Aus der Kinderchirurgischen Klinik und Poliklinik

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The functional role of TRIM71 in childhood liver cancer

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Introduction

1. Childhood liver tumors

1.1 Epidemiology

Pediatric cancers represent the highest morbidity and mortality related with disease in childhood. They encompass widely known cancer types such as leukemias, lymphomas, brain tumors, and liver cancers. Pediatric liver tumors contain two most frequently occurring groups: hepatoblastoma (HB) and hepatocellular carcinoma (HCC). HB is known to be the most common pediatric liver tumor and represents approximately 80% of the liver cancers affecting children predominantly between age of 6 months and 3 years, while HCC is found more uncommon, which about only 0.5%–1% of childhood cancers are classified as HCC. Most of HB are diagnosed under 5 years of age, whereas the patients diagnosed at over 5 years mostly have unfavorable prognosis [1]. It has an annual incidence rate of approximately 1.8 diagnosed cases per million [2] and accounts for less than 5% of pediatric malignancies [3]. It has been reported that low birth weight (<1,500g) and low birth weight (1,500g-2,500g) increases the risk of HB about 20-fold and doubles the risk of HB, respectively [4]. In HCC, contrary to adults, there are less etiologic factors that can be detected in the majority of children and adolescents. However, the probability of having HCC in chronic hepatitis B virus (HBV) carriers represent with higher prevalence of HBV infection rate, which estimated to be 10%-25% [5]. HCC patients' 3-year survival is under 20% with unresectable HCC treated with independent chemotherapy. Considering the fact that HB is the most frequent liver tumor in childhood, the following parts mainly focus on HB.

1.2 Clinical implication

Younger age at diagnosis is always predictive for better overall survival [6]. HBs are rarely composed of only one cell type, which makes their diagnosis more challenging. The tumor

is usually becoming large at diagnosis, and the alpha-fetoprotein (AFP) is nearly evaluated in all patients. This result offers the opportunity of HB be diagnosed in early stages. Recent research reported that partial hepatectomy after chemotherapy offers a good chance of survival for HB children [7]. To plan the surgery in an objective and comparable manner is always necessary, although it's still difficult to decide which patients deserve immediate surgery, while which were not [8]. Among all the staging methods, the development of the system for assessing the pretreatment extent of tumor (PRETEXT) in the liver is mentionable here (Figure. 1), which was established for better patient diagnosis to select suitable treatment options [9]. It's created by the International Childhood Liver Tumor Strategy Group (SIOPEL group) based on characterizing the location of tumors with Couinaud's system of segmentation of the liver. Although the surgical timing is mostly dependent on therapeutic schedule and patients' response, the preoperative reimaging is always necessary. As a result, staging and treatment are a crucial part of patients' management [10]. With a dreadful outcome of HB, its treatment still makes progress due to improved surgical techniques and better chemotherapy based on increasingly risk stratification. In addition, the beneficial accumulated experiences shared through international collaboration has led to an increased understanding.

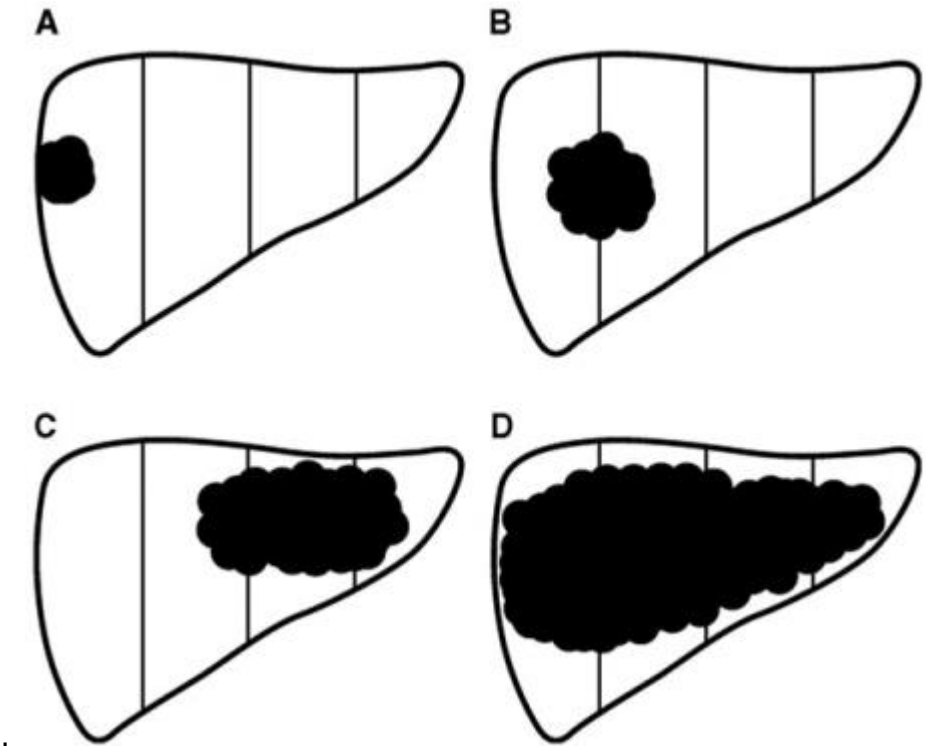

Figure.1 PRETEXT system staging the liver tumors. A: PRETEXT I, one section is involved and three adjoining sections are free. B: PRETEXT II, one or two sections are involved, but two adjoining sections are free. C: PRETEXT III, two or three sections are involved, and no two adjoining sections are free. D: PRETEXT IV, all four sections are involved. Figures are taken from Roebuck et al. [10].

2. Histopathology of HB

The various combination of epithelial and mesenchymal cell lineages characterize a rare malignant blastomatous tumor, HB, which recapitulate early phases of liver ontogenesis. HB cell types have been differentiated from immature hepatocyte precursors, including epithelial, biliary, hepatocytes, mesenchymal, and mixed epithelial-mesenchymal types. Epithelial tumors can be breaking down into fetal, embryonal, and small cell undifferentiated subtypes, while the mixed epithelial-mesenchymal group can be further classified according to their teratoid features. But, it is still not clear to attach the tumor with the proportions of fetal, embryonal and small-cell undifferentiated pattern, or a tumor that has small cell undifferentiated and fetal histology [11].

3. Molecular alteration in HB

Genetic alterations in several chromosomes were identified, for instance, the gains of chromosomes 1q, 2q, 2p, 6q, 8q, 17q and 20q, and losses of chromosome 4q [12]. Recently, Cairo and his group [13] found that gains on chromosomal region 8q and 2q were associated with a C2 subclass of HB. It is named C1 and C2 by using gene expression profiling this group that identified two subclasses of HB tumors. C1 and C2 exhibit similarity with distinct phases of liver development. Compared to the fetal-like C1 subclass, C2 conforms to an proliferate faster embryonal histotype. In addition, the group investigated that the C2 tumors display intensive nuclear accumulation of β-catenin and the cell cycle related genes are highly expressed. They introduced a 16-gene signature which classify the tumors into C1 and C2 subclasses based on the most differentially expressed genes [13]. Remarkably, the 16-gene signature can not only predicted prognosis with high accuracy, but also discriminates invasive and metastatic HB [14].

Recently, Gröbner et al. analyzed 24 types of adult cancer entities and all major childhood cancer entities and proved that HB has the lowest mutation rate among all kinds with about 4 mutations per tumor [15]. Notably, among all abnormal signaling pathways in HB, β-catenin mutations [16, 17] present with the highest rate (50-90%). Moreover, the Wnt/β-catenin signaling pathway plays a wildly known major role in hepatic-related tumor development. Other important signaling pathways in HB genesis are AKT-signaling and the *LIN28B/let-7* pathway, which are also altered in many kinds of human cancers.

4. Wnt signaling pathway

4.1 Role of Wnt signaling pathway

Wnt signaling is an essential factor in liver homeostasis as well as liver tumorigenesis. Examples for its physiological role are: i) the canonical Wnt signaling has been found to be not activated in adult liver except for hepatic lobule in centri-zonal region [18], ii) the reactivation of the Wnt pathway occurs during liver regeneration in adults [19], and iii) the β-catenin protein level increases about 2.5 fold in response to the injury induced by partial

hepatectomy during the early minutes. Moreover, Wnt signaling is involved in many human cancers, such as non-small cell lung cancer [20], colorectal cancer [21], breast cancer [22], and particularly interest liver cancer [23].

In Wnt off state, a complex including Adenomatous Polyposis Coli (APC), AXIN, casein kinase Ia (CKIa), and the glycogen synthase kinase 3β (GSK3β) leads to β-catenin phosphorylation. The β-catenin is then ubiquitinated by Beta-Transducin repeat Contaning E3 Ubiquitin Protein Ligase (βTRCP) and targeted for proteasome-mediated degradation [24]. In the absence of β-catenin, a complex containing T-cell factor/lymphoid enhancer factor (TCF/LEF) recruits Histone Deacetylases to repress target genes. When Wnt pathway is activated, the canonical Wnt signal is transduced through Frizzled (FZD) family receptors to coreceptor LRP5/6, since it induces FZD-DVL complex and LRP5/6-AXIN-FRAT complex assembly [25, 26], β-catenin then gets stabilized, shuttles to the nucleus and accumulates there. Nuclear β-catenin then complexes with (TCF/LEF) family transcription factors, at the same time with Legless family docking proteins (BCL9 and BCL9L) associated with PYGO family [27]. Finally, the TCF/LEF-β-catenin complex in the nucleus acts as effector to activate transcriptional target genes such as *MYC, AXIN2, DKK1,* and *cyclin D1* [28].

4.2 Wnt/β-catenin in hepatoblastoma

Activation of the Wnt signaling pathway (Figure.2) is prevalent in HB, occurs in both embryonal and mixed types. The Wnt signaling pathway has also been observed as a major activated pathway in both proliferative stem cells and neoplasm, which makes it intelligible that Wnt signaling has a specific role in embryonic tumors [29]. Wnt signals are shifted to canonical Wnt signaling for cell fate determination [30-33], while to the non-canonical Wnt signaling for controlling cell movement and tissue polarity. Terrada et al. [14] found that different β-catenin status correlated with HB histologic subtypes and also that *CTNNB1* mutation were occasionally absent in Wnt signaling pathway activation [34]. The *CTNNB1* mutational mechanisms that have been frequently discovered can differ,

with more than half of them accounting for deletions in HB [12, 13, 35]. Moreover, the β-catenin key working mechanism is its stability and accumulation in the cytoplasm.

