

REVIEW

Alternative Splicing in Hepatocellular Carcinoma

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SUMMARY

Alternative splicing controls key metabolic pathways important for liver development and hepatocyte homeostasis. Global changes in RNA binding proteins yield novel tumor-associated transcripts and protein isoforms in hepatocellular carcinoma. Studies addressing these mechanisms illuminate new diagnostic, prognostic, and therapeutic targets for hepatocellular carcinoma.

Hepatocellular carcinoma (HCC) accounts for the majority of primary liver cancer cases, with more than 850,000 new diagnoses per year globally. Recent trends in the United States have shown that liver cancer mortality has continued to increase in both men and women, while 5-year survival remains below 20%. Understanding key mechanisms that drive chronic liver disease progression to HCC can reveal new therapeutic targets and biomarkers for early detection of HCC. In that regard, many studies have underscored the importance of alternative splicing as a source of novel HCC prognostic markers and disease targets. Alternative splicing of pre-mRNA provides functional diversity to the genome, and endows cells with the ability to rapidly remodel the proteome. Genes that control fundamental processes, such as metabolism, cell proliferation, and apoptosis, are altered globally in HCC by alternative splicing. This review highlights the major splicing factors, RNA binding proteins, transcriptional targets, and signaling pathways that are of key relevance to HCC. We highlight primary research from the past 3–5 years involving functional interrogation of alternative splicing in rodent and human liver, using both large-scale transcriptomic and focused mechanistic approaches. Because this is a rapidly advancing field, we anticipate that it will be transformative for the future of basic liver biology, as well as HCC diagnosis and management. (*Cell Mol Gastroenterol Hepatol* 2020;10:699–712; <https://doi.org/10.1016/j.jcmgh.2020.04.018>)

Keywords: mRNA; Metabolism; Cancer; Variants.

Liver cancer-associated mortality in the United States has doubled in the past 2 decades, and continues to increase at a faster rate than any other cancer type.¹ Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer.^{2,3} HCC is the end result of chronic hepatocyte injury,⁴ and generally develops in the context of cirrhosis resulting from viral hepatitis, alcoholic liver

disease, or nonalcoholic steatohepatitis (NASH).³ In the United States, chronic hepatitis C (HCV) infection still accounts for the majority of HCC cases, but the burden of HCV has decreased in recent years⁵ with the approval of all-oral antiviral regimens that have near-complete response rates.⁶ However, the proportion of NASH-related HCC cases among patients listed on the liver transplant list has increased significantly in the past few years.⁷ NASH is the most severe form of nonalcoholic fatty liver disease (NAFLD), which affects a quarter of the general population.⁸ Compared with healthy controls, patients with NAFLD are 7 times more likely to develop HCC, and this risk is higher in those with cirrhosis.⁹ With an overall 5-year survival rate of 18%, liver cancer represents a significant health burden that will become even greater in the near future because it is projected that, by 2030, it will be the third leading cause of cancer-related deaths.¹⁰

Prevention and early detection strategies for HCC are challenging to implement because of the long disease course and the high interindividual variability in tumor growth patterns, as assessed by imaging studies on large cohorts of patients.¹¹ Therefore, targeted interventions are needed urgently to extend the quality of life and increase the survival rates among HCC patients. In that respect, understanding key drivers of chronic liver disease progression to HCC can uncover novel strategies for selective targeting of HCC tumors. There are now numerous examples from the literature that highlight how alternative pre-messenger RNA (mRNA) splicing yields new proteins that have either lost a tumor-suppressor function, or have gained new oncogenic functions in HCC. This review highlights some of the major findings published on this topic within the past 3–5 years.

Abbreviations used in this paper: BIN1, Myc box-dependent-interacting protein; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; EMT, epithelial-mesenchymal transition; ESRP2, epithelial splicing regulatory protein 2; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; hnRNP, heterogeneous nuclear ribonucleoprotein; KHK, keto-hexokinase; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; MBNL, muscleblind-like; mRNA, messenger RNA; MTR4, Exosome RNA helicase MTR4; Myc, Myc proto-oncogene protein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NONO, non-POU domain-containing octamer-binding protein; PRPS1, ribose-phosphate pyrophosphokinase 1; RBP, RNA binding protein; SRSF, serine/arginine-rich splicing factor; TAZ, WW domain-containing transcription regulator protein 1; YAP, Transcriptional coactivator YAP1.

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2352-345X

<https://doi.org/10.1016/j.jcmgh.2020.04.018>

Alternative RNA Splicing Is Dysregulated in Human HCC

Post-transcriptional gene regulation, in particular alternative splicing,¹² is frequently dysregulated in cancer,¹²⁻¹⁴ in part owing to somatic changes in key genes, such as *TP53*.¹⁵ Alternative splicing is a mechanism for controlled gene expression that involves the production of multiple mRNA transcripts from a single gene.¹⁶ This mechanism is important for the abundance and diversity of protein isoforms,^{17,18} and is particularly critical during development for tissue specification.^{19,20} Alternative splicing and other post-transcriptional control mechanisms, such as mRNA turnover and translation, are integrated with gene transcription to regulate key aspects of cellular metabolism.²¹ A prime example of this is the regulation of insulin signaling by a controlled rate of insulin mRNA translation,²² glucose-mediated insulin mRNA stabilization,²³ and alternative splicing of the insulin receptor.²⁴ Insulin, in turn, controls the expression of more than 1000 liver transcripts, including both coding and noncoding RNAs.²⁵

