleton. Tyrosine kinase receptors (RTKs) upon ligand binding trigger cell features, which allow for proliferation and migration of cancer cells. Several RTKs, including the fibroblast growth factor receptor (FGFR) [50] or epidermal growth factor receptor (EGFR) families [51,52] are major inducers of actin cytoskeleton remodeling (Fig. 4). Upon FGFR activation, CCA cells exhibit higher levels of actin polymerization in the cell periphery, with the formation of pseudopodia (for example, filopodia and lamellipodia), an effect that can be abrogated by using a MEK inhibitor [50]. Similarly, the chemokine CXCL12 (or SDF-1) induces pseudopodia and CCA cell invasion through its receptor CXCR4, MEK1/2, and PI3K pathways [10]. Also, downstream PI3K, the mTOR pathway appears critical for the formation of migratory protrusion structures. Everolimus, a potent inhibitor of mTOR signaling, alters the actin cytoskeleton and reduces filopodia formation in CCA cells [53]. In contrast to pro-invasive stimuli, $\Delta^9$-tetrahydrocannabinol (THC), the principal active component of cannabinoids, has an opposing effect, decreasing actin polymerization in CCA cells, and inhibiting migratory and invasive features [54]. These signaling pathways often affect Rho GTPases (RHOA, CDC42 and RAC1), which are key regulators of actin nucleation, polymerization, and contractility. Rho GTPases directly affect ARPs (as the nucleation factor N-WASP) or indirectly by activating the kinase ROCK. Subsequently ROCK phosphorylates myosin light chain phosphatases and actin-regulating LIM kinases. Interestingly, Rho

GTPases themselves are deregulated in CCA. RAC1 is differentially expressed between 2 CCA subgroups in a subclass of CCA patients with better prognosis [55]. Mechanical stress caused by increased tissue stiffness stimulates the expression of RHOA and RAC1 (in both CCA and HCC cells) inducing migratory and invasive phenotypes [56], which can be abolished by pamidronate treatment, a drug used for treatment of osteoporosis [57].

*Nuclear actin and chromatin integrity*

Nuclear actin is the `cytoskeleton´ in the nuclear matrix, which participates in DNA repair, genome integrity, chromatin remodeling, and transcriptional regulation. The nuclear polymerization of actin has long been debated as it in its polymerized form cannot be stained by phalloidin [58]. In fact, the polymerized form of actin in the nucleus differs from the cytoplasmic conformation and accounts only for a small proportion of the primarily monomeric actin in the nucleus.

Monomeric actin and ARPs take part in modifying and remodeling chromatin. A key chromatin-remodeling complex is the ATPase-dependent Switch/Sucrose Non-Fermentable (SWI/SNF) complex. This complex is responsible for opening the chromatin by removing or repositioning histone octamers, allowing for active DNA repair, regulating transcription, and controlling genome stability by ATP hydrolysis [59,60]. In mammalian, three SWI/SNF complexes exist: (1) BAF (BRG1-associated factor complexes containing ARID1A), (2) PBAF (polybromo BRG1-associated factor containing PBRM1), and (3) ncBAF (noncanonical BAF). Using cryo-electron microscopy of *S.cerevisiae* BAF and human PBAF has shown that both complexes contain an actin-related subunit, which is located between the ATPase domain and the core complex, including Swi1 (ARID1) or PBRM1 [59,60] (Fig. 5). The actin-related subunit is essential for the structural complex integrity, binding of actin filaments for nucleosome recruitment and regulation of the ATPase activity of BRG1 [61]. The structure consists of one monomeric actin molecule and the oncogene *ARP4* (or *ACTL6A/BAF35*) [59,60,62,63]. In hepatocellular carcinoma (HCC), overexpression of ARP4 is linked to poor prognosis and involved in activating Notch signaling thereby increasing cell migration, invasion and EMT *in vitro*
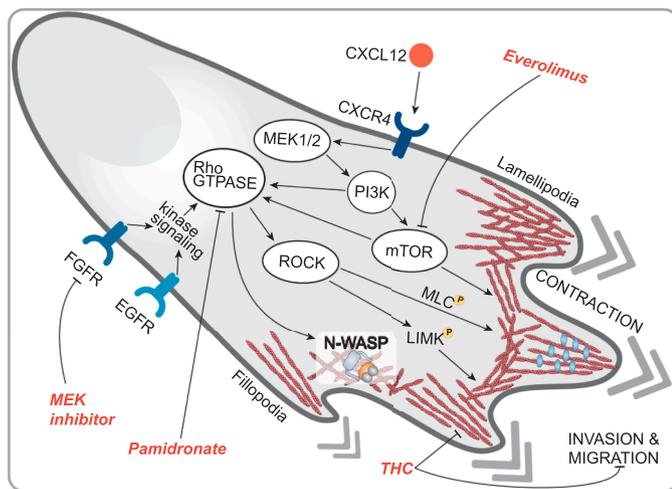