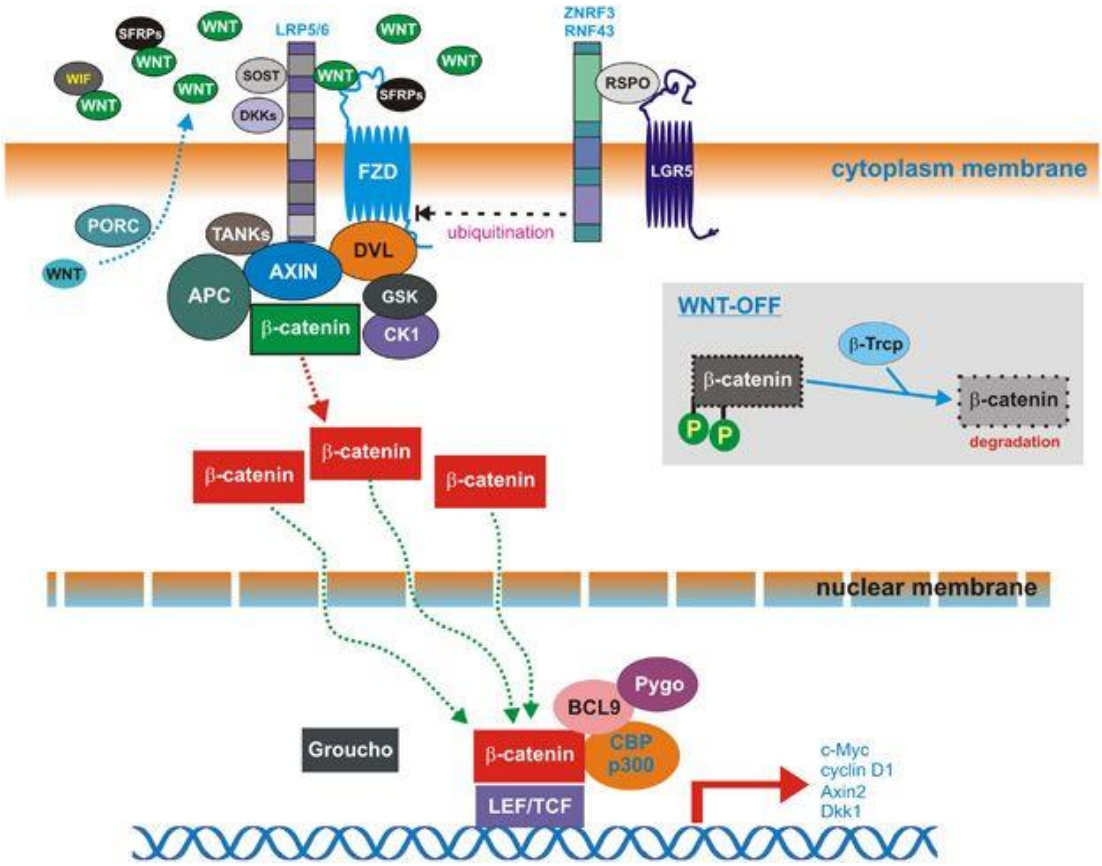

Figure. 2 The canonical Wnt signaling pathway. Wnt off state: The ubiquitin-dependent degradation of β-catenin phosphorylated by the destruction complex (*AXIN-APC* complex). Subsequently, *TCF* transcription factors are repressed and then Wnt target gene expression is inhibited. Wnt on state: β-catenin is predominantly accumulated in the cytoplasm and translocated into the nucleus, where it binds to *LEF/TCF* and further activates expression of Wnt target genes, such as *c-Myc, cyclin D1, Axin2,* and *Dkk1*. Figure is taken from Yang et al. [36].

5. The *LIN28B/let-7* network

5.1 *LIN28B/let-7*

LIN28 is a RNA binding protein has two forms: *LIN28A* and *LIN28B*, located on chromosome 1 and 6, respectively [37]. *LIN28A* is a translational enhancer, while *LIN28B* is a nuclear primary inhibitor of miRNA biogenesis. *LIN28B* is known as a RNA binding protein that influences metabolism, stem cell maintenance, and oncogenesis. It is alone sufficient to drive cancer [38]. *LIN28B* has been proven to be associated with a more aggressive, embryonic tumor type characteristic of an oncofetal gene expression program [39]. This study also reported that the overexpression of *LIN28B* is sufficient for initiating HB and HCC in murine models.

The miRNA pathway evolutionarily plays vital roles in various aspects of biology. There is a highly conserved miRNA *let-7* belongs to the initial class of miRNAs, which targets the translation of RNA binding protein *LIN28B*. The ongoing research has uncovered extensive co-regulation between these genes [40]. Due to the differential subcellular localization of *LIN28A* and *LIN28B*, let-7 blocking process in different steps of the miRNA-processing pathway were proved by human cancer cell lines [41]. However, the steps where *let-7* is blocked are controversial. Several studies that already demonstrated the strong correlation of *LIN28B* overexpression and *let-7* loss with poor prognosis [42]. *Let-7* miRNA, as a tumor suppressor, targets several oncogenes such as *MYC, HMGA2* and *cyclin D1* [43]. As one of the *let-7* targets, *LIN28B* precise mechanism responsible for *let-7* inhibition is controversial. *LIN28B* command its biological functions upon repressing the *let-7* miRNA family as well as modifying these mRNAs translation efficiency. Although the inhibition of *LIN28B* can regress established xenograft human tumors in mice, further studies are still needed to precisely define the responsible mechanisms of *LIN28B*-mediated *let-7* repression.

5.2 *TRIM71*

One important protein that regulates *LIN28B/let7* signaling is *TRIM71 (Tripartite Motif Containing 71)*, also named *LIN41* (*Abnormal Cell LINeage 41*). It is a protein-coding gene that encodes an E3 ubiquitin-protein ligase that binds to miRNAs [44]. *TRIM71* belongs to the *TRIM-NHL* family, containing a RING domain, a CC domain, a BB domain, and a NHL domain. Its functional activity as E3 ubiquitin ligase is associated with the RING domain. Moreover, the NHL domain is related to its miRNA binding activity [45]. *TRIM71* binds to a miRNA, complexes with *AGO2*, which participates in post-transcriptional repression of transcripts, such as *CDKN1A*. *TRIM71* can additionally participate in post-transcriptional mRNA repression by a miRNA independent mechanism [46]. By repressing *CDKN1A* expression, the G1-S transition was stimulated to promote rapid embryonic stem cell self-renewal. It has been reported in mammalian cells that *TRIM71* could be taken instead of *MYC* in the canonical reprogramming complex containing *OCT4, SOX2*, and *KLF4*, to invert fibroblasts into induced pluripotent stem cells (iPS) [47]. It was demonstrated in previous studies that direct binding of *MYC*, the key downstream target of Wnt signaling, to the *TRIM71* promoter enhances *TRIM71* expression, which suggests *TRIM71* as a *MYC* effector [48].

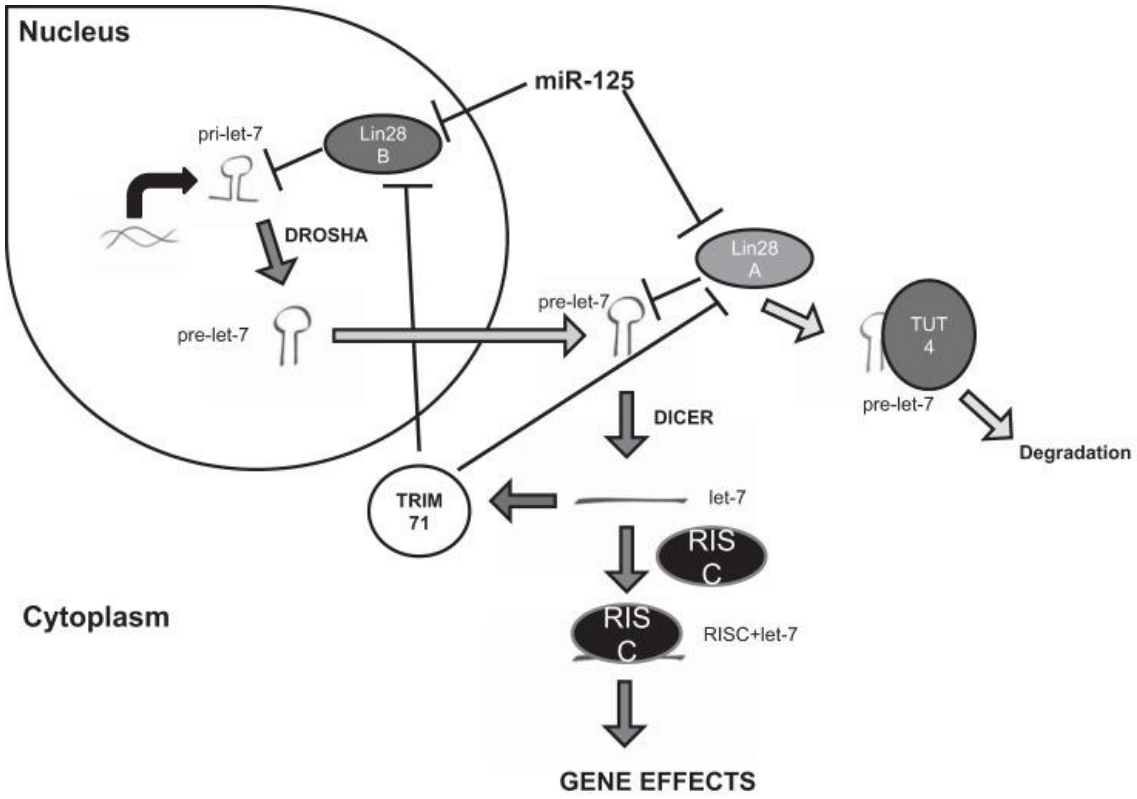

Figure. 3 *LIN28* affects *let 7* maturation via several methods. *TRIM71* inhibits *LIN28A*, which binds to *pre-let 7* for preventing cleavage by Dicer. *TRIM71* can also inhibit *LIN28B*, that can bind to *pri-let 7* in the nucleus thereby preventing cleavage by Drosha. Figure from McDaniel et al. [49].

Yin et al. claimed that *TRIM71* is the top modulator of the *LIN28B-let 7-HMGA2* pathway, and therefore serves as an essential factor in tumor formation, tumorigenesis, and malignancy [50]. By performing knock-down experiments in Caco-2, Tera-1, NCI-H1299, and NCI-H460 cancer cell lines using siRNA specific for *TRIM71*, they found that *TRIM71* inhibits *LIN28B*. Also, *TRIM71* can be activated by up-regulation of *let-7*, which inhibits both *LIN28A* and *LIN28B* in the feedback mechanism (Figure.3)[51]. *Let-7* high expression is associated with decreased *LIN28 A/B* expression. Interestingly, this inhibition of *LIN28 A/B* can be accomplished by *let-7* through activation of *TRIM71*, which targets both forms of *LIN28* for ubiquitin-mediated proteasomal degradation [52].

Interestingly, *TRIM71* was identified as a novel oncogene in human HCC and associated with poor prognosis [48], which was deduced from several findings: *TRIM71* is frequently overexpressed in HCC, *TRIM71* enhances tumor growth, proliferation, and tumorigenicity of HCC, and *TRIM71* can activate *let-7* dependent oncogenesis. According to this paper,

TRIM71 could serve as an ideal target for liver cancer treatment. However, so far there are no studies that describe a potential role of *TRIM71* in other types of liver cancer. An involvement of *TRIM71* in HB genesis and a possible connection of the Wnt signaling pathway and the *LIN28B* pathway need to be elucidated.