Most multi-exon human genes undergo alternative splicing, and different tissues possess unique splicing signatures.²⁶ Of the more than 13,000 protein-coding genes expressed in the adult human liver, more than >80% were found to undergo alternative splicing to produce 4 or more transcripts.²⁷ Skipped exon and alternative use of the first exon are the most common splicing events in the liver.^{27,28} Compared with normal liver, there is a high degree of differential splicing in primary HCC tumor tissues and many of these changes correlate with HCC patient survival.^{27,29} A growing number of studies have shown that altered splicing programs in HCC tumor cells give rise to novel protein isoforms that often have distinct, and sometimes opposing, functions from their canonical counterparts³⁰⁻³⁸ (Table 1). In a global sense, genes regulating the cell cycle, cell proliferation, DNA repair, metabolism, and the epithelial-mesenchymal transition (EMT) are differentially spliced in HCC tumors compared with nontumor adjacent tissue.^{27,28}

Among these pathways, metabolism-related genes are the most common, particularly those that are associated with carbohydrate processing. Furthermore, some splicing events are linked strongly to the etiology of HCC³⁹ and can be a source of novel biomarkers of disease activity.⁴⁰ For example, a splicing switch in the fibroblast growth factor receptor 2 is associated with the presence of hepatitis B virus or HCV infection, and the tumor-specific fibroblast growth factor receptor 2 splice variant isoform correlates with tumor size.⁴⁰

Changes in RNA-Binding Proteins and Splicing Factors in HCC

One potential mechanism underlying the vast changes in splicing programs between tumor and nontumor tissue in HCC is the differential expression of RNA binding proteins (RBPs)²⁷ and splicing factors.⁴¹ In HCC tumors, 231 RBP-encoding genes were found to be up-regulated, whereas 55 were downregulated relative to nontumor tissue.²⁷ Interestingly, a few RBP-encoding genes appear to serve as master regulators of many cellular pathways. For example, *SNRPA* and *RALY* each regulate more than 1000 splicing events and are predicted to impact up to 30 different pathways in HCC.²⁷ This is not surprising because *SNRPA* encodes a component of the spliceosome, the complex molecular machinery that catalyzes pre-mRNA splicing.⁴² The precise function of *RALY* is not known, but this gene encodes a heterogeneous nuclear ribonucleoprotein (hnRNP) that may be a potential oncogene.⁴³ Interestingly, there are also a number of RBP genes that were found to regulate fewer splicing events, but those events were linked to a large number of pathways. For example, the gene *RBM24* was associated with 200 splicing events, but linked to more than 20 cellular pathways.²⁷ *RBM24* encodes an RNA binding protein that is both a target and regulator of the tumor-suppressor p53,⁴⁴ which frequently is mutated in human HCC^{45,46} and known to limit tumor progression in mice.⁴⁷ In addition to its function as a tumor

Table 1. Examples of Novel Splice Variants Expressed in HCC and Associated Cellular Pathways

Gene	Protein	Splice variant isoform	Major associated pathway	Literature source
<i>BIN1</i>	Myc box-dependent-interacting protein-1	Long variant BIN1L	Regulation of membrane signaling and Myc	Malakar et al, Cancer Res 2017
<i>CCDC50</i>	Coiled-coil domain-containing protein 50	Short variant CCDC50S	Ubiquitin-proteasome, cytoskeleton	Wang et al, Hepatology 2019
<i>INSR</i>	Insulin receptor	Short variant IR-A	Insulin signaling	Chettouh et al, Cancer Res 2013
<i>KHK</i>	Ketohexokinase	Variant KHK-A	Fructose metabolism	Li et al, Nat Cell Biol 2016
<i>NF2</i>	Merlin	Short variant Δ2-4Merlin	Hippo signaling/cell growth and proliferation	Luo et al, Nat Commun 2015
<i>NT5E</i>	Ecto-5'-nucleotidase (CD73)	Short variant CD73S	Extracellular adenosine production	Snider et al, Mol Biol Cell 2014
<i>NUMB</i>	Protein numb homolog	Long variant PRR (L)	Tissue morphogenesis	Lu et al, Hepatology 2015
<i>RCAN1</i>	Calcipressin-1	Variant isoform 4	Regulation of transcription	Jin et al, Gastroenterology 2017
<i>TLL1</i>	Tolloid-like protein 1	TLL1 short variant	Extracellular matrix and cell differentiation	Matsuura et al, Gastroenterology 2017

suppressor, recent work has shown that wild-type p53 also can act in an oncogenic manner by regulating metabolic reprogramming of HCC cells.⁴⁸ It remains to be determined if *RBM24*-regulated pathways in HCC can be linked to its effects on p53. Collectively, these transcriptome-wide studies show that alternative splicing is involved in the rewiring of cellular metabolism and other critical pathways in the liver, and that aberrant splicing in HCC tumors can be traced to several master regulators involved in RNA processing.

