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Posttranscriptional destabilization of the liver-specific
long noncoding RNA HULC by the IGF2 mRNA-binding protein
1 (IGF2BP1)
Post-transcriptional destabilization of the liver-specific long non-coding RNA

*HULC* by the IGF2 mRNA-binding protein 1 (IGF2BP1)

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Abbreviations used in this paper: HCC, Hepatocellular carcinoma; ncRNA, non-coding RNA; RNA, Ribonucleic Acid; HULC, Highly Up-regulated in Liver Cancer; CREB, cAMP responsive element binding protein; IGF2BP, IGF2 mRNA-binding protein; qRT-PCR, quantitative Reverse Transcription - Polymerase Chain Reaction; GFP, Green Fluorescent Protein; siRNA, small interfering RNA; miRNA, microRNA

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ABSTRACT

Selected long non-coding RNAs (lncRNAs) have been shown to play important roles in carcinogenesis. Although the cellular functions of these transcripts can be diverse, many lncRNAs regulate gene expression. In contrast, factors that control the expression of lncRNAs remain largely unknown. Here, we investigated the impact of RNA binding proteins on the expression of the liver cancer-associated lncRNA HULC (Highly Up-regulated in Liver Cancer). First, we validated the strong up-regulation of HULC in human hepatocellular carcinoma. To elucidate post-transcriptional regulatory mechanisms governing HULC expression, we applied an RNA affinity purification approach to identify specific protein interaction partners and potential regulators. This method identified the family of IGF2BPs (IGF2 mRNA-binding proteins) as specific binding partners of HULC. Depletion of IGF2BP1, also known as IMP1, but not of IGF2BP2 or -3, led to an increased HULC half-life and higher steady-state expression levels, indicating a post-transcriptional regulatory mechanism. Importantly, HULC represents the first IGF2BP substrate that is destabilized. To elucidate the mechanism by which IGF2BP1 destabilizes HULC, the CNOT1 protein was identified as a novel interaction partner of IGF2BP1. CNOT1 is the scaffold of the human CCR4-NOT deadenylase complex, a major component of the cytoplasmic RNA decay machinery. Indeed, depletion of CNOT1 increased HULC half-life and expression. Thus, IGF2BP1 acts as an adaptor protein that recruits the CCR4-NOT complex and thereby initiates the degradation of the lncRNA HULC. Conclusion: Our findings provide important insights into the regulation of lncRNA expression and identify a novel function for IGF2BP1 in RNA metabolism.
INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and the third most common cause of cancer mortality (1). Representing a multifactorial genetic and epigenetic disease with a complex etiology, the major cause of HCC is a long-term liver injury caused by, e.g. infection with hepatitis B or C virus, alcoholic liver disease, aflatoxin exposure or a variety of inherited metabolic diseases (2). Although numerous small and high-dimensional profiling analyses have been performed in human hepatocellular carcinoma (for review see (3)), the molecular mechanisms and factors involved in liver carcinogenesis are still not fully understood. Recently, it has been uncovered that the human genome encodes much more information than previously anticipated. The vast majority (70-90%) of the human genome sequence is pervasively transcribed into RNA, while only a small fraction (~2%) contains information for protein-coding genes (4-7). Thus, the largest fraction of the human genome encodes ncRNAs (non-coding RNAs). Some of these transcripts are highly conserved, show regulated and tissue-specific expression, and exert critical functions in the cell (8-13). Mechanistically, some ncRNAs were shown to have a strong impact on the regulation of gene expression (14-18) or post-transcriptional processing (19, 20). Moreover, several ncRNAs are deregulated in human diseases including cancer, influence disease onset as well as progression, or can be of prognostic value (21-23). Thus, studying long ncRNA expression, regulation and function in human liver cancer is essential to fully understand the underlying molecular mechanisms.