6. Aim of the study

In this study, we mainly aim to characterize the role of *TRIM71* in HB and HCC, the two major childhood liver tumor types. We wanted to:

- characterize the biological function of *TRIM71* in liver tumors on the cellular level
- explore the clinical relevance of *TRIM71* for varying outcomes of HB patients
- describe the molecular mechanisms how *TRIM71* integrates into the Wnt signaling pathway

Materials

1. Cell lines

HepT1, Homo sapiens, HB	Pietsch et al. [53]
HUH6, Homo sapiens, HB	JCRB, Osaka, Japan
Hep3B, Homo sapiens, HB	ATCC, Manassas, USA
HUH7, Homo sapiens, HCC	ATCC, Manassas, USA

2. Cell culture materials

RPMI	Invitrogen, Karlsruhe, Germany
PS (10,000 U/ml)	Invitrogen, Karlsruhe, Germany
DPBS	Invitrogen, Karlsruhe, Germany
FCS	Invitrogen, Karlsruhe, Germany
Trypsin-EDTA 0.05%	Invitrogen, Karlsruhe, Germany
Costar Stripette Serologic Pipettes 5ml	Corning GmbH, Wiesbaden, Germany
Costar Stripette Serologic Pipettes 10ml	Corning GmbH, Wiesbaden, Germany
Costar Stripette Serologic Pipettes 25ml	Corning GmbH, Wiesbaden Germany
Biosphere Filtertips 1-10 µl	Sarstedt AG & Co., Nümbrecht, Germany
Biosphere Filtertips 10-100 µl	Sarstedt AG & Co., Nümbrecht, Germany
Biosphere Filtertips 100-1000 µl	Sarstedt AG & Co., Nümbrecht, Germany
Cell culture T-Flasks 25m^2	NUNC, Langenselbold, Germany
Cell culture T-Flasks 75m^2	NUNC, Langenselbold, Germany
Falcon tubes 15ml	Greiner bio-one, Frickenhausen,Germany
Falcon tubes 50ml	Greiner bio-one, Frickenhausen,Germany
DNase and RNase free	NUNC, Langenselbold, Germany
Petri dishes 100mm	NUNC, Langenselbold, Germany
6-well Plates, non-pyrogenic	NUNC, Langenselbold, Germany
12-well Plates, non-pyrogenic	NUNC, Langenselbold, Germany

24-well Plates, non-pyrogenic	NUNC, Langenselbold, Germany
96-well Plates, non-pyrogenic	NUNC, Langenselbold, Germany
Cell scraper	Sarstedt AG & Co., Nümbrecht, Germany
Glastips	Sarstedt AG & Co., Nümbrecht, Germany

3. Bacteria

Escherichia *coli* DH5α	Invitrogen, Karlsruhe, Germany

4. Plasmids

pcDNA3-FLAG-TRIM71	Prof. Dr. Jong Heon Kim, Goyang, Korea
pEGFP-N1	ClonTech, Mountain View, CA, USA
pUC19	Prof. Dr. Heiko Hermeking, LMU Munich
pRTR	Prof. Dr. Heiko Hermeking, LMU Munich
pRTR-FLAG-TRIM71	M. Med. Ting Jiang, this study

5. Enzymes

RnaseA	Sigma-Aldrich, St.Louis, MD, USA
DNaseI	Sigma-Aldrich, St.Louis, MD, USA
ProteinaseK	Sigma-Aldrich, St.Louis, MD, USA
Restriction endonucleases	New England Biolabs GmbH, Frankfurt, Germany
T4 DNA ligase	New England Biolabs GmbH, Frankfurt, Germany
Klenow Fragment	New England Biolabs GmbH, Frankfurt, Germany
Sfill	New England Biolabs GmbH, Frankfurt, Germany
ScaI	New England Biolabs GmbH, Frankfurt, Germany
XbaI	New England Biolabs GmbH, Frankfurt, Germany
KpnI	New England Biolabs GmbH, Frankfurt, Germany

NotI	New England Biolabs GmbH, Frankfurt, Germany
SacI	New England Biolabs GmbH, Frankfurt, Germany
SuperScript II Reverse Transcriptase	Invitrogen, Karlsruhe, Germany
Taq DNA polymerase	Sigma-Aldrich, St Louis, MD, USA

6. Antibodies

Anti-β-catenin, Rabbit, WB	Epitomics, Burlingame, CA, USA
Anti-β-actin, Rabbit, WB	Cell signaling technology, Danvers, USA
Anti-LIN41, Sheep, WB	R&D SYSTEMS, Frankfurt, Germany
Anti-Flag, Mouse, WB	Sigma-Aldrich, St.Louis, MD, USA
Anti-mouse HRP, WB	Sigma-Aldrich, St.Louis, MD, USA
Anti-rabbit HRP, WB	Sigma-Aldrich, St.Louis, MD, USA
Anti-sheep HRP, WB	Novex, Thermo Fisher, OR, USA

7. Kits

Quick Blunting Kit	New England Biolabs GmbH, Frankfurt, Germany
QIAprep Spin Miniprep Kit	QIAprep Spin Miniprep Kit, Frankfurt, Germany
QIAquick Gel Extraction Kit	QIAprep Spin Miniprep Kit, Frankfurt, Germany
QIAprep Spin Midiprep Kit	QIAprep Spin Miniprep Kit, Frankfurt, Germany
QIAquick PCR Purification Kit	QIAprep Spin Miniprep Kit, Frankfurt, Germany

8. Buffer and solutions

RIPA Protein Lysate Buffer

- 1% NP40
- 0.5% sodium deoxycholate
- 0.1% SDS

- NaCl 150 mM
- 50 mM TrisHCl (pH 8.0)

Tris-glycine –SDS Running Buffer 10X

- 14.4% glycine
- 3% Tris base
- 1% SDS
- pH 8.3-8.7

PBS-T Buffer 1X

- 50% 2xPBS
- 0.1% Tween 20

Blocking Solution

- 3-5% dry milk in ddH_2O

Laemmli Buffer 2X

- 125 mM TrisHCl (pH 6.8)
- 4% SDS
- 20% glycerol
- 0.05% bromophenol blue (in H_2O)
- 10% beta-mercaptoethanol

MTT-1 Solution

- 4 mM HCl

- 0.1% NP40
- Adjust with PBS

MTT-2 Solution

- 10% SDS
- 37% HCl

Freezing Medium

- 48.8% FCS
- 39% DMEM
- 12.2% DMSO

9. Primers

Name	*Ref Seq No.*	*Forward*	*Reverse*
TRIM71	NM_001039111.2	GCTGTGGAAGGTAGAAAAGATCC	GCTTGTTGAGGTTTTGCCG
TRIM71-seq	NM_001039111.2	GGTGGCTGACAAGGACATC	GCTTGTTGAGGTTTTGCCG
LIN28B	NC_000006.12	AGCCCCTTGGATATTCCAGTC	AATGTGAATTCCACTGGTTCTCCT
IGF2BP1	NC_000017.11	CCAAGAGACCAGACCCCTGA	AGGATGTCTCGGATCTTCCGT
HMGA2	NC_000012.12	ACCTAGGAAATGGCCACAACA	GCGCCCCCTAGTCCTCTTC
DKK1	NM_012242.2	GTCACGCTATGTGCTGCCC	GTTTCCTCAATTTCTCCTCGGA
CTNNB1	NM_001904.2	ACGTCCATGGGTGGGACA	CTAGGATGTGAAGGGCTCCG
AXIN2	NM_004655.2	TATCCAGTGATGCGCTGACG	TGTTTCTTACTGCCCACACGAT

LGR5	NC_000012.12	ACAGCAGTATGGACGACCTTCA	CAGGTCTTCCTCAAAGTCAAGCA
TBP	NM_003194.3	GCCCGAAACGCCGAATAT	CCGTGGTTCGTGGCTCTCT

10. Chemicals and Reagents

Milchpulver	Carl Roth, Karlsruhe, Germany
Dithiothreitol DTT (0.1 M)	Invitrogen, Karlsruhe, Germany
dNTPs	Thermo Fisher Scientific, Waltham, MA, USA
Magnesium chloride	Carl Roth, Karlsruhe, Germany
SYBR Green Master Mix	BIO-RAD, Stuttgart, Germany
Tween20	Sigma-Aldrich, Steinheim, Germany
MTT formazan powder	Sigma-Aldrich, Steinheim, Germany
Isopropyl alcohol	Sigma-Aldrich, Steinheim, Germany
EDTA	Carl Roth, Karlsruhe, Germany
FuGENE Transfection Reagent	Promega, Madison, WI, USA
Sodium dodecyl sulfate	Carl Roth, Karlsruhe, Germany
Water (DNase/RNase-Free)	Life Technologies GmbH, Ismaning, Germany
Crystal Violet	Carl Roth, Karlsruhe, Germany
Ethanol, absolut	Merck, Darmstadt, Germany
Chloroform	Carl Roth, Karlsruhe, Germany
Bromophenolblue	SERVA, Heidelberg, Germany
Agarose	PeQLab, Erlangen, Germany
TRI Reagent RNA Isolation Reagent	Sigma-Aldrich, Steinheim, Germany
Nonidet P40 Substitute	Sigma-Aldrich, St.Louis, MD, USA
Opti-MEM	Life Technologies GmbH, Darmstadt, Germany
Gene Ruler 1kb DNA ladder	Fermentas St.Leo-Rot, Germany
Page Ruler Prestained Protein Ladder	Fermentas St.Leo-Rot, Germany
Western Blot Protein Standard	BIO-RAD, Stuttgart, Germany
siGENOME Human TRIM71 siRNA smart pool	Dharmacon, Lafayette, CO, USA
Lipofectamine 2000	Thermo Fisher Scientific, Waltham, MA, USA

Random hexameric primers	Thermo Fisher Scientific, Waltham, MA, USA

11. Consumables

Biosphere Filtertips	Sarstedt AG & Co., Nümbrecht, Germany
Pipette tips (10ul, 100ul, 1000ul)	Sarstedt, Nümbrecht, Germany
PCR 96 Well Plates	PeQLab, Erlangen, Germany
4-20% Tris-Glyci Gels	Invitrogen, Karlsruhe, Germany
Safe-lock Eppendorf tube	Eppendorf, Hamburg, Germany
Quarz cuvette QS 10.00 mm	Hellma, Muellheim, Germany
Trans-Blot Turbo Mini PVDF Transfer Packs	BIO-RAD, Stuttgart, Germany
Multidishes Nunclon	NUNC, Langenselbold, Germany
Hyperfilm ECL	Sigma-Aldrich, St. Louis, MD, USA

12. Equipment

Table-top Centrifuge	Eppendorf AG, Hamburg, Germany
Axiovert 40 Microscope	Carl Zeiss GmbH, Oberkochen, Germany
Biofuge Centrifuge	Thermo Fisher Scientific, Inc, Waltham, USA
CF 40 Imager	Kodak, Rochester, New York, USA
Trans-Blot Turbo Transfer System	BIO-RAD, Stuttgart, Germany
Biofuge pico, Heraeus	Kendro, Langenselbold, Germany
Camera Power Shot G6	Canon, Krefeld, Germany
Centrifuge 5702	Eppendorf, Hamburg, Germany
Centrifuge J2-21	Beckman Coulter, Krefeld, Germany
Centrifuge LMC-3000	G. Kisker, Steinfurt, Germany
Heat block MR 3001	Heidolph, Kehlheim, Germany
Heatblock Thermomixer comfort	Eppendorf, Hamburg, Germany
Microwave	Panasonic, Hamburg, Germany
NanoDrop 1000 instrument	Thermo Scientific, Wilmington, USA

Shaker, Rock-N-Roller	G. Kisker, Steinfurt, Germany
Shaker, Unimax 1010	Heidolph, Schwabach, Germany
Western-Blot Detection DIANA	AGFA, Köln, Germany
Water bath GFL 1083	GFL, Wien, Austria
Vortexer Genie2	Scientific Industries, NY, USA
CO2-Incubator MCO-20AIC	Sanyo, Tokyo, Japan
Agarose gel electrophoreses apparatus	Bio-Rad, Munich, Germany
Bio Photometer	Eppendorf, Hamburg, Germany
GENios Microplatereader	Tecan, Crailsheim, Germany
Mastercycler ep gradient S	Eppendorf, Hamburg, Germany
Micro scales Te1245	Sartorius, Göttingen, Germany
XCell SureLockTM Electrophoresis Cell	Invitrogen, Karlsruhe, Germany

13. Software

ImageJ Software	NIH, Bethesda, MD, USA
GraphPad Prism 6	La Jolla, CA, USA
Microsoft Office 2010	Microsoft, Redmond, USA
Chromas	Technelysium Pty Ltd, South Brisbane, Australia

Methods

1. Cloning

The Flag-tagged full-length coding sequence of human *TRIM71* was released from the *pcDNA-TRIM71-Flag* plasmid [54] by digestion with SacI-XbaI and then integrated into the pUC-easy shuttling vector using the Quick Blunting Kit. *TRIM71-Flag* was then released from the shuttling vector by a digest with SfiI-ScaI and inserted into the pRTR (SfiI) vector. Sequence verification was performed by Sanger sequencing in the Sequence Service at the Faculty of Biology from LMU.