Alternative Splicing in Liver Development and Maturation

Pathways that normally signal during liver development are reactivated in HCC.^{49–51} Recent studies have shown significant alternative splicing changes during normal fetal-to-adult liver maturation⁵² and during injury-associated adult-to-fetal reversion in hepatocytes.⁵³ Analyses of alternative splicing events in mouse liver just before birth, shortly after birth, and in adulthood found that the most dramatic splicing changes (affecting >500 genes) occurred at the switch between the prenatal and postnatal periods, and that many of these genes encoded cytoskeleton and chromatin modification regulators.⁵² Furthermore, comparison of different cell types between P0 and adult mouse showed that more than 50% of postnatal splicing transitions in the liver occurred specifically within hepatocytes.⁵² Many of the genes showing a hepatocyte-specific exon inclusion or exclusion during the transition from fetal to adult liver also are known to be functionally involved in HCC, including *Camkk2*,⁵⁴ *Kras*,⁵⁵ *Pla2g6*,⁵⁶ *Usp4*,⁵⁷ *Vps29*,⁵⁸ and *Rpa3*.⁵⁹ Among these genes, developmentally regulated splicing of *Kras* is particularly interesting because of its known involvement in oncogenic hepatocyte signaling.⁶⁰ Although *KRAS* mutations in human HCC are not common, the Ras signaling pathway is hyperactivated,⁶¹ in part via the splicing factor hnRNP A2.³¹ Furthermore, Ras signaling contributes to HCC tumor growth by suppressing the anti-tumorigenic transcription factor *KLF6*.⁶² It is important to note that, at the mRNA abundance level, 3000–5000 mouse liver genes change during the prenatal to postnatal transition periods,^{52,63} which is under the control of the methyltransferases *EZH1* and *EZH2*.⁶³ Therefore, alternative splicing could be considered an important fine-tuning mechanism, rather than a major switch during postnatal liver maturation.

A potential caveat is that only approximately 40% of developmentally regulated, alternatively spliced mouse genes were found to be regulated similarly in human liver.⁵² This is an important consideration because there are human-specific splicing events known to be up-regulated in HCC. For example, the production of catalytically impaired splice variants of the nucleotide-regulating enzymes ecto-5'-nucleotidase (*NT5E*)³⁵ and kynurenine formamidase (*AFMID*)³⁶ occurs only in humans. In light of that, the most effective strategies for identifying tumor-promoting oncofetal splice variants and mechanisms can come out of integrating data from human HCC cell-based models^{64,65} and

tissue specimens⁶⁶ with the most appropriate animal models.^{67–69}

Up-regulation of the Oncofetal Splicing Factor Muscleblind-Like 3 in HCC

Muscleblind-like (MBNL) proteins are encoded by 3 genes (*MBNL1–3*) and regulate RNA splicing in a tissue-specific manner.^{70,71} The splicing factor MBNL3 is highly expressed in fetal liver and in HCC, but not in normal adult liver.⁷² Up-regulation of MBNL3 in HCC is considered to be a result of increased activity of several transcription factors, including *NANOG*, *OCT4*, and *SOX2*.⁷² Increased MBNL3 was associated with the differential splicing of the long non-coding RNA *PXN-AS1* in HCC tumors.⁷² Specifically, MBNL3 promoted the inclusion of exon 4, resulting in the generation of a long isoform (*PXN-AS1-L*). The *PXN-AS1-L* isoform had an opposing function to the short isoform *PXN-AS1-S*, which is expressed in normal liver.⁷² *PXN-AS1-L* bound to the 3'-untranslated region of the paxillin-encoding *PXN* gene, leading to *PXN* mRNA stabilization and increased paxillin expression.⁷² The opposite was true for *PXN-AS1-S*, which inhibited *PXN* mRNA translation and paxillin expression. Paxillin is known to promote HCC cell migration and metastasis, particularly when it is phosphorylated by JNK.⁷³ Therefore, the increased paxillin expression in HCC may be part of a splicing program to promote HCC metastasis (Figure 1). In support of that, MBNL3 expression, *PXN* exon 4 retention, and paxillin expression are correlated positively with each other and with poor HCC patient survival.⁷²

Epithelial Splicing Regulatory Protein 2 Splicing Factor Regulates Hippo Signaling in the Liver

Hepatic epithelial injury triggers a strong regenerative response through a variety of mechanisms, including hepatocyte renewal, inflammation, and extracellular matrix remodeling.^{74,75} An essential component of liver regeneration under physiologic and pathologic conditions is the remarkable plasticity of hepatocytes, which are able to dedifferentiate and become progenitor-like.⁷⁶ Furthermore, recent work has shown that the transforming growth factor β signaling pathway is critical for liver regeneration after partial hepatectomy, in part via activating hepatocyte EMT reprogramming in concert with the transcriptional co-activator YAP.⁷⁷ However, YAP-dependent signaling also can lead to improper regeneration of hepatocytes, marked by overactivation of fetal signaling pathways, thereby contributing to acute liver failure.⁷⁸ YAP is a mechanosensitive target of the Hippo signaling pathway, which regulates organ size and tissue regeneration,^{79,80} and is dysregulated in HCC.^{81–84} Furthermore, Hippo signaling in the liver is under the control of the master splicing regulator epithelial splicing regulatory protein 2 (*Esrp2*),⁵² suggesting that alternative splicing is an important component of regenerative responses in the liver.