The long ncRNA HULC (Highly Up-regulated in Liver Cancer) is one of the first strongly overexpressed non-coding transcripts to be identified in human HCC (24). The HULC gene is located on chromosome 6p24.3 and is conserved in primates.
Transcription of *HULC* yields a ~500 nt long, spliced and polyadenylated ncRNA that localizes to the cytoplasm where it has been reported to associate with ribosomes (24). *HULC* expression has been described to be regulated by the transcription factor CREB (cAMP responsive element binding protein) in Hep3B cells (25). In addition, the HBx protein has been linked to the activation of *HULC* expression in HepG2 cells via interaction with CREB (26). Elevated *HULC* levels in HBx expressing HepG2 cells induce a higher proliferation rate and tumor growth and lead to a downregulation of the tumor suppressor p18. Moreover, *HULC* might function as a miRNA sponge for miRNA-372 and thereby could regulate gene expression at a post-transcriptional level (25). While it is clear that *HULC* plays an important role in liver carcinogenesis and acts as an oncogenic ncRNA, the regulatory mechanisms controlling *HULC* expression are largely unknown.

Our aim was to determine regulatory mechanisms that control this ncRNA, highly expressed in HCC. We hypothesized that RNA binding proteins could have an impact on *HULC* expression and set up an RNA affinity purification assay to identify specific protein interaction partners of *HULC*. In this study, we identified the IGF2BP (IGF2 mRNA-binding protein) family of RNA binding proteins as specific interaction partners of *HULC* in human liver cancer cells. *HULC* was discovered as the first IGF2BP substrate that is not stabilized or translationally regulated, but destabilized via CNOT1-mediated deadenylation recruited by IGF2BP1.
Tumor Material and Patient Characteristics

Sixty human HCCs were analyzed for HULC expression using microarray analysis. Median age at surgery was 57 years (range 16-78), and the male/female ratio was 4:1. All diagnoses were confirmed by histological reevaluation, and use of the samples was approved by the local ethics committee. The cohort contained a balanced repertoire of relevant underlying etiologies: HBV (n = 15), HCV (n = 12), alcohol (n = 10), cryptogenic (presumably mostly NAFLD; n = 19) or genetic hemochromatosis (n = 3). The patients’ characteristics are shown in Suppl. Table 1.

In vitro Transcription

For in vitro transcription, the Megascript T7 kit (Life Technologies, Carlsbad, USA) was used according to the manufacturer’s recommendations. Briefly, 1 µg linearized plasmid template was used and reactions were incubated for 16 h in the presence or absence of Biotin-16-UTP (Epicentre, Madison, USA). Ratio between UTP and Biotin-16-UTP was 20:1. Reaction was stopped by addition of 1 µL Turbo-DNase. RNA was precipitated with LiCl. RNA integrity and size were controlled using agarose gel electrophoresis.

RNA affinity purification

Beads were pre-blocked with 1 mg/mL BSA (Roche), 0.2 mg/mL yeast tRNA (Roche) and 0.2 mg/mL Glycogen (Carl Roth, Karlsruhe, Germany) in low salt wash buffer (20 mM Hepes, pH 7.9; 100 mM KCl; 10 mM MgCl₂; 0.01% NP40; 1 mM DTT) before addition of RNA. RNA was incubated with 50 µL Streptavidine-Sepharose beads (GE Healthcare, Little Chalfont, England) in 500 µL HS-WB300 (20 mM Hepes, pH7.9;
300 mM KCl; 10 mM MgCl₂; 0.01% NP40; 1 mM DTT) for 4 h. Unbound RNA was washed away with 3x 1 mL HS-WB400 (20 mM Hepes, pH7.9; 400 mM KCl; 10 mM MgCl₂; 0.01% NP40; 1 mM DTT). Cytoplasmic cell extract (2-3 mg) was added and incubated over night at 4°C. The next day, the extract was removed and beads were washed 6 times with 1 mL HS-WB400. Beads were resuspended in 50 µL 6 M urea; 1 mM DTT; 0.01% NP-40 and incubated at room temperature for 30 min. in a shaking block at 900 rpm. Then, the supernatant was transferred into a new tube and proteins were precipitated with 5 volumes of pre-chilled acetone for 1 h at -20°C. Proteins were pelleted via centrifugation at 13000 g at room temperature. Pellets were washed twice with 1 mL 80% ethanol, dried for 5 min. and resuspended in 20 µL protein sample buffer.