Sequencing primers:

TRIM71-seq forward, 5'-GGTGGCTGACAAGGACAATC-3'

TRIM71-rev reverse, 5'-CGACGACGTGGACGTCTGCG-3'

2. Transformation

E.coli aliquots were thawed on ice and 100ng of plasmid DNA was added to bacteria. Without vortexing, cells were incubated on ice for 30mins, then heat-shocked at 42°C for 40seconds and put back on the ice for 2mins. 250ul pre-warmed SOC medium was added and incubated at 37°C for 1hour with 500rpm rotation. Bacteria were seeded on agar plates containing either ampicillin or kanamycin antibiotics. Plates were incubated at 37°C overnight.

3. Purification of plasmid DNA from E.coli

Small amounts of plasmid DNA were isolated using the mini-prep plasmid DNA extraction kit. Single colonies from agar plates were inoculated in 5ml LB-medium, supplemented

with the respective antibiotics. Overnight cultures were pelleted by centrifugation at 8,000rpm for 10mins at room temperature. Pellet was resuspended by adding 250ul of Buffer P1 and transferred to a 1.5ml tube. Next, 250ul Buffer P2 was added and mixed thoroughly by inverting the tubes 4-8 times until the solution becomes clear. Then, 350ul Buffer N3 was added, mixed thoroughly and centrifuged for 10mins at 13,000rmp in the microcentrifuge. Supernatant from the last step was applied to the QIAprep spin column, spinned and washed by 500ul Buffer PB. Flow-through after centrifuging was discarded and the spin column again washed with 750ul PE. Residual wash buffer was removed by centrifuging, and the DNA eluted into a new 1.5ml tube using Buffer EB. To yield a larger amount of DNA, the Midiprep system and bacteria cultures of 150ml to 200ml were used.

4. RNA-extraction and reverse transcription

All investigated cells were harvested after 48hours seeding. Total RNAs were isolated from cells using TRIzol reagent according to the manufacturer's protocol, RNA measurement was performed by nanodrop. cDNAs were synthesized from 2ug RNA with random hexameric primers and SuperScript II.

5. Real-Time qPCR

For each qPCR profiling, 40ng of cDNA-synthesis reaction was used with 500nM of primer pairs and universal SYBR green supermix in 20ul reaction volume. Cycling consisted of 40 cycles with denaturation at 95°C for 15 seconds, annealing at 59°C for 15 seconds, and elongation at 72°C for 30 seconds proceeded in Mastercycler. Each experimental data was assayed in duplicate. Relative expression of candidate genes was calculated using the △△Ct method and *TBP* as a housekeeping gene [55].

6. Protein extraction and western blot

Cells were scraped from the cell culture plates with a scraper after 48h treatment, washed twice with ice-cold PBS, and lysed with RIPA lysis buffer. Protein concentration was determined at absorbance mode with 595nm wavelength using Bradford assay. Approximately 20-40ug proteins were loaded into 4-20% gels and separated by electrophoresis at 200V/110mA. Afterwards, gels were electroblotted onto PVDF membranes. The membranes were blocked in 5% milk/PBS-T solution for at least 2 hours, followed by overnight incubation with primary antibodies against selected proteins. After that, blots were washed with PBS-T for 3 X 10mins and incubated at room temperature for 1hour with peroxidase-conjugated anti-rabbit or anti-sheep IgG antibody. Ultimately, using enhanced chemiluminescence reaction blots were detected and ß-actin served as the loading control. Western blot band intensity was quantified by ImageJ software. The ratio of values of candidate proteins was normalized to housekeeping protein β-actin.

7. Cell culture

Two human hepatocellular carcinoma cell lines (HUH7 [56, 57] and Hep3B [58]), and two human hepatoblastoma cell lines (HUH6 [59] and HepT1 [53]) were used in this study. Cells were grown in RPMI supplemented with 10% FCS and 1% PS and incubated at 37°C in a humidified incubator with 5% CO_2. Cells were passaged every 2-4 days in order to avoid confluence. For long term preserving the cell lines, they were regularly maintained in a liquid nitrogen tank.

8. Transfection

Overexpression experiments were performed with FuGENE HD Transfection Reagent. On the day of transfection, cells were trypsinized, counted and expected cells were transferred to a plate at the confluence at 70%-80%, incubated overnight. The plasmid DNAs were mixed with FuGENE at ratio of 1:3 diluted with RPMI, the amount of plasmid

DNAs referred to the FuGENE® HD Protocol Database website. Transfection complex was then dropped into the cells in medium (containing FCS and PS). The total transfection solutions in 50ml falcon were fully mixed on the shaker for 5mins at 150rpm in room temperature [60]. Cells were seeded into the plates afterward and medium changed on the next day.

Knockdown experiments were performed either by electroporation or lipofectamine2000 using the siGENOME SMARTpool si-TRIM71 (Target Sequence: GGAGGAGGGUAGAGCGCUA, AGAAGUAGUGCUAGCCGA, CUUGGGAUGUGGCGGUGAA, CACCAAGGCCACAGGCGAU). For electroporation, objective HUH6 and HUH7 cell number in RPMI medium without PS at the density of 6666.66 cells/µl together with 1% siRNA were transferred to electroporation cuvettes and electroporated at 350V for 10ms. For the lipofectamine2000 method (6-well plate were used here) oligomer-Lipofectamine2000 complexes were prepared with 100pmol siRNA oligomer in 250ul RPMI (without serum), and diluted 5µl lipofectamine2000 in 250µl RPMI (without serum). The two complexes were then incubated for 5mins at room temperature separately. Combined the diluted oligomer with lipofectamine 2000 complexes gently and incubated for 20mins at room temperature. The total complexes were added to each well containing cells and medium. And cells were incubated at 37°C for 24-96 hours until assays were ready. The medium was changed after 4-6 hours.

9. Proliferation assay

5,000 cells/well were seeded into 96-well plates and then transfected either with plasmid or siRNA as indicated above. MTT1 solution was added into each well (10µl/well) and incubated at 37°C for 4hours. Then MTT2 solution was added to each well (100µl/well). Remember the time point of adding two solutions and proceeded at the same time in following days. After the last time of processing MTT2, the plates were kept at room temperature overnight in the dark and the absorbance at 595nm was measured next day. Each experiment was realized 2 times and each condition was done in duplicates.

10. Scratch (Migration) assay

Cells were seeded at a density of 70%-80% per 6-well plate. A scratch was made into the confluent cell layer with a 10µl sterile pipette tip and the medium was changed for eliminating scratched cells. Pictures were collected from the scratch every 24h with the camera attached to the microscope. The gaps' area was quantified by ImageJ.

11. Colony formation assay

Both control and treated cells were seeded in 6-well plates with 5,000 cells per well. Medium (containing 10% FCS and 1% PS) was changed every second day. Cells were collected after 10 days and then fixed with 99% methanol 2 times for 5mins at room temperature. Afterwards, cells were stained with crystal violet (0.5% crystal violet mixed with 20% methanol) for 1 hour. Colonies were counted with ImageJ.

12. Statistical analysis

Results are given as mean ± standard error of the mean (SEM). All statistical comparisons were done using Student's T-test cooperating with Welch's correction and one-way ANOVA test. F-test was taken for expression analyses. Correlation analyses were made by Pearson correlation. Kaplan-Meier estimates of specific survival time were compared using the log-rank Mantel- Cox test. For all comparisons, the criterion for significance was $p < 0.05$ (significant) and $p < 0.01$ (highly significant).

Results

1. *TRIM71* is highly expressed in hepatoblastoma

To clarify the expression of *TRIM71*, RT-PCR was performed in 10 normal and 31 tumor samples as well as 4 liver tumor cell lines. We found *TRIM71* significantly upregulated in HB tumor samples and 4 tumor cell lines compared with normal liver samples (Figure 4). HUH6 and HUH7 cells showed relatively high, while HepT1 and Hep3B relatively low RNA levels.

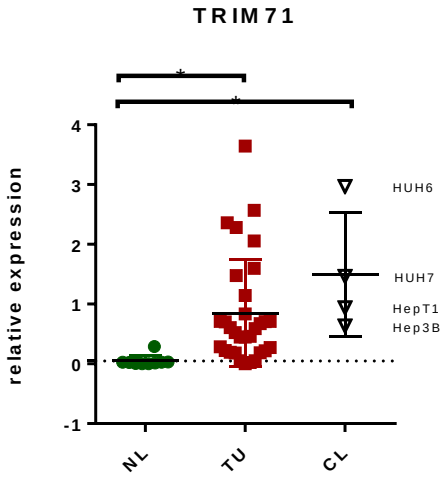

Figure. 4 Expression of *TRIM71* in hepatoblastoma, normal liver and liver tumor cell lines. *TRIM71* mRNA levels were shown as the relative expression in 10 normal liver samples (NL), 31 liver tumors (TU), and 4 cell lines (CL). The analysis was performed by RT-PCR and normalized with the housekeeping gene *TBP*. Groups were compared by Student T-test and significance was considered if *p < 0.05.

2. *TRIM71* overexpression inhibits cell proliferation in human HB cell lines

MTT assay was applied to further investigate the function of *TRIM71*. We performed gain-of-function experiments in HepT1 and Hep3B cell lines, which we know are *TRIM71* low expressing cells (Figure.5). Likewise, loss-of-function experiments were operated in

HUH6 and HUH7 cell lines, which express high levels of *TRIM71*. Interestingly, *TRIM71* overexpression suppressed the viability of HepT1 and Hep3B cells, although this was only significant in HepT1, while its downregulation stimulated cell proliferation at least in HUH6 cells. These results indicate that *TRIM71* can inhibit cell proliferation in liver tumor cells.