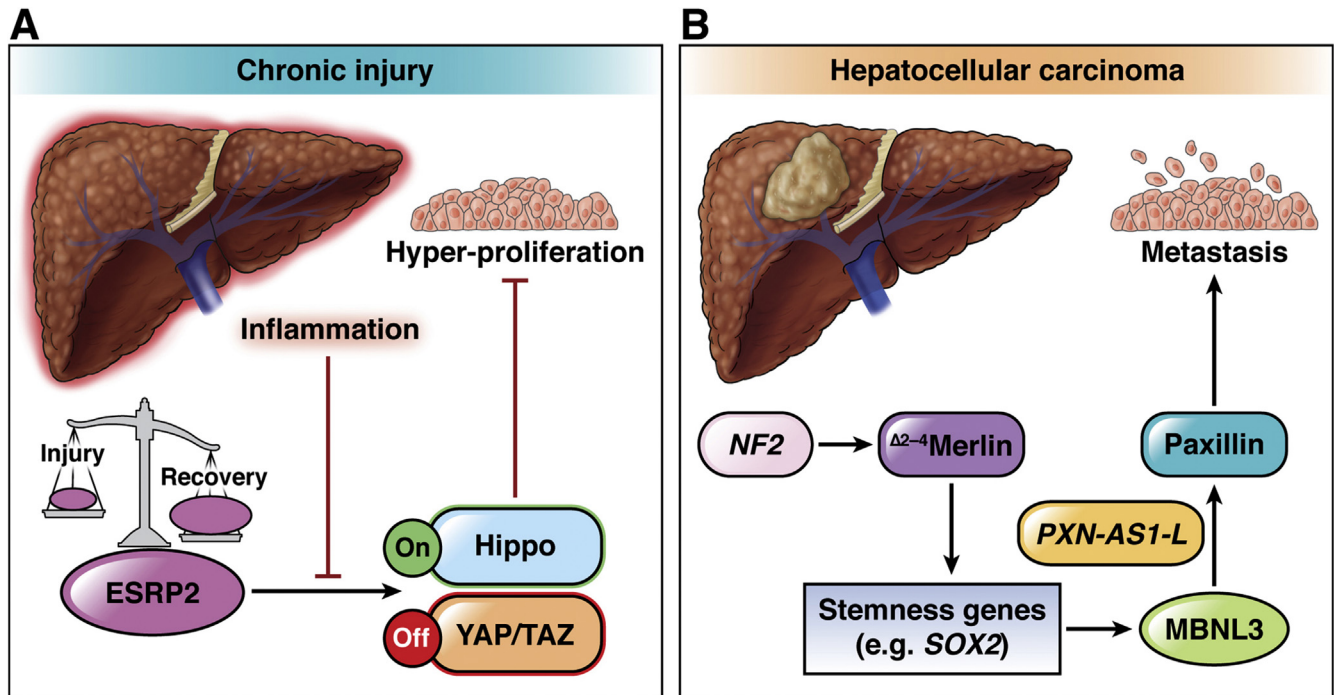


Figure 1. Alternative splicing rewires Hippo signaling during chronic liver injury and HCC. (A) The splicing regulator ESRP2 is important for the maintenance of a differentiated adult hepatocyte population via activation of the Hippo pathway. When Hippo signaling is active (On), the transcriptional co-regulators YAP and TAZ, which promote cell proliferation, are degraded (Off). This pathway is modulated during liver injury and recovery via the dynamic regulation of ESRP2 expression. Proinflammatory cytokines, such as tumor necrosis factor α and interleukin 1 α , promote down-regulation of ESRP2 in human hepatocytes. The absence or down-regulation of ESRP2 leads to altered splicing and hypoactivation of Hippo kinases. This promotes the expression of YAP/TAZ target genes, resulting in hepatocyte hyperproliferation and hepatomegaly. (B) The gene *NF2*, which encodes the protein merlin, is a direct target of ESRP2, which is implicated in HCC via a mechanism involving the production of a tumor-promoting protein variant (Δ^{2-4} Merlin). The Δ^{2-4} Merlin variant up-regulates the expression of stem cell transcription factors (stemness genes) such as SOX2. Expression of SOX2 and other stemness genes induces MBNL3, a splicing factor that is expressed in fetal liver and HCC. A major target of MBNL3 is *PXN-AS1*, a long noncoding RNA that regulates expression of the protein paxillin. Alternative splicing of *PXN-AS1* promotes the production of the long variant *PXN-AS1-L*, which stabilizes *PXN* mRNA, leading to increased paxillin expression. Paxillin regulates cell adhesion and migration and promotes HCC metastasis.

Although *Esrp2* regulates the splicing of approximately 20% of mouse liver genes, *Esrp2*^{-/-} mice develop normally, and at 4 months of age do not show any differences in their liver-to-body weight ratios, metabolic homeostasis, or signs of liver injury when compared with wild-type mice.⁵² While it appears that *Esrp2* is not essential for liver development, it is possible that it could regulate HCC metastasis. This has not been reported to date *in vivo*, but it would be of interest to examine because ESRP2 is able to support cell-cell adhesion and attenuate the motility of cancer cells *in vitro*.⁸⁵

Esrp2 is an important stress response factor that changes dynamically during liver injury and recovery, and it is involved in adult-to-fetal reversion in injured hepatocytes⁵³ (Figure 1). Livers from *Esrp2*^{-/-} mice were marked by the presence of small immature hepatocytes that produced less albumin and showed evidence of hyperproliferation.⁵² Furthermore, *Esrp2* was down-regulated significantly in mice challenged with the hepatotoxicant 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), and restored during the recovery period after DDC challenge.⁸⁶

This is likely an adaptive response to injury because DDC-treated *Esrp2*^{-/-} mice presented with significantly more hepatocyte proliferation and increased hepatomegaly.⁸⁶ In the absence of *Esrp2* (triggered by DDC feeding or genetic deletion), there was an adult-to-neonatal isoform switching of several Hippo pathway genes, including *Yap1*, *Nf2*, *Csnk1d*, and *Tead1*.⁸⁶ The resulting protein variants down-regulated Hippo signaling, allowing mature hepatocytes to exit their quiescent state and become proliferative⁸⁶ (Figure 1).