RNA stability analysis
HepG2 cells were transfected with siRNAs as stated above. After 48 h, alpha-amanitine (AppliChem, Darmstadt, Germany) was added (10 µg / mL f.c.) and cells were harvested at the indicated time points. All experiments were done in biological triplicates.

RNA-Protein-Interaction analysis
Detailed methodological information on RNA affinity purification and co-immunoprecipitation experiments including a protocol for cytoplasmic extract preparation, RNA in vitro synthesis and Biotin labeling, SDS-PAGE and coomassie staining as well as IGF2BP cloning can be found in the Supplementary Experimental Procedures.
RESULTS

**HULC is overexpressed in HCC and correlates with staging and grading**

*HULC* was shown to be overexpressed in human HCCs using an HCC-specific cDNA microarray (25). To validate these previous findings in an independent, larger patient cohort, we performed unbiased microarray analysis of 60 HCC and 7 normal liver samples using the Agilent SurePrint G3 Human Gene Expression array. We identified *HULC* as the second most highly up-regulated non-protein-coding gene in HCC (Fold change = 6.51, p = 3.3x10^{-5}, T-Test) ([Fig. 1A](#)). Only the *ERBB2* pseudogene showed a stronger up-regulation in human HCCs (Fold change = 8.23, p = 4.6x10^{-7}; data not shown). We confirmed the overexpression of *HULC* in HCC by qRT-PCR (quantitative Reverse Transcription-Polymerase Chain Reaction) analysis in a subset of 34 tumor samples and 6 normal livers ([Fig. 1B](#)) significantly correlating with the microarray data (R=0.452, Spearman). The respective patient data of this subset are enclosed in **Table 1**. The relative expression level of *HULC*, as determined by qRT-PCR, was about 8-fold higher in HCC samples than in normal liver tissue ([Fig. 1C](#)). Interestingly, we detected a significantly higher expression level of *HULC* in low grade and low stage tumors ([Fig. 1D](#)). However, *HULC* expression did not correlate with age, sex, tumor size, or haemangiosis (Table 1). *HULC* expression was previously shown to be induced by the viral HBx protein (26) and increased in HBV-producing cells (27). Thus, we tested whether *HULC* levels correlated with different tumor etiologies (Table 1). However, there was no significant correlation between *HULC* expression and HBV or HCV infection (Mann-Whitney U, p = 0.078 (HBV vs. non-HBV); p = 0.220 (HCV vs. non-HCV)), the average *HULC* level was even lower in HBV-infected patients than in other HCC samples ([Fig. 1E](#)).
**HULC interacts with IGF2 mRNA binding proteins**

After transcription, a ncRNA will likely associate with proteins to form a ribonucleoprotein complex that will govern ncRNA stability, degradation, and function. Thus, post-transcriptional regulators could interact with HULC and contribute to its regulation and consequently its functional impact. Therefore, we aimed at the identification of interacting proteins as potential regulators using an RNA affinity purification approach. An overview about the method is given in **Fig. 2A**. We used cytoplasmic extracts prepared from Huh7 HCC cells and incubated these with a 500 nt long, *in vitro* transcribed and biotinylated HULC RNA. An RNA molecule of the same length but unrelated in sequence was used as a negative control. Proteins associated with HULC or the control RNA were eluted, separated on a polyacrylamide gel and visualized with sensitive coomassie blue staining (**Fig. 2B**). Multiple proteins with an observed molecular weight of ~70 kDa were specifically pulled down with HULC (**Fig. 2B, box**). Subsequent mass spectrometry analyses identified several protein interaction partners. Among the top five candidate proteins, we reproducibly identified three members of the IGF2 mRNA-binding protein family, namely IGF2BP1, IGF2BP2 and IGF2BP3, also known as IMP1, IMP2 and IMP3 (**Suppl. Table 5**). Specific binding of IGF2BP1, IGF2BP2 and IGF2BP3 was confirmed by Western blot analysis (**Fig. 2C**). We validated the interaction between IGF2BPs and HULC also in HepG2 cells (**Fig. 2D**). HnRNP A1, an unrelated RNA binding protein, and Vinculin, a protein associated with the cytoskeleton, were included as controls for specificity.