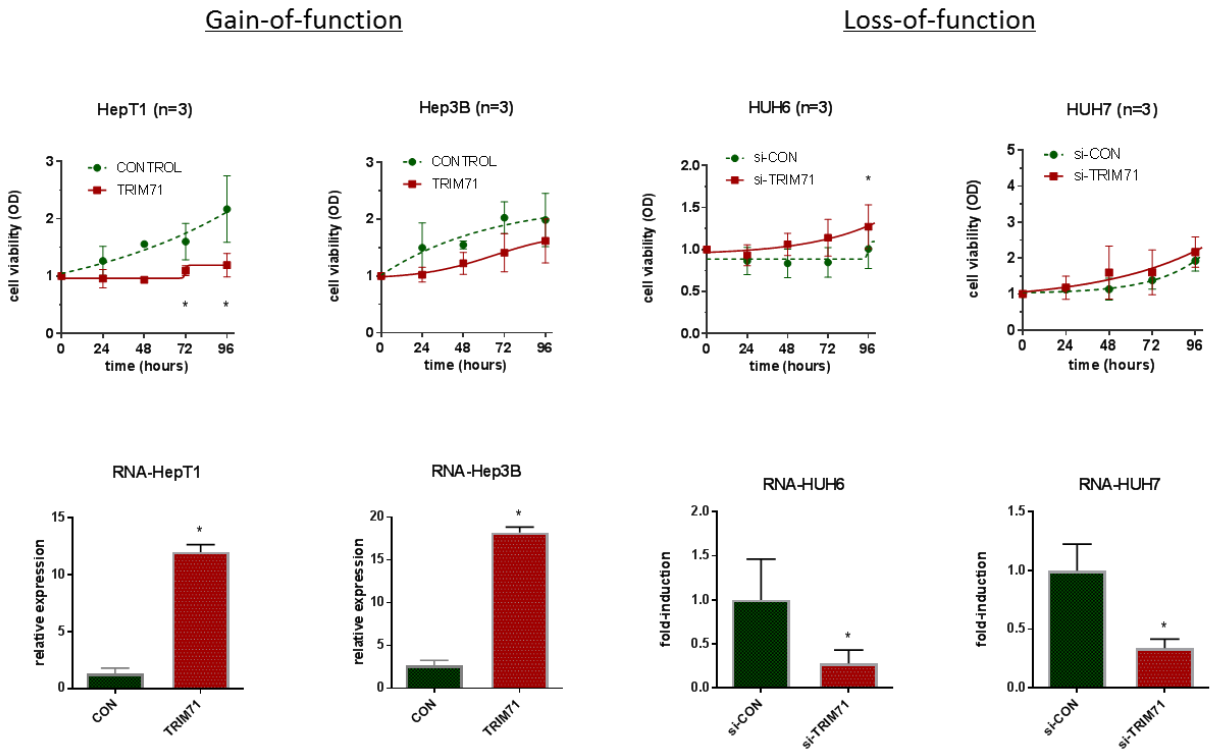

Figure. 5 *TRIM71* induces growth inhibition in HB cells. Cell viability assays in human HB and HCC cell lines. HepT1 and Hep3B cells were transfected with *TRIM71* expression and control plasmid. HUH6 and HUH7 were treated with *TRIM71* knockdown by siGENOME Human *TRIM71* smart pool. Cell viability was measured by MTT assays at the time points indicated. The mean and SEM of 3 independent experiments was calculated (lower row). The transfection efficiency was tested by measuring the relative *TRIM71* expression normalized to the house-keeping gene *TBP* by RT-PCR. Significance levels are as followed: *p < 0.05.

3. *TRIM71* does not affect apoptosis in pediatric hepatic tumors

In order to assess if the lower proliferation rate provoked by *TRIM71* is based on modulation of apoptosis, the apoptotic marker PARP was detected by western blot. Cells

were treated with *TRIM71* overexpression and knockdown and protein lysates were collected after 2 days. There is no difference between each pair of control and treated samples. Thus, it seems that the apoptotic machinery is not associated with *TRIM71*-induced growth variations (Figure 6).

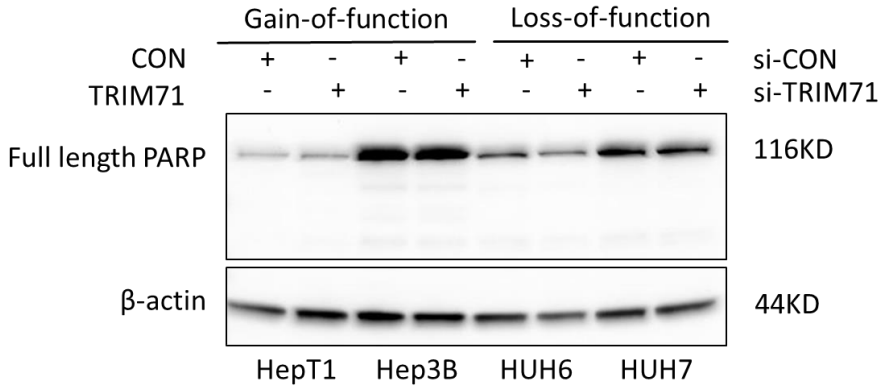

Figure.6 Hepatic cells show no apoptosis depending on *TRIM71* induction or reduction. Apoptosis was analyzed by detecting PARP protein with western blot. All 4 cell lines were treated either with *TRIM71* overexpression or knockdown, and protein was isolated after 2 days. Western blot was done against PARP, and β-actin served as a housekeeping protein.

4. *TRIM71* does not affect hepatic cell migration

The cell migration ability was determined by monitoring the time cells need to close a gap that has been introduced by scratching a confluent cell layer. *TRIM71* overexpression and knockdown showed no significant difference in time of gap closing. Hence, these data suggest that *TRIM71* does not influence cell growth by impacting migration (Figure 7).

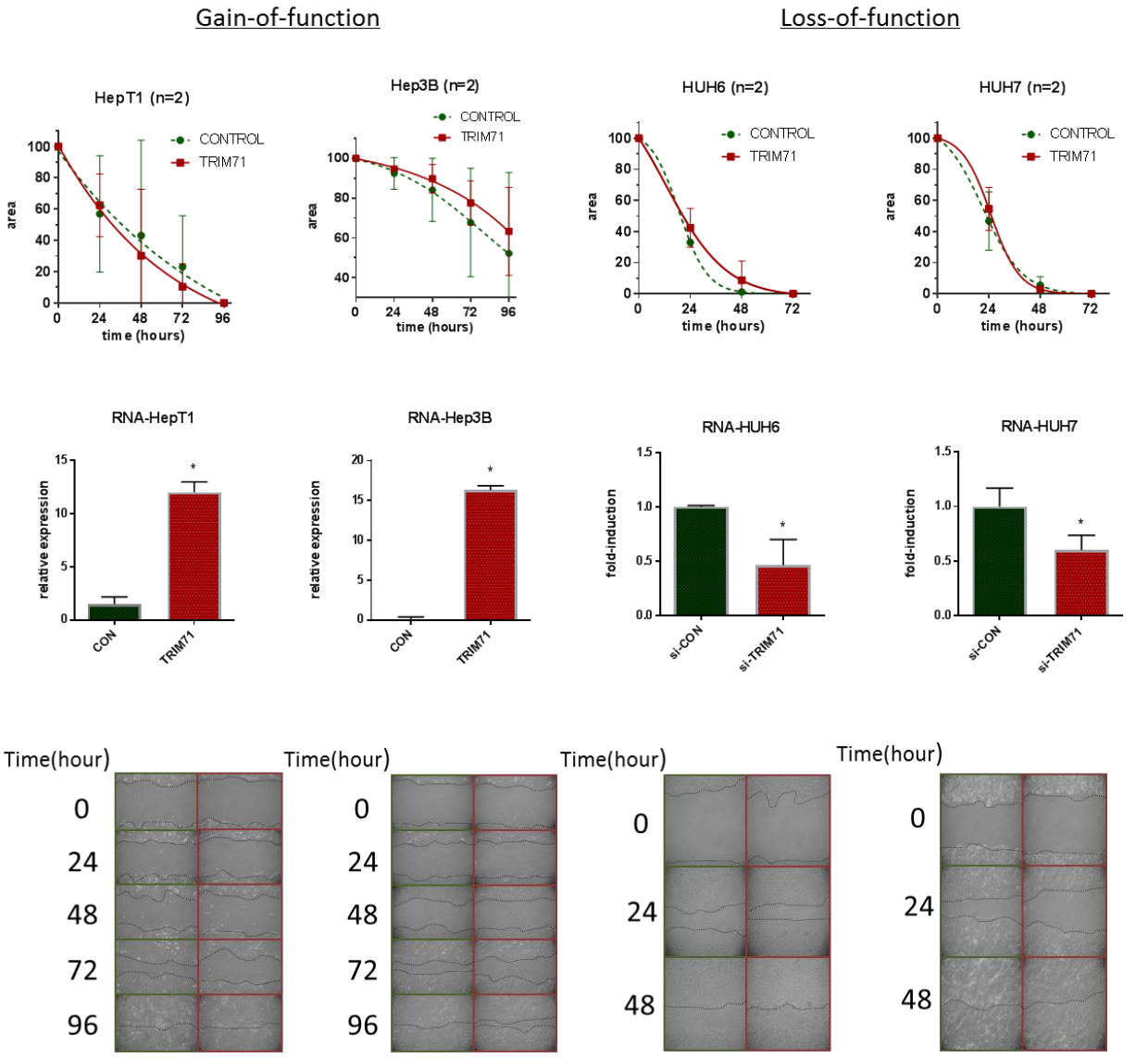

Figure. 7 *TRIM71* has no impact on hepatic cell migration. Four cell lines (HepT1, Hep3B, HUH6, HUH7) were treated either with *TRIM71* expressing plasmids or knockdown-mediating siRNA and the gap was measured at the indicated time points (top panel). mRNA expression performed with RT-PCR normalized by the housekeeping *TBP* (middle panel). Pictures were taken every 24 hours at the same point and the area calculated by ImageJ. Representative pictures of the scratch assays are show in the bottom panel. Differences in the time points are based on the varying growing speed of the different cell lines. Significance levels are as followed: *p < 0.05.

5. *TRIM71* does not interfere with E-cadherin

TRIM71 showed no impact on migration as described above. E-cadherin (CDH1) is known to play a main role in cell adhesion and is essential for preventing cell migration. Thus, we analyzed CDH1 at the protein level by western blot. It is obvious that CDH1 was unchanged upon *TRIM71* induction or reduction. Of note, CDH1 showed stronger signals in HepT1 and HUH6 cells, the two HB cell lines (Figure.8).

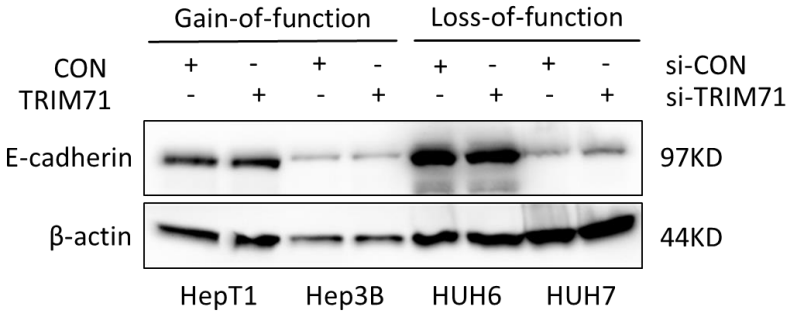

Figure. 8 E-cadherin western blot showed no difference upon *TRIM71* modulation in HB and HCC cell lines. E-cadherin abundance was determined in *TRIM71* induced and reduced conditions. Western blot was performed after 2 days with antibodies against E-cadherin/CDH1 and β-actin as a loading control.

