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REVIEW ARTICLE

The regulation and functions of ACSL3 and ACSL4 in the liver and hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is a heterogeneous disease that often features dysregulated tumour lipid metabolism. ACSL3 and ACSL4 are two homologous long chain acyl-coenzyme A synthetases (ACSL) that preferentially catalyse the activation of monounsaturated and polyunsaturated fatty acids, respectively. Both enzymes are frequently overexpressed in HCC, and multiple reports have implicated ACSL4 in tumour progression. Increased expression of these isozymes in tumour cells can upregulate lipid metabolism through de novo lipogenesis, fatty acid β-oxidation and acyl chain remodelling of membrane phospholipids. We describe the subcellular functions of ACSL3 and ACSL4 in hepatocytes, and the transcriptional, epigenetic and post-translational mechanisms underpinning their regulation. We discuss the evidence that these enzymes can modulate hepatocarcinogenic signalling by oncoproteins, cell death by apoptosis or ferroptosis, and protein degradation through the ubiquitin-proteasome pathway. In addition, we survey how knowledge in this area may inform new approaches to the diagnosis and treatment of HCC and deepen our understanding of how lipid metabolic reprogramming can promote hepatic tumour growth.

KEYWORDS
ACSL3, ACSL4, ferroptosis, hepatocellular carcinoma, lipid metabolism, liver

1 | INTRODUCTION

Altered cellular energetics is one of the hallmarks of cancer[1,2] and intratumoural lipid metabolism tends to be markedly changed in hepatocellular carcinoma (HCC)[1,3–5] where it manifests as altered intracellular levels triacylglycerol (TAG), phospholipids, cholesterol and ceramide.[3,6–19] HCC is one of the world's most common cancers[20,21] and can develop from chronic liver diseases that feature dysregulated lipid metabolism, inflammation and hepatocellular death. This pathological sequence is illustrated well in the case of non-alcoholic fatty liver disease (NAFLD) which can lead to non-alcoholic steatohepatitis (NASH)[21,22] and ultimately NASH-HCC.[23] Rewired lipid metabolism in HCC can also be characteristic of different oncogene driver mutations. For example, hepatomas with a CNNTB1 mutation encoding β-catenin are generally 'addicted' to mitochondrial fatty acid β-oxidation (FAO)[24] and have low levels of

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Abbreviations: ACSL, long chain acyl-coenzyme A synthetase; CoA, coenzyme A; FAO, fatty acid mitochondrial β-oxidation; GADD45B, DNA damage-inducible gene 45β; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; m^6A, N^6-methyladenosine; MAM, mitochondrial-associated membrane; miRNA, microRNA; MUFA, monounsaturated fatty acid; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic hepatosteatosis; PPARα, peroxisome proliferator-activated receptor delta; PPARδ, peroxisome proliferator-activated receptor delta; PUFA, polyunsaturated fatty acid; rRNA, ribosomal RNA; SREBP, sterol regulatory element-binding protein-; TAG, triacylglycerol.

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intracellular TAG. This contrasts with tumours driven by the mTOR pathway,[7,9] where de novo lipogenesis and intracellular lipid accumulation are required for the transition from steatosis to HCC.[9,22,25] As aberrant lipid metabolism in HCC requires a supply of fatty acids, there has been interest recently in two structurally homologous long chain acyl-coenzyme A synthetase (ACSL) enzymes,[26,27] ACSL4[28,29] and ACSL3,[30] which are often overexpressed in HCC (Table 1).[16,31–40] These enzymes catalyse fatty acid activation through coenzyme A (CoA) addition and can potentially modulate lipid metabolism,[16,41–51] cell death and proliferation,[16,35,37,38,40,52,53] oncogenic signalling[16,31,37,54] and even the dynamics of oncoprotein degradation.[31] Furthermore, immunohistochemical analysis of ACSL4 expression in HCC (Table 1) is emerging as a predictive biomarker for drug sensitivity,[32,33,38] patient survival[36] and as a useful tool for identifying different molecular subtypes of the disease.[35,38,55]

Of particular relevance to liver disease,[56] ACSL4 is required for ferroptosis,[57–60] an iron-dependent cell death pathway,[32,59,61] mechanistically different to apoptosis or pyroptosis, that involves the large-scale peroxidation of polyunsaturated acyl chains of plasma membrane phospholipids.[62] In ferroptosis, ACSL4 enzymatic activity generates activated polyunsaturated fatty acids (PUFA), such as arachidonoyl CoA,[29] that are required for the synthesis of plasma membrane phospholipids containing unsaturated acyl chains.[63] ACSL4-dependent ferroptosis is clinically important in HCC as it can potentiate hepatocellular death[64–66] during liver injury prior to tumourigenesis, and it has been implicated in tumour regression mediated by the multi-kinase inhibitor sorafenib[32,33,38,67–70] and the immunotherapies[71–76] that are now considered first-line treatments for this disease.[77,78] ACSL3 expression in HCC is relevant to this topic[34,79] but has not been investigated extensively, even though accumulating evidence indicates that the incorporation of ACSL3-activated monounsaturated fatty acids (MUFA) into membrane phospholipids can inhibit ferroptosis[33,80,81] and induce a ferroptosis-resistant state.[33,82–85] Moreover, in non-hepatic malignancies such as pancreatic ductal adenocarcinoma[86] and KRAS-positive lung cancer,[54] increased ACSL3 expression can promote tumour growth via mechanisms that do not involve either ACSL4 or ferroptosis.