As an independent approach to verify the interaction between HULC and IGF2BPs *in vivo*, we performed RNA immunoprecipitation assays. FLAG-tagged IGF2BP1, IGF2BP2, IGF2BP3, or GFP (Green Fluorescent Protein; neg. control) were transiently overexpressed in HepG2 cells and immunoprecipitated with an anti-FLAG
antibody (Fig. 2E). After isolation of the co-purifying RNA, the enrichment of selected transcripts was measured via qRT-PCR. Thereby, we confirmed the specific enrichment of both HULC and a bona fide target of IGF2BPs, IGF2 mRNA (Fig. 2F). No enrichment of HULC was seen in GFP pull downs. The highly abundant 5.8S rRNA (negative control) was not enriched in any of the purifications. Thus, we identified the IGF2 mRNA binding proteins as specific interaction partners of HULC. Furthermore, we characterized the interaction between HULC and IGF2BP1 in more detail and could show that also endogenous, non-tagged IGF2BP1 specifically bound to HULC (Suppl. Fig. 1A). To identify the site of interaction, we performed an in vitro binding assay using recombinant human IGF2BP1 and in vitro transcribed HULC full-length or fragmented RNA (Suppl. Fig. 1B and C). The assay revealed a direct and specific binding of IGF2BP1 to multiple sites across the non-coding transcript (Suppl. Fig. 1D).

IGF2BP1 specifically regulates HULC expression and stability

IGF2BPs are well-known RNA binding proteins that were shown to regulate translation, localization, or stability of their target RNAs (28-34). Specifically, IGF2BP1 stabilizes MYC, MDR1, and PTEN mRNAs (35-37). To determine whether HULC expression was controlled by IGF2BPs, we specifically depleted IGF2BP1, IGF2BP2, or IGF2BP3 from HepG2 cells using siRNAs (small interfering RNAs) (Fig. 3A, B). The knockdowns were efficient as analyzed by qRT-PCR (Fig. 3A), and specific to each of the IGF2BP family members as shown by Western blot analysis (Fig. 3B). Interestingly, the knockdown of each IGF2BP alone led to an enhanced HULC expression. The strongest increase was observed after IGF2BP1 depletion, which was highly significant compared to control siRNA or IGF2BP2 and IGF2BP3 siRNA transfections (Fig. 3C). To distinguish between a transcriptional and a post-
transcriptional mechanism, we specifically blocked RNA Polymerase II transcription with alpha-amanitin. This experiment revealed a strong impact of IGF2BP1 on HULC RNA stability (Fig. 3D). While the half-life of HULC was between 7.0 h and 7.7 h after control siRNA and IGF2BP2 or IGF2BP3 siRNA transfection, the half-life was almost twice as long when IGF2BP1 was depleted (13.3 h ± 1.5 h). The stabilizing effect was also seen after IGF2BP1 depletion with a second independent siRNA (Suppl. Fig. 2A). Moreover, transient overexpression of IGF2BP1 in HepG2 significantly decreased HULC expression levels (Suppl. Fig. 2B and C).

This suggested that IGF2BP1, but neither IGF2BP2 nor IGF2BP3, regulated HULC post-transcriptionally. To our knowledge, HULC was the first IGF2BP1 target RNA that was destabilized by this protein. Hence, we wanted to elucidate the mechanism of HULC destabilization by IGF2BP1.

IGF2BP1 interacts with CNOT1, a component of the CCR4-NOT deadenylase complex

Intracellular RNA degradation occurs via two major pathways starting from the 5’end or the 3’end of the RNA, respectively and could involve miRNAs (38). HULC was previously shown to be part of a negative feedback loop acting as a sponge for microRNA-372 (25). Thus, we tested whether IGF2BP1 depletion influences mature miR-372 expression in HepG2 cells, but we could not detect a significant downregulation of miR-372 (Suppl. Fig. 3A). In addition, we could not observe a downregulation of HULC upon miR-372 overexpression in three different liver cancer cell lines (Suppl. Fig. 3B). These findings implicate an alternative, miR-372-independent regulatory mechanism.