6. *TRIM71* overexpression stimulates the capacity of hepatic tumor cells to produce colonies

TRIM71 has been recently shown to play an essential part in cell self-renewal [61]. Thus, clonogenic assays were performed after *TRIM71* overexpression and knockdown to determine the ability of liver tumor cells to produce cell colonies. Our experiments clearly show that the human hepatic cell lines with *TRIM71* overexpression have a stronger self-renewal capacity as control transfected cells. In contrast, knockdown of *TRIM71* led to a significant suppression of the colony producing capacity (Figure.9).

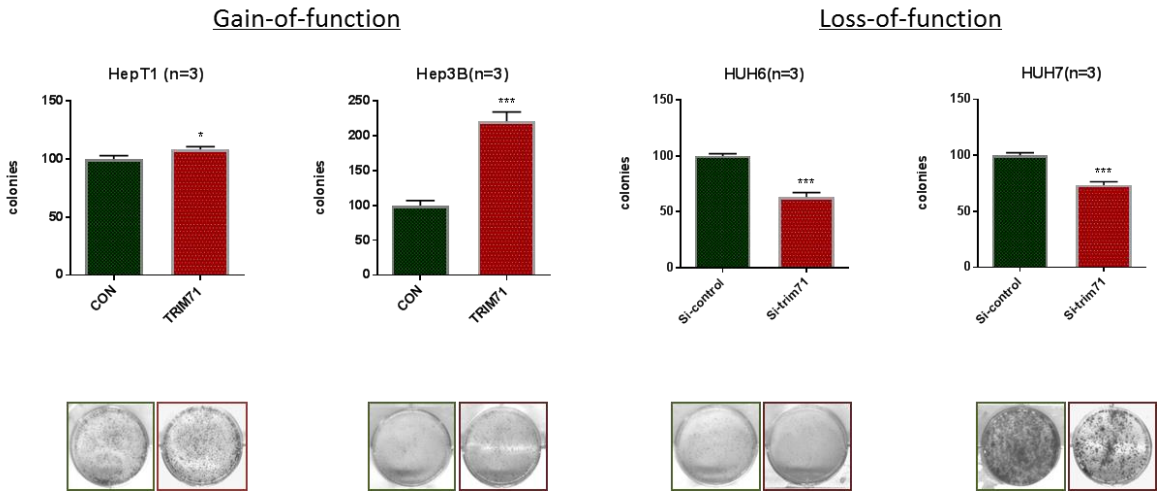

Figure. 9 Low expression of *TRIM71* reduces the capacity of producing colonies. The impact of *TRIM71* on colony formation was investigated by clonogenic assays. Four cell lines were treated with transient transfection of either an overexpression plasmid or knockdown-mediating siRNAs. Colonies were fixed and stained after 10 days of incubation. Each column represents the number of colonies of the treated cells normalize to the control cells. Significance was considered at: *p < 0.05, **p < 0.01, ***p< 0.001.

7. Genes of the *let-7/ LIN28B* network are highly expressed in HBs

To further investigate if genes of *let7/LIN28B* network are concomitantly altered with *TRIM71* in their expression, mRNA levels of *LIN28B*, *HMGA2* and *IGF2BP1* were evaluated by RT-PCR (Figure.10). Compared with normal liver tissue, all 3 genes are highly expressed. Of note, *HMGA2* and *IGF2PB1* expression was also significantly higher in liver tumor cell lines.

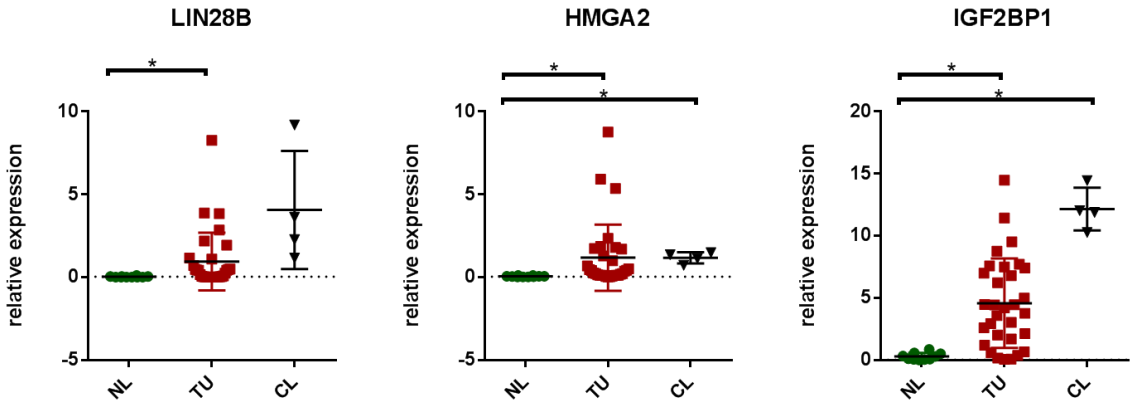

Figure. 10 Expression analysis of *let-7/LIN28B* network genes in HB and HCC. mRNA expression analysis performed by RT-PCR that showed relative candidate gene expression normalized to *TBP* housekeeping gene in 31 primary tumors (TU), 10 normal livers (NL) and 4 tumor cell lines (CL). Groups were compared using Student T-test and significance was considered at: *p < 0.05, ** p < 0.01.

8. *TRIM71* is highly correlated with l*et-7/LIN28B* network genes

The correlation analysis was performed to verify the hypothesis of a close relationship between the *let-7/LIN28B* network genes and *TRIM71*. Indeed, we found that all 4 genes are highly positively correlated with each other, which might indicate that they all play a predominant role in the mechanisms of liver cancer (Figure. 11).

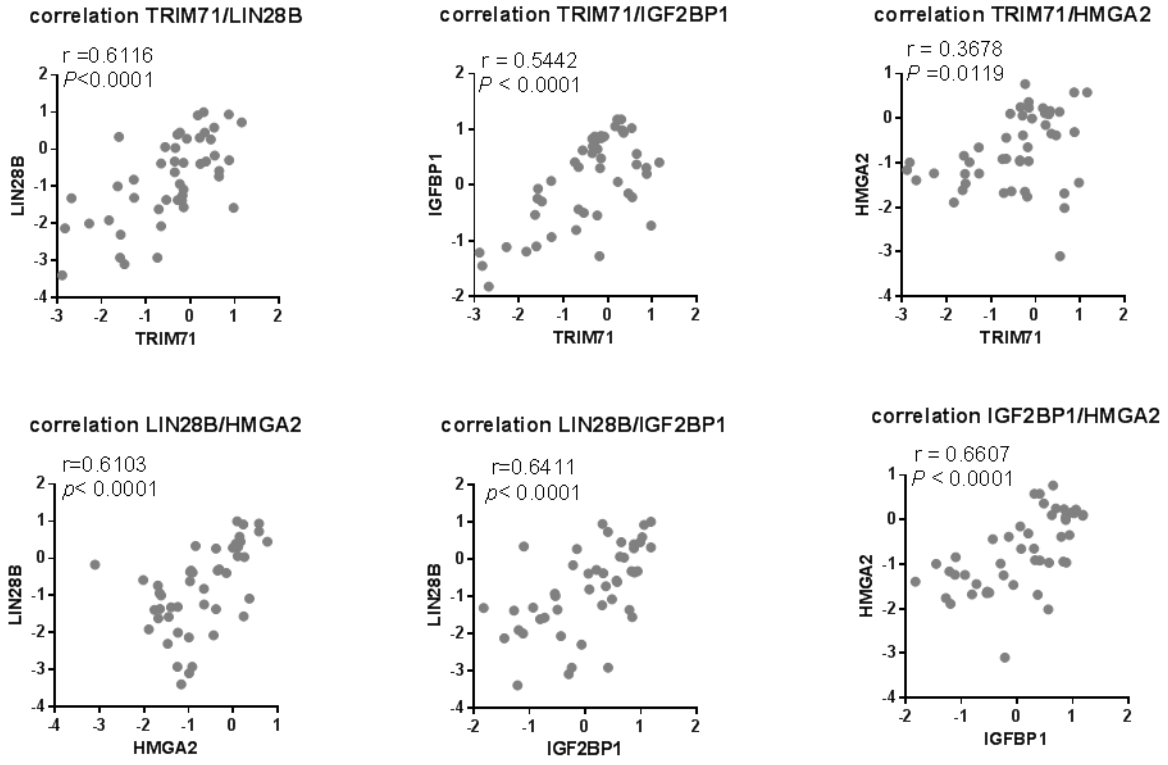

Figure. 11 The correlation analysis of *let-7/LIIN28B* network genes. The correlation analysis was applied to mRNA expression levels in each combination of the four genes using Pearson correlation. Coefficient r values were calculated, and values close to 1 indicate perfect correlation. P-values were calculated and significance was considered at *p < 0.05, ** p < 0.01, *** p < 0.001.

9. *TRIM71* overexpression is a general phenomenon in hepatoblastoma and only associated with *CTNNB1* mutations

In a next step, we stratified the 31 tumor samples according to specific clinical and biological features such as sex, age, PRETEXT, vascular invasion, metastasis, multifocality, 16 gene signature, and *CTNNB1* mutation status (Table 1). In our cohort, females (51.6%) and males (48.4%) were nearly equally distributed and most patients had an onset of disease within 36 months (74.2%). The proportion of patients with large tumors (PRETEXT IV) was 74.2%), with metastasis 35.5%, with multifocal tumor growth 38.7%, and with vena cava and portal vein invasion at the time of diagnosis of 12.9% and 16.1%, respectively. As expected, most tumors had *CTNNB1* mutations (83.9%). The recently

discovered 16-gene signature, which is known to be associated with prognosis [62], was found to show the C1 type in 61.3%, and the C2 type in 38.7% of patients.

By comparing the expression level of *TRIM71* between the defined clinical groups, we found no statistically relevant differences for any clinical parameter (Figure.12), which defines *TRIM71* overexpression as a general marker for hepatoblastoma. In line with this, there was no difference in the prognostic 16-gene signature related to their *TRIM71* expression (Figure.12), as well as in the outcome of patients, as shown by Kaplan-Meier analyses for overall and event-free survival (Figure.13). However, we found that there is a trend (P=0.0611) toward higher *TRIM71* expression in cases with *CTNNB1* mutations (Figure.14). Interestingly, this was also true for *LIN28B* (P=0.0701) and *HMGA2* (P=0.1027) expression, whereas for *IGF2BP1* (P=0.0188) this association was even significant (Figure.14).