**Fig. 4. Overview of receptor tyrosine kinase (RTK) and cytokine receptor signaling affecting Rho GTPases in CCA triggering the migration and invasion of CCA cells.** RTKs (fibroblast growth factor receptor FGFR, epidermal growth factor receptor EGFR) and cytokine signaling (CXCL12, CXCR4) activate a kinase signaling cascade, Rho GTPases and mTOR signaling and subsequently trigger N-WASP and ROCK activation. The resulting elevated levels of F-actin induces the formation of lamellipodia and filopodia and actin-myosin mediated contraction, increased migration, and invasion (cellular phenotypes are highlighted in capital letters). The RTK signaling can be inhibited on several levels in the signaling cascade by MEK inhibitors, Pamidronate, Everolimus and THC (highlighted in red).
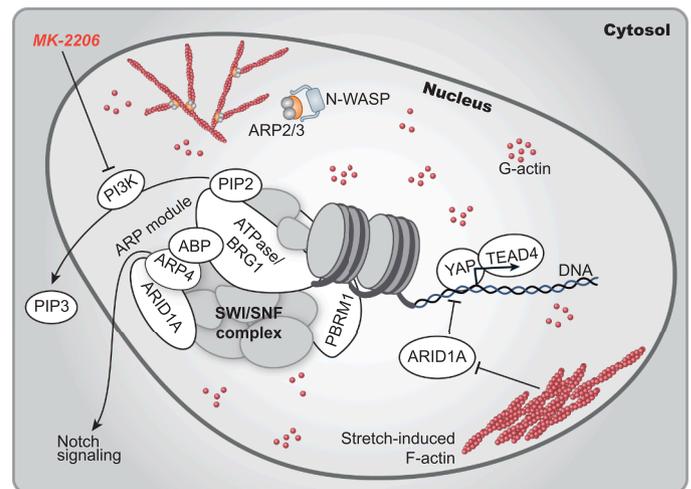


**Fig. 5. Role and function of intracellular actin in chromatin remodeling Switch/Sucrose Non-Fermentable (SWI/SNF) complex in CCA.** The chromatin remodeling complex SWI/SNF consists of the ATPase BRG1, the proteins PBRM1/ARID1A and is stabilized and linked to nuclear actin by the actin-related protein (ARP) module consisting of ARP4 and monomeric actin. Loss-of-function mutations of SWI/SNF components or activation of Notch signaling by ARP4 overexpression are linked to worse survival in CCA. Loss of ARID1A function by stretch-induced nuclear F-actin induces YAP/TEAD4 signaling, expression of stem-like genes and increased sensitivity to the PI3K inhibitor MK-2206 (highlighted in red).