Hence, we hypothesized that IGF2BP1 might associate with components of the RNA decay machinery to mediate RNA degradation. To pursue this hypothesis, we
transfected HepG2 cells with FLAG-tagged IGF2BP1 or GFP as a control. After anti-FLAG immunoprecipitation, we tested whether IGF2BP1 interacted with XRN1 or CNOT1 by Western blot analysis (Fig. 4A). XRN1, the major cytoplasmic 5’-3’-exonuclease, did not co-purify with IGF2BP1. In contrast, CNOT1 showed specific binding to IGF2BP1, but not to GFP (Fig. 4A). CNOT1 is the scaffold protein of the CCR4-NOT complex, an important deadenylase responsible for poly(A) tail shortening and inducing 3’-5’-decay of numerous RNAs in the cytoplasm (39). Thus, IGF2BP1 interacted with a central component of the RNA decay machinery.

Depletion of CNOT1 stabilizes HULC

Interaction with CNOT1 might be crucial for the destabilizing effect of IGF2BP1 on HULC. Consequently, depletion of CNOT1 should increase the half-life and steady-state expression level of HULC. To test this hypothesis, we depleted CNOT1 in HepG2 cells with two independent siRNAs and analyzed the CNOT1 expression both at the RNA and protein level. The knockdown was highly effective with both siRNAs (Fig. 4B). In both cases, the steady-state levels of HULC were strongly elevated (>2-4-fold) after CNOT1 depletion (Fig. 4C). SiRNA 1. which was slightly more effective in reducing CNOT1 levels (Fig. 4B), also had a greater effect on HULC expression (Fig. 4C). Furthermore, blocking transcription after depletion of CNOT1 revealed a strong impact of CNOT1 on HULC RNA stability (Fig. 4D). The half-life of HULC was significantly prolonged with both siRNAs targeting CNOT1 (up to 25.9 h ± 7.1 h) compared to control siRNA (5.6 h ± 0.9 h). Interestingly, the depletion of IGF2BP1 and CNOT1 simultaneously had no additive effect on HULC up-regulation indicating that both proteins are mechanistically linked to each other (Suppl. Fig. 4). Thus, we propose a model in which HULC expression is negatively regulated via binding to
IGF2BP1. By associating directly or indirectly with CNOT1, IGF2BP1 recruits the CCR4-NOT deadenylase complex onto its RNA substrate (Fig. 4E).

**DISCUSSION**

To understand the mechanisms underlying hepatocarcinogenesis, a large number of genetic and epigenetic profiling studies had been conducted (3). These studies mainly focused on the role of protein-coding genes and did rarely include long, non-protein-coding transcripts. Our study validated the significant up-regulation of *HULC*, a liver-enriched IncRNA in human HCC samples. Moreover, we showed for the first time that the expression of *HULC* is significantly higher in low stage and low grade tumors, which points towards a functional role of *HULC* in early steps of tumor development. Chronic inflammation, caused e.g. by viral infections or alcohol abuse, is a critical factor that triggers liver carcinogenesis. In our analysis, we could not detect a positive correlation with HBV or HCV infections. This is surprising in light of recent reports that established a link between *HULC* expression and HBV status, and showed that the HBx protein up-regulates *HULC* via CREB (26, 27). Based on the necessarily limited size of every patient cohort, we cannot formally exclude the possibility of a correlation with viral infections, but we do not see any trend towards *HULC* induction in primary patient samples infected with HBV. Previously, no direct association with HBV or HCV infection in patient samples was shown, but only cell culture models were used to establish this connection. Future studies with larger patient cohorts may further detail the correlations with different etiologies.