The mouse model of DDC liver injury reflects some aspects of chronic hepatocyte stress associated with alcoholic hepatitis, such as hepatomegaly and the presence of Mallory-Denk bodies.⁸⁷ Recent work has extended on the findings in *Esrp2*^{-/-} mice to provide evidence for ESRP2 function in human alcoholic liver injury.⁵³ Hyun et al⁵³ showed that ESRP2 is down-regulated significantly in severe human liver injury associated with alcoholic hepatitis, potentially via transcriptional down-regulation by the inflammatory cytokines tumor necrosis factor α and interleukin 1 β (Figure 1). In addition to reduced overall

expression, ESRP2 protein was mislocalized to the cytoplasm in liver tissue from alcoholic hepatitis patients.⁵³ This resulted in hypoactivation of Hippo kinases and depression of the downstream transcriptional co-activators YAP and TAZ, which are known to promote cell proliferation.^{88,89} Ultimately, in the absence of ESRP2, there was reversion of splicing to a fetal-like program that produced functionally compromised, proliferative immature hepatocytes that could lead to hepatic insufficiency associated with alcoholic hepatitis. It would be interesting to determine how this mechanism applies to the spectrum of alcoholic liver disease. Furthermore, as downstream effectors of Hippo signaling, YAP/TAZ have been linked to HCC development via several key pathways, including inflammation,⁹⁰ metabolic reprogramming,^{91,92} and chromosomal instability.^{93,94} Therefore, this splicing switch potentially could be harnessed to mitigate the risk of HCC development because patients with alcohol-related HCC in general have a worse prognosis compared with other etiologies.⁹⁵

Aberrant Splicing of a Core Hippo Pathway Component in HCC

The tumor-suppressor gene *NF2* encodes the moesin-ezrin-radixin-like protein merlin, an upstream regulator of Hippo signaling that is mutated in patients with neurofibromatosis type 2, a rare disease involving benign tumors of the nervous tissue.⁹⁶ Inactivating mutations in *NF2* also occur in HCC.⁹⁷ Recently, it was shown that merlin directly contributes to HCC metastasis via a dominant-negative splice variant isoform⁹⁸ (Figure 1). Canonical merlin and the closely related ezrin, radixin, and moesin proteins function primarily at the plasma membrane to assemble multiprotein complexes of receptors, adapter proteins, and Rho guanosine triphosphatase modulators, which associate with the cortical cytoskeleton.⁹⁹ Liver-specific deletion of *Nf2* in mice leads to a hyperproliferative response in the progenitor cell population and development of both cholangiocarcinoma and metastatic HCC via overactivation of the epidermal growth factor receptor.¹⁰⁰ Although it was recognized more than 2 decades ago that aberrant splicing of *NF2* via exon skipping promoted merlin inactivation,¹⁰¹ only recently was a specific merlin splice variant (of >9 different isoforms) implicated in HCC.⁹⁸ Exclusion of exons 2, 3, and 4 led to the production of the Δ^{2-4} merlin variant, which acted in a dominant-negative fashion to canonical merlin to promote tumor metastasis in HCC.⁹⁸ Expression of the Δ^{2-4} merlin variant, shown by the use of an isoform-specific antibody, was increased significantly in human HCC tumors and portal vein tumor thrombi relative to nontumor tissue.⁹⁸ This was in stark contrast to canonical merlin, which was found to be expressed most highly in nontumor liver tissue. Canonical merlin (but not Δ^{2-4} merlin) inhibited HCC cell migration and expression of EMT markers (such as *TWIST* and *SNAIL*), while Δ^{2-4} merlin promoted the expression of stemness genes, such as *EpCAM*, *SOX2*, and *KLf4* (Figure 1) and supported the formation of HCC cell spheroids in culture.⁹⁸ The latter function was attributed to the inability of Δ^{2-4} merlin to support plasma membrane

anchoring of β -catenin and ezrin, radixin, and moesin proteins, resulting in decreased expression of β -catenin at the plasma membrane.⁹⁸

The RNA Binding Protein SLU7 Controls Hepatic Metabolism

The RNA binding protein SLU7 acts as a stabilizing component of the spliceosome to ensure fidelity in splice-site recognition.¹⁰² SLU7 is normally expressed in the nuclei of mature hepatocytes, but is down-regulated in HCC tumors.¹⁰³ Knock-down of *SLU7* perturbed nearly 600 splicing events and also led to major gene expression changes in the human PLC/PRF/5 hepatoma cell line.¹⁰³ Among the genes that were affected, both at the level of splicing and expression, were many lipid and carbohydrate metabolism regulators, implicating SLU7 in metabolic homeostasis in hepatocytes. In support of that, diminished hepatic expression of *Slu7* in mice (via adenovirus-mediated knockdown) was correlated strongly with down-regulation of the rate-limiting gluconeogenic genes *Pepck* and *G6pc*.¹⁰³ This resulted in decreased hepatic glucose production after pyruvate or glucagon injection, and blunted hepatic insulin responses during fasting/refeeding.¹⁰³ *Slu7* depletion also up-regulated the expression of *Hk2* and *Pkm2*, which are linked to aerobic glycolysis and a tumor-like metabolic state. Interestingly, SLU7 regulates the splicing of *Sirt1*,¹⁰⁴ which encodes the HCC-promoting¹⁰⁵ NAD⁺-dependent sirtuin-1 deacetylase enzyme. Therefore, the metabolic reprogramming that accompanies HCC development¹⁰⁶ may be linked, at least in part, to the loss of the RNA binding protein SLU7.

Precisely how the loss of *Slu7* leads to altered metabolism is still an open question. However, one potential mechanism is via its ability to regulate the alternative splicing of a key splicing factor: *SRSF3*¹⁰⁷ (Figure 2). The serine/arginine-rich splicing factors (SRSFs) are a conserved group of nuclear RNA binding proteins that regulate multiple aspects of pre-mRNA splicing and are crucial for mammalian development.^{108,109} In humans, there are 12 nonredundant SRSF proteins (SRSF 1–12), and several have been implicated in HCC,^{38,110–113} as discussed in the next section.