and tumor growth and metastasis *in vivo* [63]. In CCA, the actin-related SWI/SNF component remains unstudied. However, loss of function mutations in *ARID1A* (11–16%), *ARID1B* (5%), *ARID2* (4–6%) and *PBRM1* (21%) [64–67] are among the most recurrent mutations in CCA patients [68], which also are associated to worse prognosis [69,70]. Deletion of *ARID1A* in CCA (*in vitro* studies in HuCCT1 and RBE cells) increases migration and invasion [70]. In patients with *ARID1A* mutations [71], CCAs are often characterized as the infiltrating mass-forming type, which may suggest a role of the actin cytoskeleton. In fact, ARID1A may be linked to the regulation of actin through Yes-associated protein 1 (YAP) and PI3K/Akt signaling. ARID1A knockout mice under DDC (3, 5-diethoxycarbonyl-1,4-dihydrocollidine) diet develop CCA-like lesions with increased YAP expression [72], emphasizing why an upregulation of stem-like genes are observed in *ARID1A* knockout CCA cells [70]. Mechanistically, ARID1A binds to YAP, inhibiting the YAP/TEAD4 complex and activating transcription, a process that is regulated by mechanical stress and nuclear F-actin. Increased stretching of Hek293T cells causes actin at the nuclear border to start forming filaments and binding to ARID1A that prevents ARID1A-YAP complex formation [72]. Additionally, CCA cells expressing low levels of ARID1A are more sensitive to PI3K/Akt signaling inhibitor MK-2206 [73]. Interestingly, the PI3K substrate (PIP2) is required for SWI/SNF complex stability and binding of BRG1 to actin filaments [74]. PIP2 activates the complex N-WASP-ARP2/3 inducing actin filament nucleation and inhibiting the binding of the actin-severing protein cofilin resulting in the formation of stable actin filaments.

**Actin remodeling and signaling—what comes first?**

Recent studies elucidated the important function of the actin cytoskeleton in liver homeostasis and early tumorigenesis. This highlights that changes in the actin cytoskeleton might not solely be a consequence of aberrant signaling, but can cause altered signaling in CCA [6]. Therefore, the second part of the review will provide arguments to study the actin cytoskeleton during early stages of CCA development, which could subsequently lead to alterations in signaling and CCA progression. We will describe actin cytoskeleton rearrangements in the context of CCA risk factors particularly during infection and liver cirrhosis.

*Role of actin cytoskeleton in biliary inflammation*

Chronic inflammation of the biliary tree is a key risk factor for CCA, and mainly caused by either cholestasis or viral, parasite and bacterial infections. The primary mechanism of infection is through bile secretory function in hepatocytes and cholangiocytes and is tightly regulated by transporters localized at the apical domain. Integrity of the actin cytoskeleton and the linker proteins are critical for the functional regulation of the apical transporters. In hepatocytes, the bile canalicular lumen comprises a pericanalicular actin cortex linked to integral plasma membrane proteins by radixin, a member of the ERM (ezrin-radixin-moesin) family, which acts as a cross-linker. A similar structural architecture is present in the apical domain of cholangiocytes, with expression of the ERM cross-linker protein ezrin, a protein expressed exclusively in the biliary lineage [75]. Genetically-engineered murine knockdown models either of radixin [76] or ezrin [77] have demonstrated the role of these ERM proteins in the liver epithelium. In radixin knockout mice, a loss of microvilli, a structure characterizing the canaliculi membrane of hepatocytes, along with an apical loss of multidrug resistance-associated protein 2 (MRP2) [76] cause hyperbilirubinemia in mice, a phenotype equivalent to Dubin-Johnson syndrome in humans. Ezrin-deficient mice develop a severe intrahepatic cholestasis following a dysregulation of the bile fluidity into the bile duct epithelium [77]. Ezrin deficiency in cholangiocytes results in loss of apical expression of several key transporters (such as cystic fibrosis transmembrane conductance regulator (CFTR), anion exchange protein (AE-2), and aquaporin-1 (AQP1)) and of ERM-binding phosphoprotein

of 50 kDa (EBP50/NHERF-1), a PDZ-scaffold protein highly expressed in bile duct cells [77,78]. Ezrin-deficient mice have no prominent alterations in the cholangiocytes neither of microvilli nor their primary cilia structure. However, mutation in the above transporters may have consequences on the actin cytoskeleton and associated proteins. CFTR is a chloride transporter anchored at the apical plasma membrane to the actin cytoskeleton via EBP50 and ezrin [79]. In CFTR-defective cholangiocytes, the F-actin cytoskeleton is defective and EBP50 is mis-localized into the cytosol, resulting in destabilization of apical membrane organization and function [80]. The consequence of CFTR disorganization is an activation of proinflammatory signaling, simulated through a Src-dependent kinase feedback loop and subsequent activation of Toll-like receptor 4 (TLR4) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kβ) upon endotoxin exposure.