After confirming the high up-regulation of *HULC* in liver cancer, we wanted to explore the regulation of this transcript in human liver cancer cells. First, we could not verify the previously described regulation of HULC by miR-372 in three different liver
cancer cell lines. Thus, we performed RNA affinity purification experiments to identify RNA-binding proteins that bind and potentially regulate \textit{HULC} post-transcriptionally. Through this approach, we identified a novel and unexpected function of the well-known RNA-binding protein IGF2BP1. IGF2BP1 acts as a \textit{trans}-acting factor that represses \textit{HULC} stability and expression. Moreover, IGF2BP1 associates with CNOT1 and thereby, brings \textit{HULC} into close proximity to the CCR4-NOT deadenylase complex, which initiates RNA degradation from the 3’end (39). After the initial deadenylation of \textit{HULC}, its final degradation may occur via the 3’-5’- or the 5’-3’-exonucleolytic pathways.

Surprisingly little is known about the general role of RNA decay in the context of cancer. While factors such as miRNAs and AU-rich element binding proteins are known to specifically target mRNAs for degradation, we are still far away from a comprehensive understanding of the network that controls the stability of individual RNAs. Here, we discovered that IGF2BP1 might act as an adaptor protein that helps to destabilize \textit{HULC} in human liver cancer cells. However, the regulatory mechanisms governing the expression, activity, localization and RNA binding capacity of IGF2BP1 are mostly unknown. Derived from PAR-CLIP data to identify RNA substrates of the IGF2BP family, a potential RNA recognition consensus element has been proposed (40). This short CAUH (\(H = A, U, \text{or} C\)) motif is present in \textit{HULC} RNA ten times, distributed over the whole transcript and might represent a part of the binding site for the IGF2BPs that can associate as homo- or heterodimers (see Suppl. Fig. 1). However, this very short element lacks specificity - stochastically, it should be found every 85 nucleotides - so that additional, so far undiscovered bindings motifs are likely (41, 42). It will be of future interest to elucidate the underlying control mechanisms that define whether an RNA is bound, stabilized or destabilized by IGF2BP1 and which signaling pathways induce, control and limit the
interaction and subsequent RNA degradation of its targets, notably of \textit{HULC}. This is especially important since we did not find any negative correlation between \textit{IGF2BP1} and \textit{HULC} expression at the mRNA level (data not shown). Hence, the regulation of \textit{HULC} in primary liver cancer might be independent of IGF2BP1-mediated post-transcriptional regulation and mainly controlled at the transcriptional level - or so far undetermined inhibitory mechanisms (e.g. post-translational modifications) might affect the activity, localization or binding of IGF2BP1 proteins to \textit{HULC} transcripts in primary human HCC.

IGF2BP1 is a known oncofetal protein linked to several malignant human diseases: Its expression is induced in human malignant melanomas or colorectal carcinomas with activated WNT/ß-Catenin/TCF signaling (43, 44). High IGF2BP1 expression is a poor prognostic marker in high-stage and high-grade ovarian carcinomas and lung cancers (45-47). This study has unraveled that IGF2BP1 can also destabilize client transcripts. Hence, it opened up a new field of potential IGF2BP targets and IGF2BP-mediated silencing effects. Future studies may determine whether other IGF2BP1-bound transcripts - both coding and non-coding - are destabilized and degraded via the CNOT1 pathway in HCC or other tumor entities. Our study has revealed a novel mechanism that will help to fully establish the function of IGF2BP1 as a gene regulator in human cancer.
REFERENCES


FIGURE LEGENDS

Figure 1: Differential expression of *HULC* in HCC and normal liver patient samples. (A) Microarray analysis of 60 HCC and 7 normal liver samples showing significantly increased *HULC* expression in HCC. The horizontal line represents the mean of non-tumor samples. (B) Validation of differential *HULC* expression using qRT-PCR. *PPIA* was used as reference gene. (C) Boxplot analysis of differential *HULC* expression in HCC and normal liver showing a significant higher expression in tumor samples (*p*<0.001; Mann-Whitney U test). (D) Correlation analysis of *HULC* expression with tumor grade and stage. (E) Correlation analysis of *HULC* expression with tumor etiology.