Table. 1 Clinical and biological features of patients

Parameter	Numbers (%)
Sex	Male=15(48.4), Femal=16(51.6)
Age	<36m=23(74.2), >36m=8(25.8)
PRETEXT IV	Small=23(74.2), Large=8(25.8)
Vena cava invasion	No=27(87.1), Yes=4(12.9)
Portal vein invasion	No=26(83.9), Yes=5(16.1)
Metastasis	No=20(64.5), Yes=11(35.5)
Multifocality	No=19(61.3), Yes=12(38.7)
16-gene signature	C1=19(61.3), C2=12(38.7)
CTNNB1 mutation	Wt=5(16.1), Mu=26(83.9)

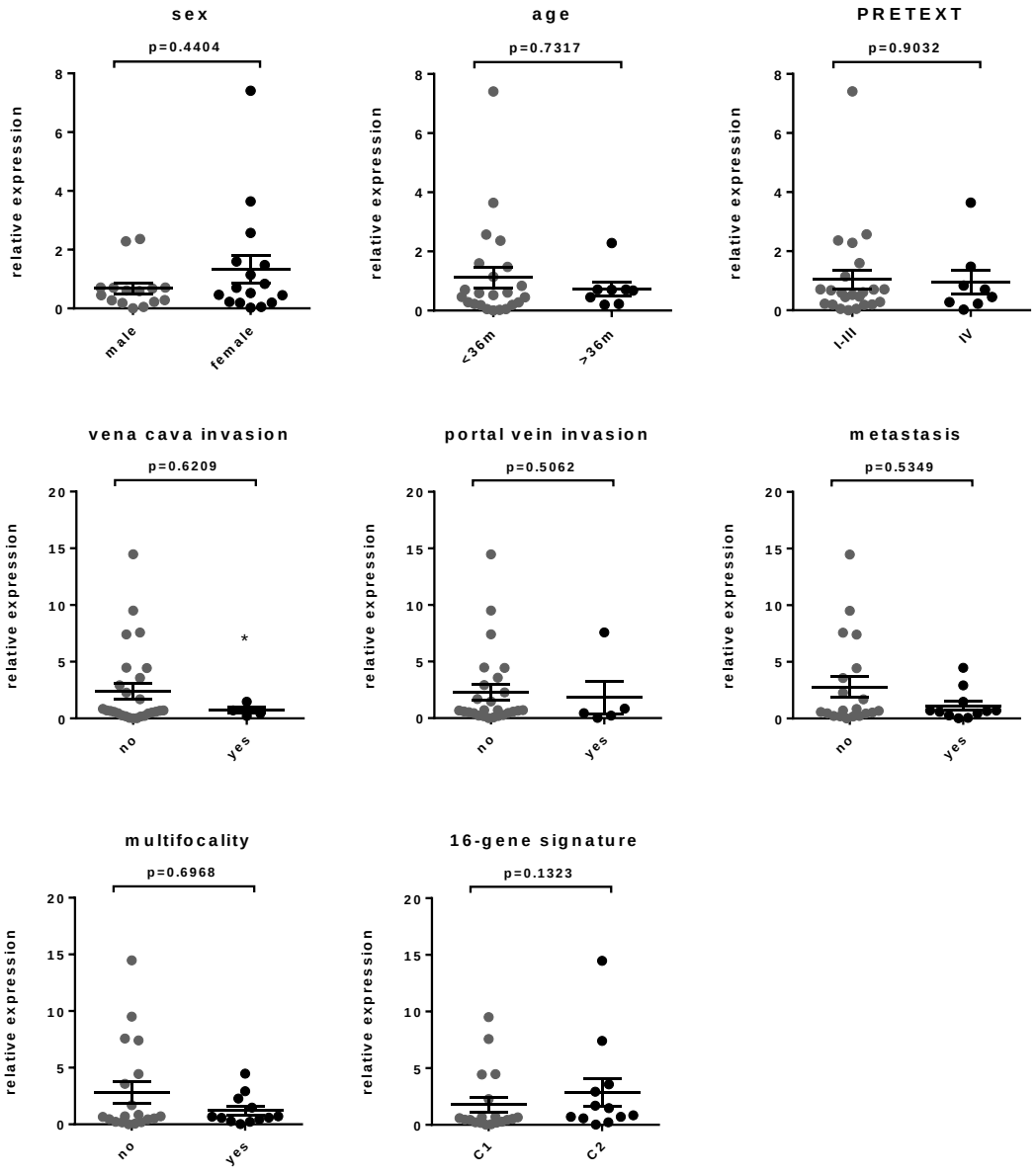

Figure. 12 *TRIM71* expression in clinical and biological defined subsets of patients. Relative genes expression of *TRIM71* in 31 primary tumors were grouped according to their clinical and biological characteristics. Groups were compared by Mann-Whitney test and the P-values are given.

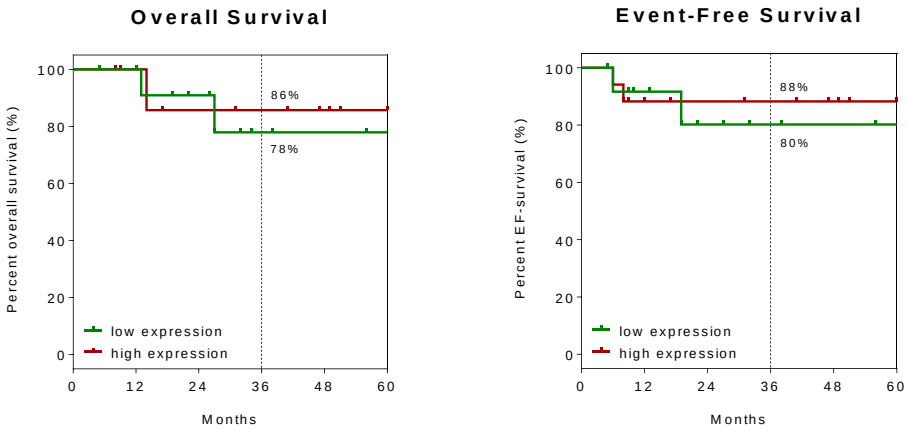

Figure. 13 Kaplan-Meier analyses for overall and event-free survival of *TRIM71.* The high expression is defined as > mean+5-fold standard deviation of 10 normal liver samples. Months stand for the time after diagnosed.

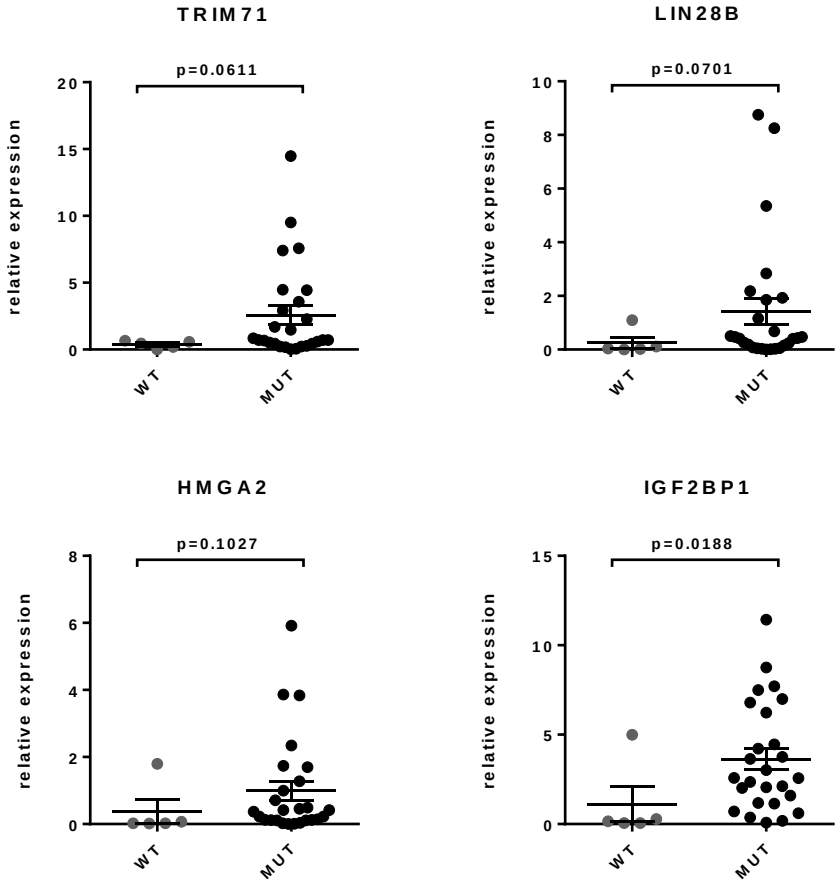

Figure. 14 Gene expression dependent on the *CTNNB1* mutation status. Relative gene expression of candidate genes in 31 primary tumors stratified according to their *CTNNB1* mutational status (wt = wild type, mut = mutated). Groups were compared by Mann-Whitney test and the P-values are given.

10. β-catenin accumulates upon *TRIM71* induction in HB cells

Wnt activation is an important mechanism that drives development of liver tumors [63]. As the β-catenin protein is the central component of Wnt signaling and high *TRIM71* expression is associated with *CTNNB1* mutations (Figure.14), we investigated if β-catenin is altered by *TRIM71* modulation by doing western blot. In HepT1 cells, *TRIM71* induction increased β-catenin protein levels, while in HUH6 cells *TRIM71* knockdown led to a reduction of β-catenin, indicating that *TRIM71* influences β-catenin accumulation.

However, this scenario was not seen in the two HCC cell lines Hep3B and HUH7, which are known to be wildtype for β-catenin, as opposed to the two HB cell lines HepT1 and HUH6 that harbor mutations in the β-catenin gene (Figure.15).

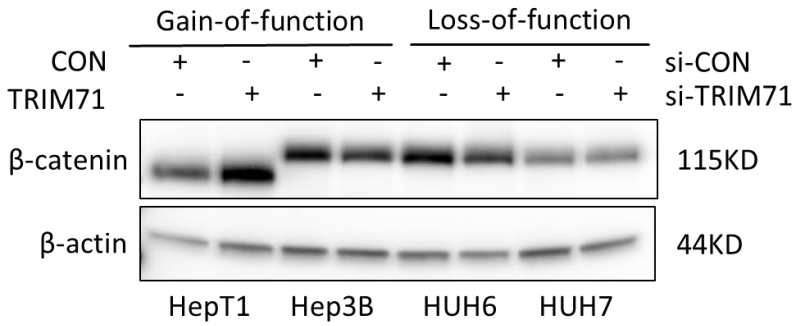

Figure. 15 *TRIM71* overexpression accumulates β-catenin in HB cell lines. To determine the β-catenin abundance in *TRIM71* induced and reduced conditions, western blot was performed 2 days after treatment. The western blot analysis was done by β-catenin antibody and β-actin served as a loading control.

11. Wnt target gene and *LIN28B/let7* pathway expression upon *TRIM71* level modulation

To further analyze the association between *TRIM71* and Wnt signaling as well as the *TRIM71*-associated *LIN28B/let-7* pathway, RT-PCR was performed with three independent experiments from each cell line upon *TRIM71* overexpression and knockdown (Figure.16). The Wnt target genes *AXIN2, LGR5,* and *DKK1* as well as *CTNNB1* itself showed no significant changes upon either *TRIM71* knockdown or overexpression. Identically, the *LIN28B/let-7* pathway genes *LIN28B, HMGA2,* and *IGF2BP1* also remained unchanged (Figure.16). However, it is not surprising considering that *TRIM71* mainly regulates its targets post-transcriptionally.