There have been several recent reviews detailing the involvement of ACSLs in cancer[27,87,88] and ferroptosis in liver disease.[66,89–91] Therefore, the scope of this review extends beyond ferroptosis to encompass the array of biochemical, cellular and pathological changes in liver cells associated with increased expression of either ACSL3 or ACSL4, and to explore their relevance to HCC. Specifically, in this review we discuss:

1. The enzymatic activities and cellular functions of ACSL3 and ACSL4 in the liver that are relevant to HCC.
2. How expression of these enzymes can be upregulated in hepatocytes.
3. Emerging evidence for a pathological role for ACSL4 in augmenting tissue damage leading to HCC through the induction of ferroptosis.
4. Findings that increased ACSL4 expression in HCC can promote both lipogenesis and oncogenic signalling.
5. Recent insights into how knowledge of increased ACSL3 and ACSL4 expression may lead to novel biomarkers for different molecular subtypes of HCC and the possible development of new ACSL-targeted therapies for the treatment of HCC.

Lay summary

Hepatocellular carcinoma (HCC) is a primary liver cancer that can arise from number of chronic conditions where there is long-term damage to liver cells. In healthy individuals the liver has an important role in regulating fat metabolism. Recent work has shown that fat metabolism in HCC tumours is altered, and this may help cancer cells divide and survive leading to tumour growth. This article focuses on two related enzymes called ACSL3 and ACSL4 that are present at high levels in HCC and are important for rewiring fat metabolism in tumour cells. We review how these enzymes are involved in lipid metabolism and cell survival pathways, and how emerging knowledge about their roles in liver cancer may potentially lead to new tools for diagnosing different subclasses of HCC and new treatments for this disease.

Key points

1. Lipid metabolism is often altered in liver tumours.
2. ACSL3 and ACSL4 are enzymes involved in fatty acid activation and are frequently overexpressed in HCC.
3. ACSL4 overexpression can modulate cell death processes such as apoptosis and ferroptosis, and promote tumour growth in HCC.
4. ACSL4 is an emerging drug target in cancer.

2 | BIOCHEMICAL AND CELLULAR FUNCTIONS OF ACSL3 AND ACSL4 IN THE LIVER

ACSL3 and ACSL4 are two homologous proteins from the acyl CoA synthetase long chain (ACSL) family which also includes the ACSL1, ACSL5 and ACSL6 isozymes.[92] All ACSL isoforms catalyse a similar general reaction: the ATP-dependent thioesterification of substrate long-chain fatty acids, which are typically 16–20 carbons in length, with CoA to form fatty acyl CoA molecules and ADP.[93–95] This reaction is known as fatty acid activation and it represents a key energy-requiring step that prevents fatty acids from exiting the cell and commits their entry into intracellular metabolic

TABLE 1 Examples of studies investigating ACSL3 and ACSL4 expression in HCC versus normal liver tissue

Protein	References	Result
ACSL4	Sung et al., 2003[40]	Increased mRNA expression in 40% of HCC vs paired normal liver (*n* = 12)
ACSL4	Liang et al., 2005[197]	Increased mRNA expression in 80% of HCC vs paired normal liver (*n* = 16)
ACSL4	Hu et al., 2008[52]	Increased mRNA expression in HCC vs paired normal liver (*n* = 40)
ACSL4	Sun and Xu, 2017[36]	Increased protein IHC staining plus mRNA levels in HCC vs paired normal liver (*n* = 116)
ACSL3 & ACSL4	Ndiaye et al., 2020[34]	Increased protein IHC staining in HCC vs normal liver (*n* = 157)
ACSL4	Chen et al., 2020[31]	Increased protein IHC staining in HCC vs paired normal liver (*n* = 87)
ACSL3 & ACSL4	Chen et al., 2016[39]	Increased mRNA levels in HCC in the Oncomine database
ACSL4	Wang et al., 2020[37]	Increased protein and mRNA levels in HCC vs paired normal liver
ACSL4	Yu et al., 2022[198]	Increased protein and mRNA expression in HCC (mining online databases)

pathways such as FAO.[92,96] The main enzymatic differences amongst the ACSL isoforms lie in their isoform-specific affinities for different structural classes of long-chain fatty acid substrates. Combining a range of evidence from both in vitro and biological studies[30,42,58,59,80,81,94,97–100] makes it possible to infer that ACSL3 has a high affinity for the saturated palmitic and stearic acids, and also for oleic acid—a monounsaturated fatty acid (MUFA); whereas ACSL4 preferentially activates polyunsaturated fatty acids (PUFA) and in particular arachidonic acid. Intracellular levels of ACSL3 and ACSL4 determine the cytosolic concentrations of their respective free fatty acid substrates and their incorporation into more complex membrane phospholipids or storage triacyclglycerols.[71,80,99–102] In the liver, these isoform-specific substrate preferences are pathologically important; for example, in the activation of hepatic stellate cells[102]—a cellular process required for fibrosis, and implicated in the pathway that leads from uncomplicated steatosis to liver cirrhosis and HCC.[22] Activation of stellate cells relies specifically on the ACSL4 isoform to activate arachidonic acid which is subsequently sequestered in PUFA-enriched TAGs.[102]