Figure 2: Identification of IGF2 mRNA-binding proteins as interaction partners of *HULC*. (A) Protein interaction partners were detected using RNA affinity purification, incubating cell lysates with *in vitro* transcribed, biotinylated RNA and analyzing differential bands with mass spectrometry. (B) Representative SDS-PAGE gel after coomassie blue staining shows a prominent band at about 70 kDa, representing mainly IGF2 mRNA-binding proteins (IGF2BPs, see also Supplementary Table 4). (C) Validation of specific binding of IGF2BPs to biotinylated *HULC* in Huh7 by Western blotting. (D) Validation of specific binding of IGF2BPs to biotinylated *HULC* in HepG2 cells by Western blotting. (E) Representative Western blot analysis after FLAG-immunoprecipitation. HepG2 cells were transiently transfected for 72 h with FLAG-tagged GFP (negative control), IGF2BP1, IGF2BP2 or IGFBP3, respectively. (F) Analysis of co-purified RNA and respective enrichment as determined by qRT-PCR after anti-FLAG immunoprecipitation validating specific binding of *HULC* RNA to IGF2BPs. All immunoprecipitation experiments were done.
in biological replicates (n=3). Given is the mean and the respective standard error of the mean (±SEM).

**Figure 3: Impact of IGF2BP1 depletion on HULC expression and stability.** (A) IGF2BP RNA expression in HepG2 cells 72 h after siRNA transfection as determined by qRT-PCR. Shown is the remaining expression level relative to the respective IGF2BP level in cells transfected with control siRNA. Shown is the mean of at least three independent experiments (±SEM). (B) Validation of the specificity of individual siRNAs against IGF2BP1, IGF2BP2 and IGF2BP3 at the protein level as determined by Western blot. Shown is a representative western blot of three independent experiments. Vinculin expression was detected to verify equal loading. (C) HULC expression after IGF2BP knockdown. Depletion of IGF2BP1 had the strongest effect and significantly increased steady-state HULC expression as determined by qRT-PCR. Shown is the mean of at least three independent experiments (±SEM). Expression was normalized to PPIA and the siControl sample was set to 1.0. (D) HULC stability analysis in HepG2 cells after alpha-amanitin treatment. Cells were transfected with siRNAs against IGF2BPs and 48 h later, a time course for RNA stability was started by adding the RNA-Polymerase II inhibitor. Cells were harvested at the indicated time points. Expression levels were normalized to “0 h” and RNU6 was used as reference gene. HULC half-life nearly doubled upon IGF2BP1 knockdown compared to knockdown of IGF2BP2, IGF2BP3 or control siRNA. Shown is the mean of at least three independent experiments (±SEM).
Figure 4: IGF2BP1 interacts with components of the RNA decay machinery. (A) Overexpression of FLAG-IGF2BP1 or FLAG-GFP for 72 h in HepG2 cells. Subsequent immunoprecipitation revealed a specific interaction of IGF2BP1 with CNOT1 but not XRN1. Shown is a representative Western blot of three independent experiments. (B) Efficient knockdown of CNOT1 at RNA (upper panel) and protein level (lower panel) was achieved in HepG2 cells using two independent siRNAs. Expression of GAPDH is used to verify equal loading of the Western blots. Shown is a representative Western blot of three independent experiments. (C) Depletion of CNOT1 increased HULC expression as measured via qRT-PCR. PPIA was used as reference gene in qRT-PCR. Shown is the mean of at least three independent experiments (±SEM). (D) Analysis of HULC RNA stability using alpha-amanitin treatment after CNOT1 knockdown in HepG2 cells. HULC RNA half-life was drastically increased by CNOT1 knockdown compared to control siRNA transfection. Shown is the mean of at least three independent experiments (±SEM). RNU6 was used as stable reference gene. Expression is shown relative to control siRNA transfection and “0 h”. (E) Proposed model of HULC expression and stability regulation. IGF2BP1 binds HULC and recruits it to the CCR4-NOT1 complex, initiating deadenylation and degradation via the 3'-5' degradation pathway.
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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

M.H., T.G. and S.D. designed and executed the experiments, analyzed the data and wrote the manuscript. H.U., M.G. and E.F. performed experiments and analyzed the data. S.O. and G.S. contributed to design of experimentation and helped with interpretation of results. B.S. and R.G. performed microarray expression analysis. T.L., K.B. and P.S. provided tissue samples and helped with data interpretation.
Table 1: Correlation analysis of *HULC* expression and patient’s characteristics

