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One stone, two birds: circACTN4, a nexus for a coordinated activation of Hippo and Wnt/β-catenin pathways in cholangiocarcinoma

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Abbreviations: CCA, cholangiocarcinoma; circRNA, circular RNA; EVs, extracellular vesicles; miRNA, microRNA; RBP, RNA binding protein.

Journal Pre-proof

Cholangiocarcinomas (CCA) gather a group of tumors arising along the hepatobiliary tree. CCA is a rare cancer, but its incidence has been increasing over the last decade. CCA is associated with a poor prognosis and limited effective therapeutic options [1]. Innovative strategies are therefore urgently needed to identify novel therapeutic targets and clinically relevant biomarkers to ultimately improve the survival of patients with CCA [1]. Circular RNAs (circRNAs) are splicing products forming a continuous loop by joining the 3' extremity of a downstream exon with the 5' extremity of an upstream exon. Although circRNAs are mainly outlined for their ability to sponge microRNAs (miRNAs) and RNA binding proteins (RBPs), their modes of action remain largely unknown [2]. Latterly, circRNAs emerged as new regulatory molecules contributing to human carcinogenesis with a promising potential as biomarkers [2]. However, very few studies reported the role of circRNAs deregulated in CCA so far [3-7]. Notably, an increased expression of circ-CCAC1 has been identified by circRNA expression profiling in tumor tissues and bile-resident extracellular vesicles (EVs) from patients with CCA [7]. CCA-delivered exosomal circ-CCAC1 was reported to be transferred to endothelial cells to disrupt endothelial barriers and induce angiogenesis [7]. Mechanistically, circ-CCAC1 increased cell permeability by sequestering enhancer of zeste homolog 2 (EZH2) in the cytoplasm, thereby increasing SH3GL2 expression to suppress intercellular junction proteins [7]. Besides this well-designed functional study, most of the published reports highlighting circRNAs in CCA solely focused on the clinical relevance of their expression. Thus, a comprehensive analysis of their functional role in the gene regulatory networks contributing to CCA onset and progression has been barely addressed so far. In addition, 80-90% of reports focused on the sponging capacity of circRNAs as a mechanism of action. We would like to point out that circRNAs are more than simple sponges for miRNAs. Indeed, emerging data from the literature demonstrate that circRNAs take active part of complex transcriptional and post-transcriptional regulatory systems.

In this issue of *Journal of Hepatology*, Chen and colleagues illustrate this concept by investigating the function and the clinical relevance of circular RNA ACTN4 (circACTN4) in

intrahepatic CCA (iCCA). They demonstrate that circACTN4 is induced in iCCA and promotes proliferation and invasion of tumor cells *in vitro* and *in vivo*. Importantly, the study implies circACTN4 as a signaling nexus allowing a coordinated activation of Hippo and Wnt/β-catenin pathways, notably by acting on different targets in the cytoplasm and the nucleus **(Figure 1)**. Indeed, circACTN4 is reported i) in the cytoplasm, to sponge miR-424-5p, which could target the oncogenic transcriptional coactivator Yes-associated protein 1 (YAP1) from the Hippo pathway and ii) in the nucleus, to recruit Y-Box Binding Protein 1 (YBX1) transcription factor to initiate the transcription of *Frizzled-7* (*FZD7*), a positive regulator of the Wnt/β-catenin signaling pathway [8]. CircACTN4 is also shown to favor the interaction between YAP1 and β-catenin and their nuclear accumulation, which ultimately leads to CCA cell growth and metastasis **(Figure 1)**. Thus, this study exemplifies circRNAs as sponges for miRNAs and as scaffolds to locate transcription factors to the nucleus. The data suggest that the specific location of circRNAs within the cells could play a significant role in controlling gene expression. Overall, the study by Chen and colleagues unraveled circRNAs as intracellular nodes acting simultaneously in the cytoplasm and in the nucleus through specific regulatory processes. However, this study raises unanswered questions. Notably, the dynamic regulation of circRNAs shuttling between the nucleus and the cytoplasm, in relationship to their mode of action, is an important feature which remains to be elucidated. In addition, whether or not circACTN4 acts as a scaffold by interacting with DNA and/or other co-factors to recruit YBP1 to the promoter of *FZD7* remains to be determined.