In healthy human liver, the basal levels of ACSL3 and ACSL4 protein expression are low,[26] and even difficult to detect in the case of ACSL4.[28,34,40] Whilst the ACSL3 and ACSL4 isoforms have both been implicated in hepatic cancer, it is important to note that in healthy liver neither enzyme is as abundant as ACSL1 which is considered to be the main, constitutive, hepatocyte ACSL activity, accounting for at least half of the total fatty acid activation in this organ.[103] ACSL5 is also constitutively expressed in the liver of different species and some hepatoma cell lines[48,104–108] but there is little available information on its expression pattern in human HCC other than one report that ACSL5 mRNA levels are reduced in HCC[39] and another separate study using a PTEN-null model for NASH-induced HCC in mice which demonstrated an increase in ACSL5 expression.[109] The relevance of this model to human disease needs to be further investigated as only approximately 1%–3% of HCC cases in patients are thought to be because of the complete deletion of the PTEN tumour suppressor.[110] Whilst there is some evidence that ACSL1 levels can be upregulated in HCC[111] most recent analyses have determined that ACSL1 expression in HCC is protective and associated with a positive prognosis.[112–115]

3 | SUBCELLULAR LOCALISATIONS OF ACSL3 AND ACSL4 IN LIVER CELLS

In hepatocytes and HCC cells, ACSL3[34,116–119] and ACSL4[34,106,120] have been localised to the endoplasmic reticulum and lipid droplets (Figure 1). Early subcellular fractionation studies concluded that a pool of ACSL4 was localised to peroxisomes,[120] which are important sites for intracellular lipid metabolism, but subsequent investigations did not confirm this finding.[121] ACSL3[43,122] and ACSL4[120,123] have also been localised to varying degrees at ER-mitochondria membrane contact sites (MAM) which are known to be important for substrate exchange between these organelles during lipid synthesis. Indeed, the enrichment of ACSL4 at MAMs has led to its use as a marker enzyme for the biochemical isolation of MAM fractions.[124–126]

There is accumulating evidence for a sub-population of ACSL4 present at the plasma membrane of some cell types.[34,80,101] The presence of ACSL4 at the plasma membrane may be relevant for HCC as this enzymatic pool can activate PUFA required for the synthesis of pro-tumourigenic inositol phospholipids.[101] Alternatively, plasma membrane localised phospholipids generated from ACSL4-activated PUFA may be channelled for lipid peroxidation during ferroptosis.[58,80,127]

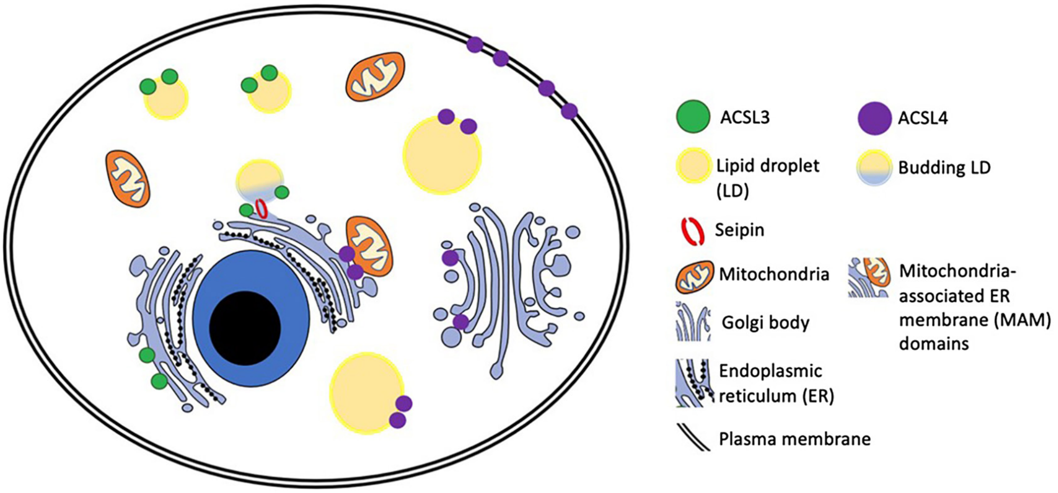

FIGURE 1 Subcellular localisations of ACSL3 and ACSL4 in hepatocytes. ACSL3 is predominantly localised to endoplasmic reticulum (ER) and the surface of lipid droplets (LD) . It is also found at mitochondria-associated ER membrane (MAM) domains. ACSL4 is present on LDs as well as at MAM domains, the *cis*-Golgi and at the plasma membrane. Figure created with Biorender.com

In hepatomas, both ACSL3 and ACSL4 have been localised to cytoplasmic lipid droplets[34] suggesting that they may be functional in these storage compartments. Evidence from different sources has revealed that ACSL3 is required for initial steps in lipid droplet biogenesis[116,118] on microdomains of the endoplasmic reticulum[119] enriched for either seipin[128] or syntaxin-17[129]—proteins that tether nascent lipid droplets through the formation of ER-lipid droplet contact sites. When lipid droplet formation is induced experimentally by adding exogenous free fatty acids, ACSL3 redistributes from MAMs in a process that involves its displacement from the resident MAM protein syntaxin-17.[43,122] ACSL3 then diffuses to sites of lipid droplet biogenesis where it generates activated fatty acids required for TAG biosynthesis.[116,118] There is evidence that ACSL3 is only required for these initial steps in lipid droplet formation at the ER and not for the subsequent expansion of lipid content as the droplet matures in the cytoplasm.[116,118] In myoblasts, ACSL3 binds to Rab18 in complex with PLIN2 on the surface of lipid droplets, and in these cells, Rab18 regulates lipid droplet size and number[130]; however, it is not yet known if this scenario also applies in hepatocytes. Structurally, ACSL3 association with both the endoplasmic reticulum and lipid droplets is thought to involve a hairpin loop-like structure in its N- terminus that facilitates its insertion into both the endoplasmic reticulum and possibly the outer phospholipid layer of lipid droplets.[116]