Other sources of chronic inflammation include parasite and bacterial infections. In these cases, inflammation is caused by secretion of excretory-secretory products and internalization of liver fluke and bacteria into cholangiocytes and hepatocytes. The role of actin cytoskeleton rearrangement in parasite internalization is well-established [81,82]. *Cryptospodium parvum* infection in cholangiocytes requires host cell actin remodeling at the attachment site. At the host-parasite interface, c-Src is activated and phosphorylates ABP cortactin [82], along with activation of the Cdc42 pathway and the nucleation machinery of ARP2/3 complex proteins. This process causes branching of actin filaments, facilitating the membrane protrusion and the parasite entry [83]. Protrusion-formation is further facilitated by water influx through the translocation of AQP1 and Na+/glucose cotransporter 1 (SGLT1) mediated by the actin myosin network regulator myosin IIB [84].

Similarly, bacterial infection of epithelial cells is associated with an actin cytoskeleton remodeling and an inflammatory response. Actin polymerization is required for infection and internalization of *Helicobacter pylori* (*H. pylori*) and *Shigella flexneri* in CCA cells [85]. Pilus-like structures of *H. pylori* interact with integrin α5β1 on the surface of the host cell both in CCA [85] and HCC [86], and induces actin polymerization to allow internalization of the bacterium [85]. Once internalized, the bacterium stimulates expression of Nucleotide-binding oligomerization domain containing 1 (NOD1), TLR4, and TLR5, as well as activates the NF-Kβ pathway and interleukin 8 (IL-8) production in CCA cells [85]. In gastric cells, at the bacterium-host cell interface, *H. pylori* activates paxillin by phosphorylation at focal adhesion and regulates cytoskeletal reorganization to form actin stress-fibers, which favors cancer cell mobility and inflammation. Activation of paxillin by *H. pylori* depends on EGFR, FAK-Src, and PI3K/Akt signaling pathways [87]. Exposure of CCA cells in culture to *H. pylori* results in phenotypic changes, including the loss of cell-cell contact, filopodia protrusion, and induction of EMT-inducing transcription factors and cancer stem cell marker CD44. These features all favor malignant transformation of the biliary epithelium and progression of CCA [88]. *H. pylori* plays a role in altering stemness via its cytotoxin-associated gene CagA by inducing the expression of reprogramming factors (Splat-like transcription factor 4 (SALL4) and Kruppel-like factor 5 (KLF5)) as well as nuclear accumulation of β-catenin. In addition, these cells also display higher expression of epithelial splicing regulatory protein (ESRP1), which is involved in upregulating alternative splicing of CD44 (CD44$^{total}$) generating the stemness marker CD44 variant 9 [89].

*Impact of liver stiffness on the function of actin in mechano-transduction*

Cirrhosis is one of the key risk factors for CCA [2]. During the change from a normal to a cirrhotic liver, the liver tissue stiffness is increasing and is used as a diagnostic measure (fibrosis F0–3; cirrhosis (F4)) [90]. Increased liver stiffness negatively affects the function of the hepatocytes and overall liver function. In fact, mimicking cirrhosis by transferring hepatocytes onto a stiff matrix leads to nuclear deformation and reduces the hepatocyte function observed by decreased mRNA