The mechanisms regulating the spatial localization of circRNAs are yet poorly understood, although this parameter is probably of special importance in determining their activity. The reason why intron-containing circRNAs are restrained to the nucleus to act as transcriptional regulators while exonic circRNAs are enriched in the cytoplasm to operate as post-transcriptional regulators by sponging miRNAs and/or RBPs is unclear [9]. Several reports explored possible mechanisms whereby circRNAs could shuttle from the nucleus to the cytoplasm. One of them

described an evolutionarily conserved pathway that controls the nuclear export of circRNAs depending on the length of the transcripts. Hence, long (> 800 nt) or short circRNAs could be translocated to the cytoplasm with the assistance of UAP56 or URH49, respectively [10]. Wang and colleagues described a nuclear export process specific for a circular repeat containing intron. They identified a NXF1:NXT1-related active process regulating the level of intronic circRNAs in the cytoplasm [11]. Another study pinpointed N^6-methyladenosine (m6A) epitranscriptomic modifications favoring the export of circNSUN2 to the cytoplasm [12]. These studies highlight the importance of better characterizing the mechanisms regulating circRNA spatial localization to better understand the underlying molecular mechanisms involved in CCA progression. In the future, the modulation of such transport mechanisms could be considered as an option to modulate circRNA availability and activity.

When it comes to circRNAs, most studies describe a cytoplasmic circRNA sequestrating a couple of miRNAs, which are therefore not available to inhibit their predicted target genes. However, it is becoming obvious from emerging functional studies that investigating the molecular mode of action of circRNAs should not be considered through a unique axis. Indeed, several reports, including the one from Chen and colleagues in this issue of *Journal of Hepatology* [8], established that circRNAs could operate through a multi-layered coordinated fashion, by acting at different levels on several gene regulatory networks. The ability of a single circRNA to concomitantly sponge miRNAs in the cytoplasm and to recruit RBPs in the nucleus to mediate RNA Pol II transcription has been also reported in gastric cancer [13]. Hence, circFAT1 exerts tumor suppressive actions by regulating the miR-548g/RUNX1 axis in the cytoplasm and by targeting YBX1 in the nucleus to inhibit its activity [13]. However, the process regulating circFAT1 spatial localization is unknown [13]. Factors regulating RNA circularization, such as QKI5 RBP, could also act at a post-transcriptional level to competitively regulate the level of canonical and back-splicing. That competition could be explained by a shared spliceosome machinery for intron and exon definition and back-splicing [14, 15]. Although initially viewed as

non-coding RNAs, growing evidence indicate that circRNAs could also be translated, notably by cap-independent-mediated mechanisms that rely on the internal ribosome entry site and m6A modifications [16]. Characterizing the function of circRNAs-derived peptides is still at its infancy, but several reports described a role as protein baits [17]. An original mode of action of circβ-catenin, a circRNA derived from β-catenin has been recently reported [18]. Circβ-catenin differs from its cognate mRNA by a new stop codon generated by the circularization junction. Thus, circβ-catenin is translated into a 370-amino acid β-catenin isoform which acts as a decoy. Indeed, this shorter isoform can stabilize full-length β-catenin by antagonizing GSK3β-induced β-catenin phosphorylation and degradation, leading to Wnt/β-catenin pathway activation and promotion of tumor cell growth [18]. In breast cancer, circSMARCA5 was shown to combine several of the functions described above. Indeed, circSMARCA5 binds its parental gene locus forming a R-loop with DNA and regulates concomitantly the splicing machinery and the transcriptional pausing, leading to the production of a nonfunctional truncated SMARCA5 protein [19]. Eventually, growing evidence suggest that circRNAs act as important cellular regulators with various modes of action. However, how many functions a single circRNA can fulfill needs to be determined. This is a fundamental point to address before thinking of circRNAs for a clinical translation as therapeutic targets, as suggested by Chen and colleagues for circACTN4.

Chen and colleagues reported in this issue of *Journal of Hepatology* that a high expression of circACTN4 in iCCA tissues was associated with a worse survival [8]. Although numerous circRNAs are already described for their potential as prognostic biomarkers, none have been proved to be worth for a clinical use. Nonetheless, circRNAs attracted a substantial interest by their presence in body fluids (*e.g.* serum, plasma, saliva) circulating either freely or encapsulated into EVs, particularly in exosomes (100-200nm EVs) [20]. Moreover, circRNAs display an expanded half-life compared to linear RNAs, suggesting they could challenge the sensitivity and specificity of standard biomarkers. Thus, measuring the level of circulating circRNAs in liquid