An unexpected insight into the functional consequences of ACSL3-induced lipid droplet formation at the ER has recently emerged from studies on the non-hepatic Hela cell line. In Hela cells, ER-associated ACSL3 can form a heterocomplex with UBA5,[131] which is a protein required for ufmylation—a type of post-translational protein modification similar to ubiquitination,[132] and also GABARAPL2—a protein required for autophagy of the ER, also known as ERphagy.[133] In these experiments, ACSL3-dependent lipid droplet formation resulted in decreased recruitment of both GABARAPL2 and UBA5, and consequently, reduced levels of autophagy and protein ufmylation. Hence, ACSL3 activity at the ER can be likened to a molecular switch that can promote either anabolic TAG synthesis when fatty acids are available or degradative ufmylation and autophagy when MUFA levels are low.[131] These observations may potentially provide a mechanistic basis for understanding the ACSL3-mediated upregulation of lipid synthesis during the ER stress response in hepatoma cells[41] that is known to modulate autophagy.[134,135] It remains to be shown if lipid droplet biogenesis and ufymylation are functionally linked via ACSL3 in hepatocytes, but it is a relevant concept to explore since both autophagy[136] and decreased ufmylation[137] have been implicated in HCC development and notably in the formation of protein-rich Mallory-Denk bodies.[138]

Compared to ACSL3, there is much less information available on the role of ACSL4 present on hepatocyte lipid droplets. However, ACSL4 overexpression in metabolic disease induces increased steatosis through inhibition of FAO,[49] and ACSL4 upregulation in hepatomas can increase lipogenesis through the indirect promotion of SREBP1 activity, indicating the existence of molecular pathways through which ACSL4 can mediate neutral lipid accumulation.[16] However, the structural basis for ACSL4 localisation to lipid droplets is not yet established and unlike ACSL3, it does not possess a putative N-terminal membrane-insertion motif. In terms of membrane association, recent evidence has shown that ACSL4 interacts with the early Golgi and ER resident protein p115 and that this protein heterocomplex is important for ACSL4 localisation to the early secretory pathway.[139] This precedent suggests that protein: protein interactions are an important determinant of the membrane targeting dynamics of ACSL4 and point to the existence of a hitherto unidentified ACSL4 protein interacting partner on the surface of lipid droplets.

4 | PHYSIOLOGICAL FUNCTIONS AND REGULATION OF ACSL3 AND ACSL4 EXPRESSION IN THE LIVER

To understand the pathological dysfunction of ACSL3 and ACSL4 in liver cancer it is useful to understand their physiological functions and expression patterns in healthy liver cells. With regards to ACSL4, studies using murine models have revealed that ACSL4 functions in hepatocytes to channel fatty acids to the lipogenic pathways that generate both phospholipids and triacylglycerol, with the latter associating with VLDL particles that can be subsequently secreted into the circulation as opposed to being stored in the hepatocytes.[99] Specifically, in a hyperlipidaemic murine model, adenoviral-mediated knockdown of hepatic ACSL4 expression to approximately half of the control levels resulted in substantial falls in the amount of triacylglycerol associated with circulating VLDL and also reduced levels of lysophosphatidylethanolamine phospholipid in liver tissue.[99] In addition, these ACSL4 liver-specific knockdown animals had reduced insulin sensitivity demonstrating a link between hepatic ACSL4 expression and glucose metabolism, at least in rodents.[99] These studies also found that in mice fed a high-fat diet,[99] hepatic ACSL4 expression resulted in increased serum levels of leukotriene B4—a pro-inflammatory cytokine derived from arachidonic acid which is associated with obesity[140] and an important factor in mediating systemic inflammation.[141] Recent work has demonstrated that systemic inflammation in HCC correlates with poor clinical outcomes for patients[142] treated with immune checkpoint inhibitors, suggesting that further work investigating the pathological significance of a hepatic ACSL4-leukotriene B4 axis may be warranted.

ACSL4 expression in hamsters, mice and human hepatocytes is regulated by the peroxisome proliferator-activated receptor delta (PPARδ) which is a nuclear receptor transcription factor (also sometimes referred to as PPARβ or PPARβ/δ[50]) that can be activated by long-chain fatty acids.[143] This PPARδ-dependent mechanism provides a potential link to rationalise an association between diet and the regulation of ACSL4 gene transcription. Whilst PPARδ is overexpressed in some HCC cases,[144] available evidence indicates that its activation favours reduced lipogenesis, and as a further complication both pro- and anti-carcinogenic effects of this transcription factor have been reported.[144–146] Thus, it is currently unclear whether the PPARδ-dependent expression of ACSL4 is important in terms of HCC development.

ACSL3 expression is also under the control of PPARδ[51] indicating that the transcription of both isozymes is controlled by the same general mechanism. It is important to note that PPARδ also induces the transcription of several other genes important for lipid metabolism such as *ACC1*.[147,148] Therefore, a more comprehensive understanding of the functions of ACSL3/4 in hepatic lipid metabolism[46] likely needs to consider alterations to the expression levels of other PPARδ regulated enzymes.