expression of albumin and hepatocyte nuclear factor 4α (HNF4α) [90]. Expression of these genes can be restored by disruption of the cytoskeleton-nuclear interaction administering actin (Cytochalasin A) and microtubule inhibitor (nocodazole), highlighting the importance of the cytoskeleton in mechano-transduction. Besides the cytoskeleton and polarity protein complexes, YAP and transcriptional coactivator with PDZ-binding motif (TAZ) are key intracellular mechano-sensing effector proteins. In healthy hepatocytes and cholangiocytes YAP/TAZ signaling is inactive and contact-inhibition by neighboring cells prevents proliferation. The Hippo signaling pathway is a major inhibitor of YAP signaling, which is orchestrated through phosphorylation of YAP by the kinases LATS1/2 [91–93]. Phosphorylation of YAP excludes it from the nucleus and prevents its function as a transcription factor. Piezo-1 (PIEZO1) is a mechano-sensitive ion channel protein overexpressed in CCA cells, which induces migration and invasion *in vitro* as well as lung metastasis *in vivo* [91]. Activation of PIEZO1 by its agonist Yoda-1 induces EMT in CCA cells through the activation of YAP and reduced LATS1/2 phosphorylation. It is unknown, if PIEZO1 is regulating the phosphorylation of LATS1/2 directly or indirectly (direct activation of YAP). In this process, the actin cytoskeleton takes solely the role of a downstream effector of the Hippo pathway. Several lines of evidence argue for a LATS1/2-independent mechanism regulating YAP activity [94]. Neither knockdown of LATS1/2 nor YAP/TAZ mutants (insensitive to LATS1/2 phosphorylation in normal cells) can rescue YAP transcriptional activity, which indicate an additional inhibitory mechanism [94]. F-actin bundles can directly inhibit YAP signaling in cells in a stress-free surrounding. Knockdown or chemical inhibition of proteins preventing the formation of strong F-actin fibers (such as cofilin, CAPZ, gelsolin and formins) cause YAP activation, whereas inhibition of ARP2/3 has only minor effects on the YAP activity [94–96]. These studies in mammary epithelial and fibrotic breast cells have been reproduced in hepatocytes. Inhibitors of F-actin bundling (Cytochalasin D, latrunculin A) and the formin inhibitor (SMIFH) all induce the activation of YAP in hepatocytes *in vitro* [97]. Overexpression of the actin bundling protein (Fascin-1) *in vivo* in the murine liver induces YAP-dependent proliferation and dedifferentiation of ductular cells into atypical cells positive for cytokeratin 19 (CK19), countering the effects of the F-actin-capping protein subunit beta (CAPZB) and ARP2/3 [96]. Similar phenotypes can be observed in mice with liver specific *Capzb* knockout [7]. Additionally, these mice have impaired hepatocyte zonation and metabolism with improved glucose tolerance and decreased expression of gluconeogenic genes, which can be rescued by YAP inactivation [7].

Single-cell RNA-sequencing has shown that during liver injury elevated mechanical tension leads to YAP activation in biliary epithelial cells (BEC) [98]. Besides, the above-mentioned F-actin bundles, actin-myosin fibers play an essential role in this process. Mechanical stress following bile duct ligation results in expansion of the apical surface of hepatocytes, increased levels of F-actin and phosphorylation of actin-myosin [97]. Stretching of CCA and HCC cells *in vitro* increases the expression of Rho GTPases and mediates the phosphorylation of myosin light chains (P-MLC) [56]. Since fasudil (inhibitor of MLC phosphorylation) can inhibit this process, both the expansion and bile canaliculi contractility are dependent on actin-myosin. Depending on the apical actin integrity, YAP is localized to the apical F-actin layer, but with increasing mechanical stress and contraction YAP is released and translocates to the nucleus. Disruption of F-actin by *Capzb* knockout increases ROCK activity and MLC phosphorylation, further inducing YAP activity and resulting in a positive feedback loop [94]. Transcriptomic analyses of YAP targets in CCA and HCC have identified a positive feedback loop on the actin network via the AMPK kinase NUAK Family Kinase 2 (NUAK-2) [8]. By combining analyses of (1) TEAD4-CHIP seq from liver of TetO-YAP mice, (2) YAP-ChIP-seq in HuCCT1 CCA cells, (3) RNA-seq of YAP/TAZ silenced mouse liver and (4) RNA-seq from TetO-YAP mice, Yuan et al. [8] have identified 14 YAP-regulated gene-targets in liver cancer. Among the identified genes