SRSF RNA Binding Proteins Differentially Regulate Hepatic Genomic and Metabolic Stability

The importance of SRSF proteins in liver biology is reflected in findings that hepatocyte differentiation during the early postnatal period and lipid homeostasis are under the control of *Srsf3*.¹⁰⁹ Liver-specific deletion of *Srsf3* in mice led to significant perinatal death, and the mice that survived had significantly lower body weight, liver weight, and liver/body weight ratios.¹¹⁴ Highly abnormal, enlarged hepatocytes with irregular nuclei were present in 1-month-old mice lacking hepatic *Srsf3*, along with a high rate of cell proliferation and apoptosis.¹¹⁴ Not surprisingly, compromised hepatocyte maturation ultimately

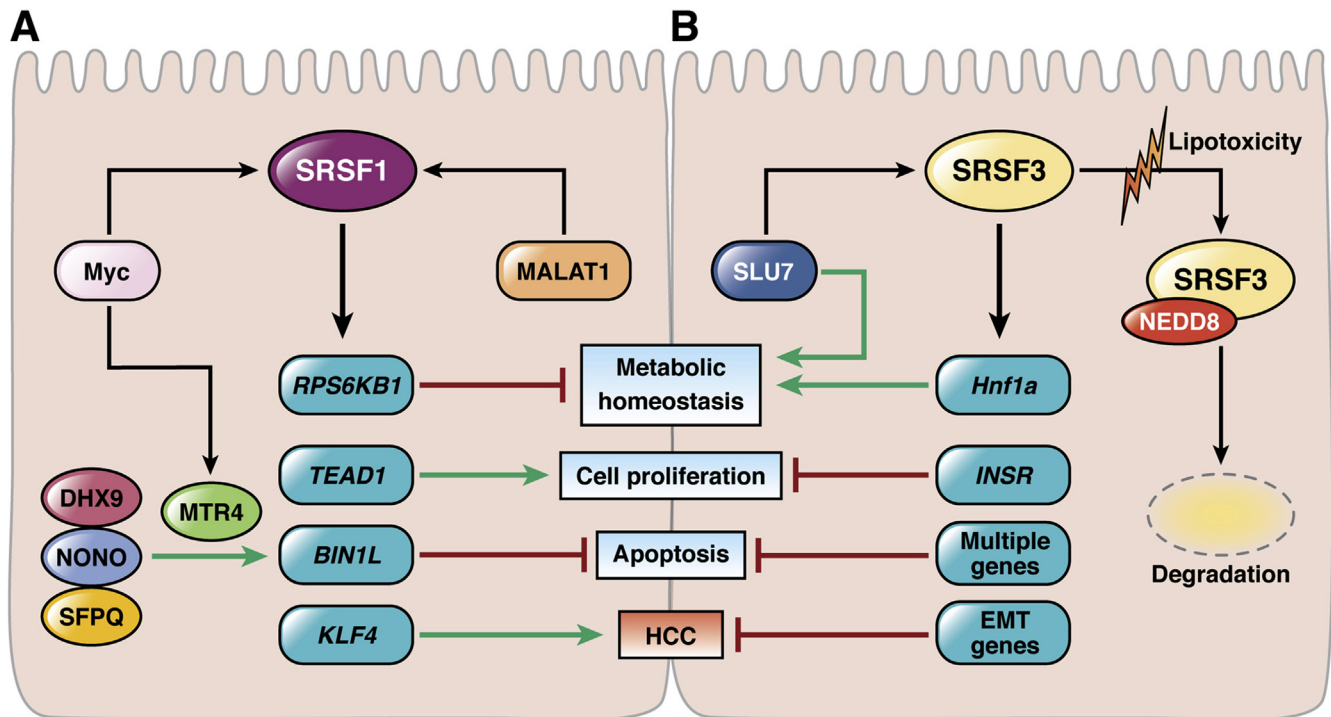


Figure 2. Splicing factors SRSF1 and SRSF3 regulate hepatocyte homeostasis and HCC development via opposing mechanisms. The alternative splicing of multiple genes involved in metabolic homeostasis, cell proliferation, and apoptosis is under the control of the master splicing regulators SRSF1 and SRSF3. (A) SRSF1 is a target of the tumor promoter Myc and MALAT1, an oncogenic long noncoding RNA that is overexpressed in HCC. SRSF1 induces metabolic reprogramming, increases cell proliferation, decreases apoptosis, and promotes HCC by regulating the splicing of several key genes, including *RPS6KB1*, *TEAD1*, *BIN1*, and *KLF4*. SRSF1 promotes exon 12A inclusion in the *BIN1* gene to form the variant BIN1L. Several other RNA regulators promote BIN1L up-regulation, including NONO, which associates with RNA helicases DHX9 and potentially MTR4, and the splicing factor SFPQ9. BIN1L is unable to block the oncogenic function of Myc, and promotes cells survival. (B) SRSF3 is a target of the RNA binding protein Slu7, and both are down-regulated significantly in HCC. SRSF3 promotes metabolic homeostasis, limits cell proliferation, blocks apoptosis, and inhibits HCC development by regulating the splicing of several key genes, including *HNF1a*, *INSR*, and several *EMT* genes. SRSF3 also supports the expression of multiple prosurvival and anti-apoptotic genes. In chronic liver disease, there is significant down-regulation of SRSF3 at the protein level. This is mediated by the ubiquitin-like modification neddylation, which involves conjugation of target proteins to NEDD8. Neddylation of SRSF3 is induced by lipotoxicity, and may represent an important mechanism for regulating SRSF3 in NAFLD and in NAFLD-related HCC.