Similar to ACSL4, most of the available information on ACSL3 function in the normal liver also derives from animal studies. In a healthy liver, the function of ACSL3 may partially overlap with ACSL4 as studies in hamsters have shown that ACSL3 is also required for VLDL generation and specifically for the synthesis of phosphatidylcholine which is the sole phospholipid on the surface of VLDL particles.[47] Phosphatidylcholine is also a major constituent of the hydrophilic phospholipid monolayer on the surface of cytoplasmic lipid droplets where ACSL3 is found in HCC.[34] However, unlike ACSL4, several reports have shown that experimentally induced expression of ACSL3 may lead to less neutral lipid storage in hepatocytes.[45,48,149] Adenoviral-mediated expression of ACSL3 in hamster liver leads to reduced TAG storage[45] as does upregulation of ACSL3 (and ACSL5) expression through oncostatin M addition.[48] In hamsters, feeding with a high-fat diet induces hepatic ACSL3 expression[46] thereby demonstrating that expression of this enzyme is also regulated in response to dietary lipid concentrations. On the contrary, high fructose diets have been shown to specifically decrease ACSL3 expression through effects on the LXR transcription factor, thus indicating the existence of alternative, diet-specific, isoform-selective modes of gene regulation.[149] LXR-mediated ACSL3 upregulation also reduces hepatic TAG which is again consistent with a function in lipid clearance or catabolism.[149] Another layer of complexity when seeking to understand the adaptive changes to ACSL3 expression in the liver stems from the observation that this enzyme can positively regulate lipid metabolism by promoting the activity of several lipogenic transcription factors including SREBP1c, LXRα and PPARγ[42] through a yet to be elucidated mechanism. Overall, these studies have found that increasing ACSL3 expression in the liver does not cause increased lipid droplet or TAG accumulations as might be expected from the very detailed cell biology studies showing that ACSL3 is required for lipid droplet biogenesis. The reasons for such conflicting observations are not immediately clear but one possible explanation is that ACSL3 can channel activated fatty acids to either lipogenic or catabolic pathways depending on the local protein co-expression network and available protein interactome similar to mechanisms determined for ACSL1.[150] Potential protein interaction partners for ACSL3 that are relevant in this context include syntaxin-17,[43] seipin[128] and CDCP1.[151] Therefore, predicting the biochemical consequences of increased hepatic ACSL3 is not a simple proposition and may be highly contingent on nutritional or signalling drivers that modulate its transcription.

5 | POST-TRANSCRIPTIONAL CONTROL OF ACSL EXPRESSION BY MICRORNAS IN HCC

Beyond gene transcription by lipogenic transcription factors, ACSL expression can be subject to epigenetic regulation through the inhibition of mRNA translation by microRNA (miRNA) binding.[87] Several miRNAs have been identified that bind to the 3′-untranslated region of ACSL4 mRNA to prevent its translation.[33,87] One such miRNA, designated miR-23a-3p, is the main miRNA species isolated from HCC tissues where its presence is strongly associated with a poor prognosis and resistance to sorafenib.[33] A recent study from Lu and colleagues demonstrated that miR-23a-3p suppressed the translation of ACSL4 mRNA, and this was the molecular mechanism underlying attenuated sorafenib-induced ferroptosis in this subclass of HCC.[33] Transcription of miR-23a-3p is regulated by ETS-1[33]; a proto-oncogene that activates transcription by the pregnane X receptor, and a proposed mediator of sorafenib resistance in HCC.[152] It is possible that the loss of ACSL4 expression induced by ETS-1, especially in the case of recurrent tumours, may represent an adaptive response to enhance cell survival by reducing PUFA-dependent ferroptosis which manifests as increased sorafenib resistance.[32]

Conversely, in a separate study, Qin and colleagues reported that inhibition of ACSL4 translation by a different miRNA, miR-211-5p, suppressed tumourigenesis and growth, indicating that ACSL4 expression alone does not necessarily predict tumour aggressiveness in all aetiologies of HCC.[35] Another complicating issue is that a single class of miRNA can alter the expression of more than one ACSL isoform; for example, miR-205 inhibits the expression of ACSL4 in HCC[153] but also increases the expression of ACSL1.[114] Hence, miRNAs—similar to the PPAR family of transcription factors can potentially generate a complex mosaic of possible outcomes for fatty acid metabolism and ACSL functions. Such inferences complicate considerations of ACSL4 as a therapeutic target in HCC since depending on the genetic landscape or aetiology of a hepatoma, both loss and gain of ACSL4 expression have the potential to affect HCC progression. In the majority of HCC cases, increased ACSL4 expression leads to tumour progression[55,127] through increased cell proliferation, decreased apoptosis[37,38,53,100] and boosted fatty acid metabolism.[55] However, as illustrated in the case of miR-23-3p, reduced expression of ACSL4 in advanced lesions may have the potential to attenuate drug sensitivity due to diminished ACSL4-dependent ferroptosis.[33]