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<td>HCV vs. non-HCV</td>
<td></td>
<td>0.220#</td>
</tr>
<tr>
<td>Alcohol vs. non-alcohol</td>
<td></td>
<td>0.190#</td>
</tr>
<tr>
<td><strong>Tumor size</strong></td>
<td>5 (2-29)</td>
<td>0.310*</td>
</tr>
<tr>
<td><strong>Grading</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well differentiated HCC</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Moderately differentiated HCC</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated HCC</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Well vs. poorly differentiated</td>
<td></td>
<td>0.016#</td>
</tr>
<tr>
<td>Moderately vs. poorly differentiated</td>
<td></td>
<td>0.016#</td>
</tr>
<tr>
<td><strong>Staging</strong></td>
<td></td>
<td>0.015§</td>
</tr>
<tr>
<td>T1</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>T1 vs. T2</td>
<td></td>
<td>0.015#</td>
</tr>
<tr>
<td>T1 vs. T3</td>
<td></td>
<td>0.027#</td>
</tr>
<tr>
<td>T1 vs. T4</td>
<td></td>
<td>0.034#</td>
</tr>
<tr>
<td><strong>Vascular invasion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>9</td>
<td>0.007#</td>
</tr>
<tr>
<td>None</td>
<td>21</td>
<td></td>
</tr>
</tbody>
</table>

Pearson correlation, # Mann-Whitney U test, § Kruskal-Wallis test
Figure 1

A

B

C

D

E

HCC normal

0

0.2

0.4

0.6

0.8

1.0

1.2

1.4

1.6

1.8

Rel. HULC Expression

10

9

8

7

6

5

4

3

2

1

0

Rel. HULC expression

2.0

1.5

1.0

0.5

0

p<0.001

T4 (n=2)

T3 (n=8)

T2 (n=12)

T1 (n=9)

poorly (n=5)

moderately (n=23)

well (n=5)

p=0.015

p=0.03

p=0.038

cryp
genetic
(n=11)

he
omatosis
(n=2)

alcohol
(n=5)

HBV
(n=7)

HCV
(n=8)

* p=0.038

Figure 1
A

**T7** Gene Of Interest

- *in vitro* transcription with Biotin-16-UTP
- coupling to streptavidine beads
- incubation with cell extract
- extensive washing, elution and precipitation of bound proteins
- SDS-PAGE, staining and ESI-MS

B

- Biotin-16-UTP
- Marker
- HULC
- HULC
- control
- Marker

- ~72 kDa

C

- Biotin-16-UTP
- Input
- HULC
- HULC
- control

- IGF2BP1
- IGF2BP2
- IGF2BP3
- hnRNPA1
- Vinculin

D

- Biotin-16-UTP
- Input
- HULC
- HULC

- IGF2BP1
- IGF2BP2
- IGF2BP3
- hnRNPA1
- Vinculin

E

- IB: FLAG

- Input
- GFP
- IGF2BP1
- IGF2BP2
- IGF2BP3

F

- Enrichment IP over input

- 5.8S rRNA
- IGF2 mRNA
- HULC

**Figure 2**
Figure 3

A

![Bar chart showing relative IGF2BP expression levels](chart_a.png)

B

![Western blot images for control siRNA, IGF2BP1 siRNA, IGF2BP2 siRNA, IGF2BP3 siRNA](chart_b.png)

C

![Bar chart showing relative HULC expression levels](chart_c.png)

D

![Graph showing % of RNA (log2) over alpha-amanitin (hours) for control siRNA, IGF2BP1 siRNA, IGF2BP2 siRNA, IGF2BP3 siRNA](chart_d.png)

**Control siRNA**: 7.7 h +/- 1.9 h
**IGF2BP1 siRNA**: 13.3 h +/- 1.5 h
**IGF2BP2 siRNA**: 7.0 h +/- 0.5 h
**IGF2BP3 siRNA**: 7.2 h +/- 2.4 h
Figure 4