led to hepatic insufficiency with impaired glucose production.¹¹⁴ At the molecular level, gene expression changes and missplicing were observed in the *Srsf3*-null livers when compared with wild-type livers. The most notable effects were observed for *Hnf1 α* , where aberrant splicing led to the exclusion of exon 2, predicted to cause non-sense-mediated decay of the transcript.¹¹⁴ Consistent with that, a number of *Hnf1a* target genes were down-regulated, including *Ghr*, leading to growth hormone insensitivity.¹¹⁴ Interestingly, 100% of the *Srsf3*-null mice that survived to 24 months developed spontaneous HCC, with lung metastasis noted in approximately a quarter of the HCC tumor-bearing mice.¹¹³ Furthermore, development of a fibrotic phenotype was noted as early as 1 month of age, and attributable in part to aberrant splicing of the *Fn1* gene.¹¹³ When the younger mice were challenged further with the profibrotic agent carbon tetrachloride, they developed precancerous lesions.¹¹³ The primed tumor phenotype in these mice was attributed to

aberrant splicing of *EMT* genes and abnormal activity of the Wnt/ β -catenin pathway.¹¹³

These results have translational importance to human HCC because SRSF3 is absent or down-regulated significantly in more than half of HCC cases.¹¹³ In cases in which SRSF3 was present in HCC, it was mislocalized to the cytoplasm, suggesting diminished activity as a splicing regulator.¹¹³ Interestingly, truncated variants of SRSF3, produced by aberrant splicing in the absence of SLU7, were found to act in a dominant-negative fashion and to interfere with proper cell division.¹⁰⁷ It remains to be determined if the truncated variants account for the mislocalization of SRSF3 in HCC cells. Down-regulation of SRSF3 protein also was noted in NAFLD livers, and found to be triggered by lipotoxicity in cell culture.¹¹⁵ SRSF3 degradation was independent of the ubiquitin-proteasome pathway, but controlled by another ubiquitin-like modification: neddylation¹¹⁵ (Figure 2). Neddylation involves conjugation of target protein lysine residues to the protein NEDD8,

although there is some cross-over between the neddylation and ubiquitination pathways.¹¹⁶ The NEDD8-mediated down-regulation of SRSF3 bears relevance to HCC because multiple components of the neddylation machinery are up-regulated and correlate with shorter survival in HCC patients.¹¹⁷

SRSF2, unlike SRSF3, is up-regulated in HCC and correlates with poor prognosis in patients.¹¹¹ Previous work has shown that *Srsf2* is essential for liver homeostasis and survival because liver-specific deletion of *Srsf2* in mice caused liver failure and death within the first 2–4 weeks.¹¹⁸ *Srsf2*-null hepatocytes showed markers of severe ER and oxidative stress, together with aberrant splicing of multiple autophagy and stress response genes.¹¹⁸ The splicing program regulated by SRSF2 in HCC tumors is not well described, although in Huh7 cells it was linked to the splicing of genes regulating the cell cycle, DNA repair, and chromatin modifications.¹¹¹ Therefore, the evidence to date suggests that SRSF2 serves both a protective homeostatic function as well as a tumor-promoting function in the mammalian liver. This dual function may be attributed, in part, to the ability of *Srsf2* to serve as a transcription factor for cholesterol and bile acid metabolism genes.¹¹⁸

Mice with a liver-specific deletion of *Srsf1* have a normal liver function and life span, suggesting that SRSF1 is dispensable for liver homeostasis.¹¹⁸ However, SRSF1 is a known transcriptional target of Myc¹¹⁹ and functions as a proto-oncogene.¹²⁰ It was shown several years ago that SRSF1 promotes HCC cell growth via the splicing of *KLf6*, acting upstream of the cell-cycle regulator p21.¹¹⁰ More recently, it was shown that expression of SRSF1 is also under the control of the long noncoding RNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), which is overexpressed in HCC¹²¹ (Figure 2). When MALAT1 levels were high, the expression and nuclear activity of SRSF1 were enhanced, and there was increased production of several splice variant isoforms of SRSF1 target genes that control apoptosis, cell proliferation, and protein synthesis.¹²¹ Specifically, MALAT1 overexpression was associated with the inclusion of exon 12A in the gene encoding the Myc suppressor box-dependent-interacting protein-1 (BIN1).¹²¹ The HCC tumor-associated BIN1 variant isoform containing exon 12A encodes a longer protein that lacks this tumor-suppressive activity against Myc.¹²² SRSF1 also regulates the Hippo signaling pathway via the inclusion of exon 5 in the gene encoding the transcription factor *TEAD1*, the primary target of YAP/TAZ transcriptional co-activators.¹²³ This also was under the control of MALAT1 and led to enhanced TEAD1-mediated cell proliferation.¹²¹ Another consequence of MALAT1 overexpression in HCC is the increased production of the isoform 2 of ribosomal protein S6 kinase $\beta 1$ (S6K1), leading to mammalian target of rapamycin complex 1 activation. The latter is of particular relevance to HCC because mammalian target of rapamycin is overactivated and represents a central target for limiting tumor growth and recurrence.¹²⁴ Collectively, these studies highlight the importance of SRSF proteins in balancing hepatic genomic and

metabolic stability, and open several potential avenues for selective targeting of tumor cells (Figure 2).