6 | REGULATION OF ACSL EXPRESSION IN HCC BY N6-METHYLADENOSINE-MODIFIED RIBOSOMAL RNA

Covalent, epigenetic, methylation of adenosine at the N6 position of RNA molecules to form N6-methyladenosine (m^6A) is a common occurrence in HCC.[154] Recently, Peng and colleagues reported that 18 s ribosomal RNA (rRNA) in hepatocytes undergoes an m^6A modification in a reaction catalysed by the METTL5-TRMT112 methyl transferase complex.[55] They demonstrated that this rRNA modification was required for ribosomal biogenesis and consequently the translation of genes involved in fatty acid metabolism including ACSL1, ACSL3, ACSL4 and ACSL5. They also reported that increased METTL5-TRTMT112 levels correlated with more advanced and aggressive HCC grades. Using cultured HCC cell lines and mouse models, METTL5 was shown to boost cell proliferation, inhibit apoptosis, and amplify fatty acid metabolism through both the FAO and de novo lipogenic pathways. Concentrating their efforts on ACSL4, the authors demonstrated that the tumourigenic and tumour progression properties of overexpressed METTL5 in HCC were mainly mediated by ACSL4 protein expression.[55] Moreover, inhibition of the hepatic expression of ACSL4 and METTL5 in mice effectively blocked HCC progression thus revealing a potential novel strategy to treat established HCC. These observations provide strong evidence that post-transcriptional regulation of ACSL expression is an important determinant in HCC promotion and progression, and that elevated ACSL4 levels are essential for upregulating fatty acid metabolism to sustain hepatoma cell survival and proliferation, mirroring to some extent, the dual catabolic and anabolic roles described for ACSL1 in promoting prostate cancer progression.[155]

7 | POST-TRANSLATIONAL REGULATION OF ACSL4 EXPRESSION AND ACTIVITY

Early studies concluded that mRNA expression levels are not always a reliable guide to ACSL protein expression in tissues,[44] prompting speculation that post-translational mechanisms are likely to be important for regulating their steady state protein levels and/or enzymatic activity. There is no published information hitherto available on post-translational regulation or covalent modifications of ACSL3, hence the focus of this section will be on the ACSL4 isozyme which can undergo several reversible, regulatory modifications in response to changes in oxygen and nutrient availability, as well as its substrate and product lipid concentrations.

In cultured liver cancer HepG2 cells, ACSL4 is constitutively ubiquitinated, and its degree of ubiquitination increases as substrate arachidonic acid levels rises.[156] Arachidonic acid-induced ubiquitination of ACSL4 targets the enzyme for proteasomal degradation, consistent with a mode of substrate-dependent, feed-forward regulation.[156] Sumoylation, the covalent addition of a small ubiquitin-like modifier protein to ACSL4 may also be important for its regulation. In cardiomyocytes, hypoxia-dependent HIF-1α activation induces deSUMOylation of ACSL4 leading to reduced protein levels and reduced ferroptosis[157]; however, it is not known if ACSL4 can be similarly regulated in hepatomas. ATP-dependent phosphorylation has also been proposed to regulate ACSL4. In adrenocortical tumour cells, ACSL4 can undergo hormone-induced phosphorylation[123] and there is now a precedent for ACSL4 activation by phosphorylation catalysed by PKCβII during ferroptosis[61]—a process likely to be relevant for precancerous liver disease.[127]

Another recently described post-translational modification of ACSL4 with potential relevance to hepatocarcinogenesis is its O-GlcNAcylation catalysed by N-acetylglucosaminyltransferase, and this is associated with ACSL4-upregulated mTOR signalling.[37] Elevated protein O-GlcNAcylation is associated with increased oncogenesis in HCC and is thought to result from aberrant glucose metabolism (eg, see[158–161]). The covalent addition of N-acetylglucosamine to a protein's serine or threonine residues can potentially modulate its phosphorylation by a regulatory kinase.[162,163] Whilst the sites of N-acetylglucosamine glycosylation on ACSL4 have not yet been identified, future work may elucidate if this modification affects the interaction of ACSL4 with PKCβII in a similar fashion to that reported for RACK1 protein in HCC cells.[164]

8 | ACSL4 INVOLVEMENT IN NAFLD AND NASH

NAFLD is the most significant growing cause of HCC in developed countries, and with the rising obesity epidemic and NASH incidence estimated to increase by 56% over the next decade,[21] there is a strong focus on understanding the pathogenesis of NASH-HCC, as well as its prevention and therapy. There is accumulating evidence that ferroptosis[165–167] and ACSL4 are involved in the development and progression of NAFLD, NAFLD-related hepatocellular damage and NASH.[49] ACSL4 mRNA expression levels rise with increasing levels of fat in the human liver[168–170] and ACSL4 protein levels are elevated in both NAFLD and NASH HCC.[49,171] A recent study also found that ACSL4-deficient mice are more resistant to developing NASH, exhibit lower serum levels of ALT and AST and show reduced fibrosis on liver histology.[49] Abemaciclib— a drug currently approved for use as a small molecule inhibitor of cyclin-dependent kinases in HER2 negative metastatic breast cancer,[172] was identified as a novel, potent, ACSL4 inhibitor which prevented excess hepatic lipid accumulation by increasing FAO in mice with NASH.[49] Similar to a previous finding where ACSL4 activity was inhibited by thiazolidinediones,[59] increased FAO induced by Abemaciclib was not accompanied by additional intracellular oxidative stress,[49] and, therefore, less likely to cause cell death. Work by Grube and colleagues using hepatocyte-specific gene knockouts for ACSL4 in murine models has revealed further information on the pathogenesis of ACSL4 in both NASH and HCC.[127] In concordance with previous observations, ACSL4 expression was found to be required for hepatocyte ferroptosis, but also led to increased apoptosis, oxidative stress and inflammation, thus establishing that ACSL4 can exacerbate liver injury through a variety of mechanisms. Significantly, ACSL4 expression was found to be dispensable for HCC tumourigenesis with similar numbers of tumours counted in both ACSL4-expressing and ACSL4 knock-out livers.[127] Hence, ablating hepatic ACSL4 expression did not prevent NASH-induced HCC in this set of experiments. However, the association between ACSL4-induced tissue damage and HCC is likely to more nuanced because in a diethylnitrosamine carbon tetrachloride, chemically induced liver injury disease model, there was increased hepatic fibrosis when ACSL4 was expressed, and under such circumstances, there was also a subsequent increase in tumour progression which manifested as larger tumours.[127] These results are noteworthy since they demonstrate that at least in these experimental models, ACSL4-dependent ferroptosis during liver injury neither initiates nor suppresses the formation of HCC but may increase tumour progression in instances where ACSL4-dependent cell death leads to fibrosis. Furthermore, the authors also considered that the effects of ACSL4 on tumour growth might be mediated through functions other than ferroptosis such as upregulated lipid metabolism, which would align with other recent work in this area.[55]