are two well-known YAP-target genes (angiomotin (*AMOT*) and *NUAK2*). Depletion or chemical inhibition of NUAK-2 by HTH-02–006 partially rescued the YAP-dependent tumorigenesis in HCC and CCA *in vitro* and *in vivo* [8]. Mechanistically, NUAK-2 phosphorylates S445 in the myosin phosphatase target subunit (MYPT1), thereby inhibiting the MLC phosphatase (MLCP). Increased levels of MLC phosphorylation and loss of actin fibers induce liver stiffness and YAP signaling, which subsequently triggers more actin fibers and actomyosin contraction [94].

In summary, increased cellular tension activates YAP and is favored by the formation of F-actin bundles and the actin-myosin network. In contrast, in healthy tissues with low contraction and space limitation, YAP activity is inhibited by ABPs, which promotes F-actin disassembly and branching. It remains unclear how the cell senses the content of F-actin. Further, it is controversial if F-actin regulation is through direct or indirect Hippo signaling or both or alternatively a secondary effect of cellular tension.

## Therapeutic opportunities for targeting actin cytoskeleton in CCA

We have highlighted actin cytoskeleton deregulation through different signaling pathways and cellular components in CCA including the primary cilium, cell-ECM interaction, intracellular receptor signaling, and intranuclear functions. These pathways encompass therapeutic options for targeting either directly or indirectly to modify the deregulation of the actin cytoskeleton in CCA.

Major proteins and signaling pathways, such as Hh and HDAC6 signaling, are linked to the primary cilium, and have shown to affect the actin cytoskeleton in CCA. Among pharmacological inhibitors, SMO antagonizes Hh signaling, some of SMO inhibitors being already approved by the US Food and Drug Administration (FDA) in other cancer types. Cyclopamine is a natural alkaloid SMO inhibitor that effectively counteracts the development digestive tract cancer and CCA [99,100]. The continuous advancement in the development of cyclopamine derivatives has resulted in the second-generation compound vismodegib (GDC-0449), which are approved by the FDA for treatment in advanced basal cell carcinoma [101]. Interestingly, vismodegib inhibits the invasion of tumor cells *in vivo* in a rat model of CCA [11], a compound deserving to be investigated in the framework of CCA.

Two HDAC6 inhibitors (ACY-1215 and tubastatin A) have both shown favorable results in CCA. ACY-1215 inhibits the proliferation of cystic cholangiocytes *in vitro* and *in vivo* [26] and has shown promising results in a multi-institutional phase Ib/II study in relapsed lymphoma [102]. Tubastatin A represses CCA growth and restores the formation of the primary cilium [26], but due to complications in the delivery method of this compound, it has not yet moved into clinical trials [103]. Lovastatin is an inhibitor of cellular-ECM interaction, which is mediated by integrins [47]. As such, lovastatin has been used as a breast cancer prevention in patients with abnormal breast ductular cytology, but in a phase II setting it showed no significant change, questioning a beneficial application in CCA [104].

Intracellular signaling inducing actin rearrangements as well as cellular motility and plasticity are often mediated by RTKs such as EGFR, FGFR, and CXCR4, to which many tyrosine kinase inhibitors (TKI) have been developed. Within the TKI category are included inhibitors against the constitutively active FGFR2 fusion protein and prevalent *FGFR* mutations as seen in a subset of iCCA patients. This category includes the FDA approved pemigatinib [105], infigratinib (phase 2) [106], and futibatinib (phase 1) [107]. Similar results have been achieved by the simultaneous inhibition of BRAF and MEK signaling combining dabrafenib and trametinib, showing a benefit in 20 out of 43 BRAF$^{V600E}$ mutated CCA patients [108]. Also, an antagonist against CXCR4 (AMD3100) approved by the FDA had minor effects on solid tumors and showed significant adverse side effects [109]. Although, AMD3100 has been developed into the structurally similar compound (BPRCX807), which in the preclinical setting prevents tumor growth,