The RNA Binding Protein NONO Controls Glucose Metabolism Genes in HCC

Interestingly, there appears to be some functional redundancy between the MALAT1-SRSF1 axis and another RNA binding protein, the non-POU domain-containing octamer-binding protein (NONO). NONO is an RNA binding protein that regulates the splicing of hepatic metabolic genes, including the glucose transporter *Glut2* and *Gck*.¹²⁵ NONO function is important for hepatic nutrient metabolism in coordination with the circadian clock.¹²⁵ In the context of HCC, NONO interacts with an adenosine triphosphate-dependent RNA helicase (DHX9) and a splicing factor (SFPQ) to promote exon 12A retention in BIN1¹²⁶ (Figure 2). This, again, leads to the synthesis of the BIN1 long protein product that lacks the ability to suppress Myc.¹²⁶ In this case, the long BIN1 isoform also stabilized the serine/threonine kinase PLK1 by preventing its degradation by the ubiquitin/proteasome system.¹²⁶ PLK1 is known to be tumor-promoting in HCC.¹²⁷ High expression of NONO was associated with poor survival and increased recurrence of HCC after surgery, while deletion of NONO reduced HCC cell growth in vitro and in vivo.¹²⁶

Another manner in which NONO could affect HCC progression is through interacting with another RNA helicase, MTR4.¹²⁸ MTR4 is a component of the nuclear exosome targeting complex, which regulates RNA turnover.¹²⁹ MTR4 is a direct target of Myc that promotes HCC cell proliferation in vitro and tumor growth in mice. Analysis of splicing in MTR4-deficient cells has shown a number of differentially spliced genes, including the metabolic regulators *GLUT1* and *PKM2*. Similar to NONO, MTR4 is up-regulated in HCC tissues and is associated with poor survival in patients.¹³⁰ Although they were shown to interact,¹²⁸ it remains to be tested whether NONO and MTR4 work in tandem to reprogram glucose metabolism in HCC.

Altered Fructose Metabolism via hnRNP H1 and H2 Proteins

Recent work has shown a novel isoform switching mechanism favoring de novo nucleotide biosynthesis at the expense of fructose metabolism in HCC.¹³¹ Kethexokinase (KHK) phosphorylates fructose to form fructose-1-phosphate, which undergoes further metabolism to generate substrates for glycolysis.¹³² The *KHK* gene contains the mutually exclusive exons 3A and 3C.¹³³ Retention of exon 3A results in the expression of KHK-A, which is associated primarily with fetal development¹³³ and has a low affinity for fructose.¹³⁴ In contrast, retention of exon 3C yields the high-affinity KHK-C that is expressed primarily in the liver and is the main isoform involved in normal hepatic fructose metabolism.¹³⁴ Splicing of *KHK* pre-mRNA is under the control of the RNA binding protein A1CF, which generates the KHK-C isoform and promotes metabolic homeostasis in the normal

liver.¹³⁵ Liver-specific deletion of *A1cf* results in complete loss of KHK-C protein, while re-expression of *A1cf* leads to KHK-C protein restoration¹³⁵ in mice. Furthermore, A1CF activity is antagonized by the RNA binding proteins hnRNPH1 and hnRNPH2.¹³⁵ These proteins belong to a large family of RNA regulators that control alternative splicing, transcription, translation, and mRNA stability,¹³⁶ and are transcriptionally up-regulated by Myc.¹³⁷ Increased expression of hnRNPH1 and H2 promotes KHK-A over KHK-C production in HCC tumor cells, leading to a reduction in fructose metabolism.¹³¹ KHK-A leads to the phosphorylation and activation of phosphoribosyl pyrophosphate synthetase 1 (PRPS1) and enhanced de novo synthesis of nucleic acids to fuel tumor cell growth and proliferation.¹³¹ PRPS1 is essential for nucleotide biosynthesis and sensitive to feedback inhibition by phosphate and nucleotide concentrations in normal hepatocytes. This feedback inhibition was lost in HCC cells and PRPS1 remained in a constant “on” state.¹³¹ High expression of Myc, hnRNPH1/2, KHK-A, and phospho-PRPS1 all were correlated with poor HCC patient survival,¹³¹ suggesting that this pathway could be targeted to restore metabolic balance.

Conclusions and Future Directions

Understanding of the cellular and molecular mechanisms involved in HCC and other forms of primary liver cancer has expanded rapidly in the past several years.⁴ These advancements have been fueled by large-scale genomic, transcriptomic, proteomic, and metabolomics studies, which have identified new players and regulatory networks.¹³⁸ Transcriptomic profiling of primary human HCC tumors coupled with mechanistic studies in cells and animal models have unveiled novel disease targets that arise in response to dysregulated processing of RNA via alternative splicing. Alternative splicing is recognized as a critical mechanism involved in the tumorigenesis process across cancer types.¹³⁹ Detailed insight into the regulation and function of novel transcript and protein variants in HCC is useful on many fronts, such as discovery of novel biomarkers for early detection and molecular targets for intervention. However, timely and effective translation of these novel molecular findings to the clinic hinges on the ability to classify patients based on the molecular features of their tumors and tailor their therapy accordingly. Although some alternative splicing pathways may be linked strongly to the etiology of HCC,¹⁴⁰ others may be related to the presence of cirrhosis and otherwise independent of the underlying major risk factor.¹⁴¹ As such, detecting and modulating alternative splicing events at the premalignant stage also could be used as an approach for HCC prevention. The latter would be an ideal scenario to lessen the global burden of this highly common and deadly cancer type.¹⁴²

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Received March 11, 2020. Accepted April 27, 2020.

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Seung Eun Lee and Natasha T. Snider performed a literature review and wrote the first draft of the manuscript and generated the figures; Karel P. Alcedo and Hong Jin Kim provided comments and edits on the first draft; and Natasha T. Snider revised and finalized the manuscript and figures.

Conflicts of interest

The authors disclose no conflicts.

Funding

This work was supported by National Institutes of Health grant DK110355 (N.T.S.) and by an institutional training grant from the University of North Carolina Cancer Cell Biology Training Program (K.P.A.).