Despite recent successes in the use of immunotherapies for the treatment of HCC, and in particular the combination of atezolizumab with bevacizumab,[77,78,173] NASH-HCC has been shown to be intrinsically resistant to immune checkpoint inhibitors because of the presence of unusually activated and phenotypically altered $CD8^{+}PD1^{+}$ T cells in the tumour microenvironment.[174–176] This T cell impairment is thought to be due to the sustained period of lipotoxicity, tissue damage and chronic inflammation that precedes tumourigenesis[174]—processes which can be aggravated by hepatocyte ACSL4 expression.[49] Whilst ACSL4 expression in NASH can result in more cell death, ACSL4 expression in tumour cells may also represent a metabolic vulnerability that can be exploited to increase the effectiveness of immune checkpoint inhibitors. This is because activated $CD8^{+}$ T cells kill tumour cells by inducing ferroptosis, and this requires tumour expression of ASCL4.[71] For example, studies on melanoma have shown that T-cell-derived interferonγ stimulates tumour cell localised ACSL4 to activate arachidonic acid. This activated PUFA is subsequently incorporated into membrane phospholipids resulting in increased tumour cell ferroptosis and therefore, enhanced immune checkpoint inhibitor sensitivity.[71] It is not yet known if HCC cells are similarly susceptible to this mode of T-cell-induced cell death but there has been significant research, and indeed some debate,[56] into the induction of ACSL4-dependent ferroptosis as a potential anti-tumour strategy for HCC[32,33,177]; or alternatively, direct inhibition of ACSL4 to block its tumour-promoting functions that do not involve ferroptosis.[16,31,37,55,127]

There is less literature available on the role of ACSL3 in pathological steatosis. There has been some speculation that ACSL3 may play a protective role in NAFLD based on its upregulation in response to oncostatin M[48,51]—a member of the interleukin (IL)-6 family of cytokines implicated in post-injury liver tissue regeneration.[178,179] Oncostatin M upregulation of ACSL3 appears to channel long-chain fatty acids, and especially palmitate, to FAO, thereby lowering both cellular and serum triglyceride levels.[48] In addition, oncostatin M receptor β knockout mice are more prone to developing hepatic steatosis and have increased inflammation of the adipose tissue.[180] Nevertheless, there is currently no direct evidence for ACSL3 involvement in NASH-HCC progression.

9 | ACSL4 STABILISATION OF C-MYC AND INCREASED SREBP1 ACTIVITY LINKS ONCOGENIC SIGNALLING WITH INCREASED LIPOGENESIS

In HCC, overexpression of ACSL4 promotes cell survival, proliferation, and lipogenesis,[16,31,37] which may indicate the existence of a common mechanism to control these functions in transformed cells. Recent evidence indicates that this functional integration may be achieved through ACSL4 stabilisation of c-Myc; the proto-oncogene transcription factor that transcribes genes required for cell cycle progression and the prevention of apoptosis, as well as SREBP1—the master transcription factor for lipogenesis.[16,31] In hepatoma cells with increased Ras signalling, ACSL4 (through a yet undetermined mechanism) alters the ERK-dependent phosphorylation of c-Myc so that it is no longer recognised by the FBW7 tumour suppressor component of the ubiquitin ligase system,[181] and is consequently not trafficked for proteasomal degradation.[16,31] This scenario wherein ACSL4 expression modulates both cell signalling and lipogenic gene transcription provides a molecular basis for understanding its pathological roles in HCC.[181] In this vein, it is relevant to note that decreasing hepatic ACSL4 expression in mice also results in reduced levels of the p53 tumour suppressor protein.[99] These findings suggest that ACSL4 may have a general role in stabilising transcription factor levels in HCC and that this regulatory function has the potential to affect tumour growth.

Another takeaway from these observations regarding c-Myc stabilisation[31] is that ACSL4 can positively modulate the RAS/ERK signalling axis, albeit through a yet unelucidated mechanism. Results from a non-biased, close-proximity, RAS protein interaction screen have revealed that ACSL4 can interact with both NRAS and KRAS[182] indicating a possible direct mechanism to consider for further investigation. This integration of fatty acid metabolism with tumour growth suggests the existence of oncogene-specific functions for ACSL4 that are triggered in cancer cells with particular mutational profiles such as the co-overexpression of Ras and/or c-Myc. This concept also implies that the tumour-promoting functions of ACSL4 in HCC cannot be explained simply through amplification of its intrinsic catalytic activity alone but can, however, be rationalised through positive effects on proteins that drive cell proliferation such as c-Myc or activate lipogenesis, such as SREBP1. To some extent, this concept echoes previous findings from mapping the protein interactome of ACSL1 where the specific cellular functions of the enzyme were found to be dependent on the dynamic and differential subcellular targeting of the protein—protein interaction complexes that ACSL1 can form.[150]