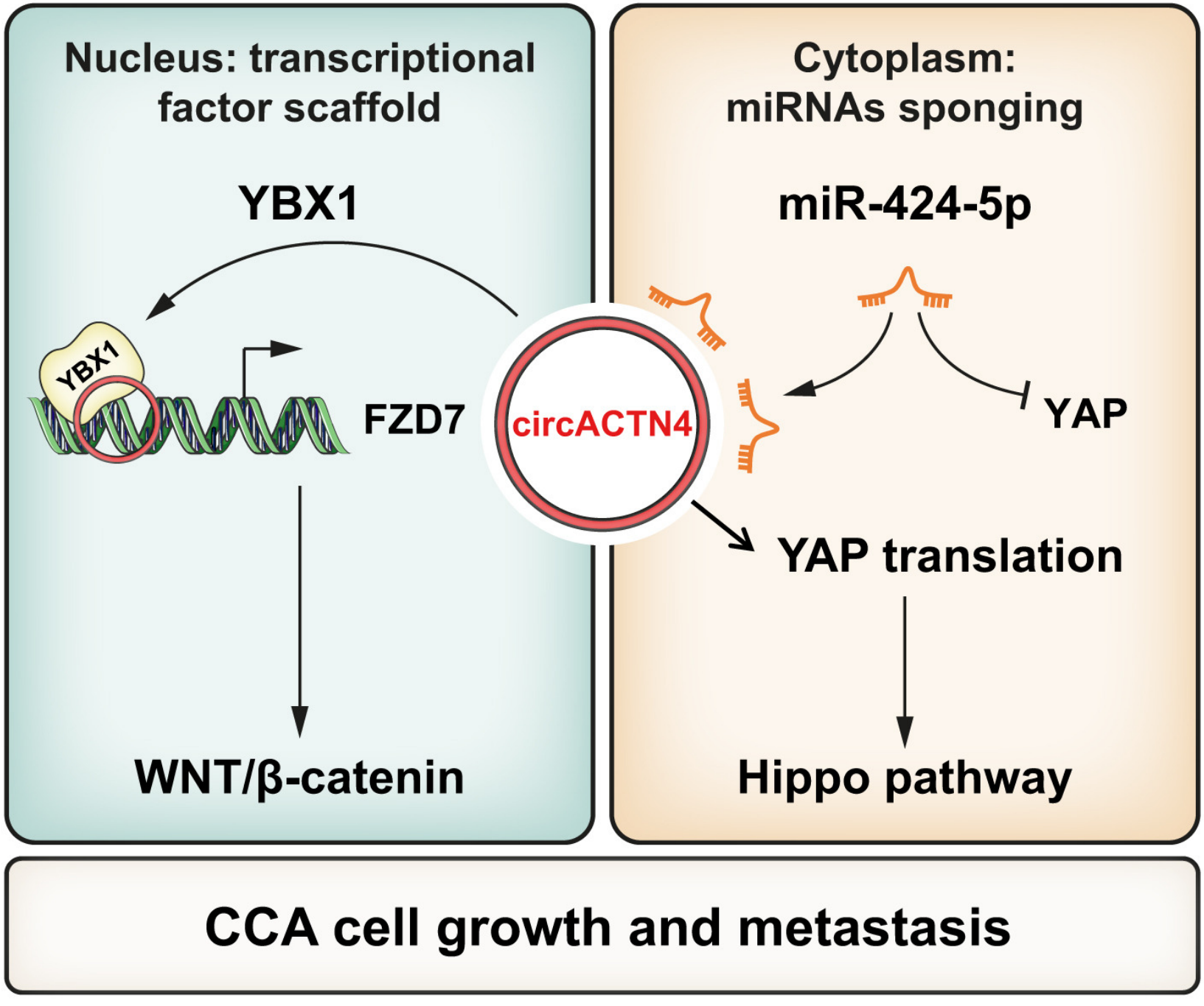
biopsies represents an appealing alternative to tissues biopsies to improve the management of patients with CCA [20].

Taken collectively, the study by Chen and colleagues provides an elegant example on how circRNAs could contribute to a coordinated activation of signaling pathways involved in CCA progression. With other pioneer studies in the field, it highlights the promising potential of circRNAs as targets to develop innovative therapeutic strategies and to identify clinically relevant biomarkers. Nevertheless, the field is still in its infancy, with many unanswered questions, notably as regard to the exact functions and modes of action of circRNAs in the gene regulatory network contributing to tumor progression. Integrating coding and non-coding RNA profiles could lead to a comprehensive picture of circRNA/miRNA/mRNA networks and to better understand the underlying molecular mechanisms involved in CCA tumorigenesis.

Figure 1. Squaring the circle: emerging roles of circRNAs in CCA. In this issue of *Journal of Hepatology*, Chen and colleagues report the role and the clinical relevance of circACTN4 in CCA [8]. The study demonstrates that circACTN4 is induced in CCA and exhibits specific but complementary functions depending on its subcellular localization. Thus, in the cytoplasm circACTN4 acts as a sponge sequestrating miR-424-5p and therefore preventing the degradation of YAP1, from the Hippo pathway. In the nucleus, circACTN4 recruits YBX1 to initiate *FZD7* transcription and contributes to activate the Wnt/β-catenin pathway. Altogether, the coordinated activation of Wnt/β-catenin and Hippo signaling pathways by circACTN4 contributes to CCA cell growth and metastasis.

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Author names in bold designate shared co-first authorship


Nucleus: transcriptional factor scaffold
YBX1
YBX1
FZD7
WNT/β-catenin
circACTN4
Cytoplasm: miRNAs sponging
miR-424-5p
YAP
YAP translation
Hippo pathway
CCA cell growth and metastasis

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