cell migration, and metastasis in HCC [110]. Finally, the benefits of the mTOR inhibitor (everolimus) is currently investigated in phase IV in liver transplants and liver tumor recurrence (NCT02081755), but results from this trial is awaited. In contrast to inhibition of RTKs, the restoration of silenced proteins like ARID1A and PBRM1, which are often mutated in CCA, is a more challenging approach and require continued investigation [111].

The above-mentioned treatment options can only affect the actin cytoskeleton in CCA by inhibition of cellular migration and invasion. Interestingly, actin rearrangements have been shown to mediate resistance to cisplatin, used in combination with gemcitabine as current standard-of-care in CCA, in several different cancer types. As such, the overall expression levels of filamin and actin are reduced in cisplatin-resistant compared to matched parental epidermoid and liver carcinoma cell lines [112]. In prostate cancer, cells surviving 24 h of cisplatin treatment were characterized by increased cellular stiffness and decreased migration rates caused by actin stress fiber formation, actin disintegration and tubulin rearrangements [113]. In addition, several transporters involved in cisplatin influx (for example, VSOR) and efflux (for example, ABC7A/B) are regulated by the actin cytoskeleton in their cellular localization and membranous activity [114].

In CCA, the current standard-of-care for patients with locally advanced tumors is chemotherapy with a combination of gemcitabine and cisplatin, resulting in a median progression free survival (PFS) of 8 months [115]. Thus, these patients might benefit from a combination with one of the above-mentioned drugs targeting the actin cytoskeleton. In contrast, direct inhibition of the actin cytoskeleton (Table 1) might help in the prevention of CCA development in individuals associated with risk factors like biliary inflammation and liver cirrhosis as emphasized in the second part of the review. Manipulation of the actin cytoskeleton and its organization highlights a potential for a novel therapeutic area often missed [116]. Targeted disruption of the actin network in tumor cells by magnetic field-responsive supramolecular assemblies increases tumor cell death and thus, reduces tumor growth *in vivo* [117]. Other promising drugs include chondramide B, miuraenamide A (derived from myxobacteria strains), and jasplakinolide that induce actin stabilization and polymerization, and reduces tumor growth and invasion (Table 1) [118,119].

### Conclusion and future perspectives

The actin cytoskeleton is significantly affected in CCA and contributes to the malignant transformation of cholangiocytes. Focusing future research on how actin affects and regulates other signaling pathways may provide more insights into the mechanisms of CCA cancer development, progression, and metastasis. Impact of the basic actin cytoskeleton components, such as actin isoforms (ARPs), ABPs, and Rho GTPases, remains largely unknown in cholangiocarcinogenesis (Table 2). Particularly, the actin isoform ACTB, known to be involved in invasive and metastatic tumors, is overexpressed in CCA [120] and may present an interesting future target. Nevertheless, studying actin remains challenging as many fixation and staining methods interfere with the actin integrity and dynamics. Fixation reagents (for example, methanol and paraformaldehyde), temperature conditions and buffer compositions can disrupt actin networks and F-actin membrane structures [121,122]. A potential solution avoiding cell fixation is live cell-imaging, but actin markers, such as LifeAct, must be optimized carefully as these markers impact actin polymerization in a concentration-dependent manner [123]. Therefore, optimization and appropriate controls are vital. For understanding the spatiotemporal organization of the actin cytoskeleton, proximity-based labeling techniques combined with proteomics are suitable to elucidate protein interactions in specific cellular organelles and subcellular localizations [124]. The actin cytoskeleton is a central pathway in the development of cholangiocarcinoma and tumor maintenance.

### Declaration of Competing Interest

JBA declares consultancy roles for Flagship Pioneering, SEALD and QED therapeutics.

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