One caveat when considering the general applicability of an ACSL4 -Ras-Myc-SREBP1 pathway in HCC is that activating RAS mutations are rarer in human HCC compared to other gastrointestinal tumours[183]; nevertheless, signalling through the wildtype RAS—ERK/MAPK pathway is elevated in many cases of HCC[183,184] because of mutations[185] or epigenetic promoter silencing of genes such as RASSF1A[186] that regulate RAS. Hence, in HCC cases where Ras signalling is elevated, ACSL4 may act as a macromolecular amplifier that can boost both oncogenic signalling and lipogenesis through positive effects on c-Myc and SREBP1-mediated transcription.

The link between ACSL4 activity and SREBP1 may also be relevant for understanding the increased expression of ACSL3 in HCC as there is evidence that SREBP1 upregulates ACSL3 transcription under pathological conditions that feature reprogrammed lipid metabolism. In lactate-rich HCC cells, SREBP1-activated transcription is elevated and this leads to a ferroptosis-resistant state attributed to increased generation of MUFA-containing phospholipids[83]: an emerging characteristic of ACSL3-mediated malignancy[81,83,187] which counteracts ACSL4/PUFA-mediated cell death.[80] Additionally, in colorectal cancer cells, TGFβ1-stimulated epithelial-mesenchymal transition requires SREBP1-dependent upregulation of ACSL3 expression,[188] as does the rewiring of macrophage fatty acid metabolism that occurs during the resolution of TLR4-stimulated inflammation.[189] Distinct from the ACSL4-stabilised RAS-Myc pathway, SREBP1-dependent changes to fatty acid metabolism can be mediated by the phosphatidylinositol 3-kinase—mTOR pathway, and this HCC mutational signature is often associated with increased lipogenesis.[7,190] Moreover, mTORC2 overexpression has been demonstrated to drive both steatosis and HCC[9] by upregulating lipogenesis and FAO (although effects on ACSL3 were not mentioned in this study).

Whilst most of the emphasis in this section has been on the relationship between SREBP1 and ACSL3 in lipogenesis, there is also a possibility that upregulated ACSL3 expression in HCC could increase the flux of fatty acids into the catabolic FAO pathway as occurs in *KRAS*-driven lung cancers, where increased ACSL3 expression leads to more FAO and, therefore, augmented cell survival and proliferation.[54] As mentioned earlier, there are some precedents for overexpression of ACSL3 in hepatocytes and HCC cell lines increasing FAO.[48] However, the relevance of these findings to HCC is not yet fully understood, and ACSL3 has so far not been implicated, for example, in FAO in β-catenin driven HCC.[24]

10 | FUTURE DIRECTIONS AND CONCLUDING REMARKS

The overexpression of ACSL4 is a robust immunohistochemical marker for HCC, and when used in combination with other co-expressed proteins such as c-Myc,[31] SREBP1[16] or DNA damage-inducible gene 45β (GADD45B),[38] it can discriminate between different molecular subclasses of HCC and may be useful for defining sensitivity to different drugs[38] or as a prognostic biomarker.[31,36] The clinical utility of ACSL3 expression is comparatively less well understood in this regard, but this isoform is also upregulated in several classes of liver tumours including cholangiocarcinoma and secondary metastases.[34] Our recent work indicated that ACSL3 and ACSL4 as combined biomarkers may be useful for identifying different types of hepatic tumours.[34]

With regard to developing drug treatments that target ACSL pathways in HCC, several studies have found that ACSL4 expression is required for the induction of ferroptosis by sorafenib and this has been proposed as the primary mechanism through which this non-specific kinase inhibitor[191] can induce cell death.[32] However, ACSL4 is not the sole molecular determinant of ferroptosis and diverse factors such as increases in intracellular lactate,[83] the cholesterol-sensing SCAP protein,[6] metallothionein-1G[192] or phosphoseryl-tRNA kinase expression[193] can all suppress this cell death pathway in HCC cells. Furthermore, co-expression of ACSL3 is known to be anti-ferroptotic in other cancers.[80,81] Hence, high levels of ACSL4 expression alone may be insufficient to ensure sensitivity to ferroptosis in HCC. Aspirin in combination with sorafenib has been suggested as a treatment for a subclass of HCC tumours that express high levels of ACSL4 alongside low levels of GADD45B—a protein which participates in the JNK pathway for apoptosis.[38] This combined therapy induced cell death via apoptosis as opposed to ferroptosis.[38] Notwithstanding known issues with acquired resistance to apoptosis in liver cancer,[194] selective inhibition of ACSL4 could be a feasible approach for the targeted treatment of some HCC subtypes. The availability of newly identified small molecule inhibitors of ACSL4[195] including Abemaciclib as a candidate treatment for NASH,[49] and PRGL493 as a potential treatment for ACSL4-expressing breast and prostate cancers,[196] opens up opportunities to investigate direct pharmacological inhibition of this enzyme in HCC.

In conclusion, ACSL3 and ACSL4 are implicated in an array of HCC functions beyond just ferroptosis and simple steatosis. Recent advances have revealed how overexpression of these isozymes in the liver can reprogramme lipid metabolism, increase oncogenic signalling, and drive tumour growth, and this knowledge is already informing new strategies for the treatment and diagnosis of HCC.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

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