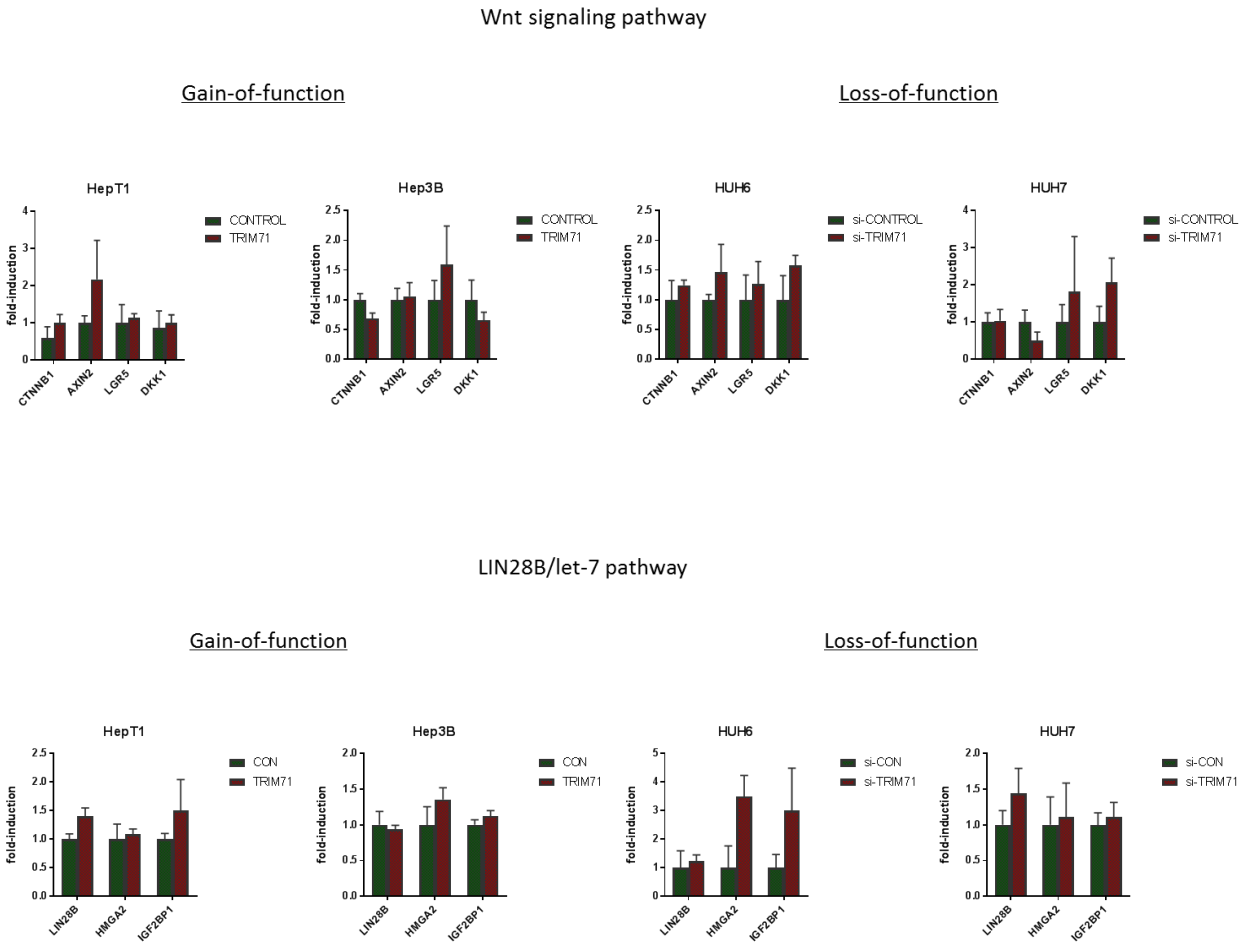

Figure. 16 Expression analysis of Wnt- and *let-7/LIN28B*-associated genes upon *TRIM71* modulation. Gene expression was analyzed by RT-PCR and relative candidate gene expression normalized to TBP housekeeping gene of 3 independent experiments after *TRIM71* overexpression or knockdown are shown (mean and standard deviation). Each sample was done in duplicates. The treated group was normalized to control and is shown as fold change. Statistical significance shown as follow: *p < 0.05, ** p < 0.01.

Discussion

1. Why can *TRIM71* only repress cell proliferation and accumulate β-catenin in HB cell lines?

Although tumors are heterogeneous, they still all share the ability to proliferate compared to the limiting growth of normal tissues. Alterations in key pathways controlling cell proliferation including Wnt signaling are mandatory for tumor establishment [64]. In our study, proliferation assays performed in four different liver tumor cell lines (2 HB and 2 HCC) clearly showed that *TRIM71* affects proliferation only in the two cell lines HepT1 and HUH6 that are *CTNNB1* mutated, where overexpression and gene knockdown resulted in either the reduction or induction of cell growth, respectively. The two HCC cell lines with wildtype *CTNNB1* instead did not respond on *TRIM71* modulation. In recent publications, *TRIM71* has been found also to inhibit tumor growth in non-small lung carcinoma [50]. Other TRIM family members have been discovered to be implicated with tumorigenesis as well, for example *TRIM8, TRIM11, TRIM16,* and *TRIM62* [65]. *TRIM2, TRIM3* and *TRIM32* and some other family genes share structural homology in C terminal like *TRIM71* [66-68]. *TRIM3* was reported with NHL domain-dependent cell proliferative inhibition, even more, the RING domain was found necessary for tumor suppression. *TRIM25* has been implicated with the proliferation of many cancer too, while often with estrogen response and through a variety of pathways for further promotion [69]. It has been published that *TRIM25* increased cell proliferation with the requirement of targeting a negative cell cycle regulator for degradation [70]. Nevertheless, in our case, *TRIM71* seems to have another unique function than other TRIM members, which mainly depend on their epigenetic modulation and E3 ligase activity or their RNA binding property. Surprisingly, this significant difference can be only seen in HB cell lines with an activated Wnt signaling pathway. So the questions arose, if *TRIM71* is a new regulator of the Wnt signaling pathway or at least related to this pathway?

The western blot of β-catenin upon *TRIM71* overexpression and knockdown in our study showed the predictive result. *TRIM71* induction causes β-catenin accumulation, while its

reduction decreased β-catenin, which was interestingly observed only in HepT1 and HUH6 cells, the two HB cell lines with *CTNNB1* mutation. A recent genome-wide expression analysis revealed that certain genes were constantly upregulated in HB tumors: *LIN28B, HMGA2, AFP* and *DLK1* [71]. The first two genes are linked to the Wnt signaling pathway and were uncovered up-regulated in HB tumor samples in our study as well. According to the literature [47, 50, 72, 73] and based on our new findings, we set up a regulatory network (Figure. 17). *Let-7* is known as a tumor suppressor, and *LIN28B, IGF2BP1,* and *HMGA2* are three downstream targets of it in cancer cells which have been proved working as oncogenes [73]. *LIN28B, IGF2BP1,* and *HMGA2* can unsurprisingly induce cancer cell proliferation and tumor growth [74, 75] or even induce aggressive phenotypes. Then, a feedback were found that *LIN28B* represses *let-7* via a *zcchc11*-independent mechanism [41], while *LIN28B* expression is only enhanced by *IGF2BP1* substantially. *Let-7-LIN28B-IGF2BP1* potentially become a triangle, that we can assume *LIN28B* itself overexpression may abolish *let-7* synthesis and cause the *IGF2BP1* stimulation. *Let-7* negatively regulates *TRIM71* has been proven in several studies [76, 77] and more importantly *TRIM71* has been investigated to suppress both *let-7* and *LIN28B* [50, 72]. It should be noted that *TRIM71* is unusual in this total interactome considering its unique position. Moreover, *TRIM71* has been proved as an oncogene in HCC [48], while during this study we found its role in HB is more diverse.

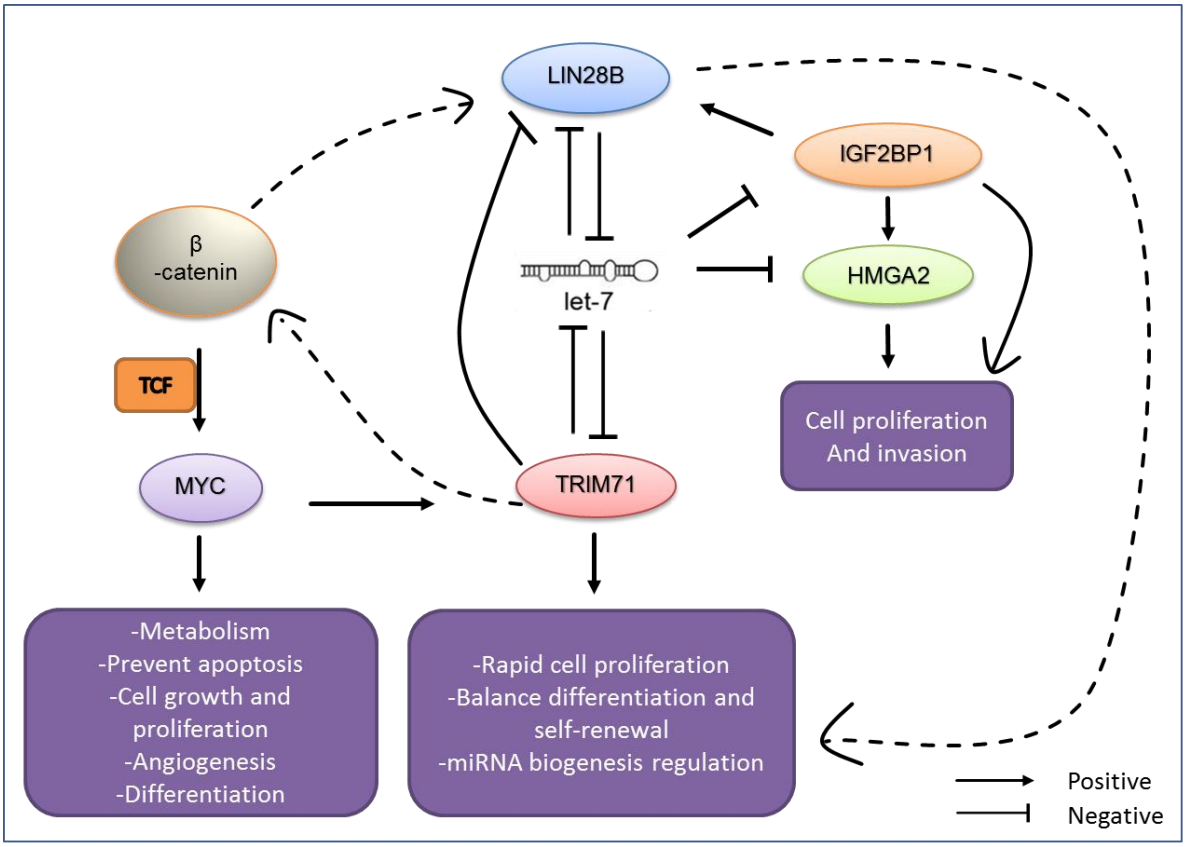

Figure. 17 The interplay of candidate genes and their functional role. Genes related to hepatic liver tumor formation are specifically listed in this functional network. *let-7* is an inhibitor of *LIN28B* in the feedback mechanism. This inhibition of *LIN28B* can be accomplished by let-7 through activation of *TRIM71*, which targets *LIN28B* for ubiquitin-mediated proteasomal degradation. *IGF2BP1* and *HMGA2* are two targets of *let-7* which have been investigated working as oncogenes. Besides, *IGF2BP1* positively regulates *LIN28B* which forms a feedback triangle between *let-7, LIN28B, IGF2BP1*. Our study found β-catenin can result in *TRIM71* accumulation. *MYC* is a Wnt target and among the most potent oncogenes associated with HCC development in mice [78]. The dashed line stands for hypothetical interactions from our study and the full lines are the proven interactions from the literature.

TRIM71, LIN28B, HMGA2, and *IGF2BP1* expression in HBs was tightly positively correlated with each other. Interestingly, these genes are all suppressed by *let-7* (Figure.17). *LIN28B*, a small RNA-binding protein, can promote proliferation and repress differentiation as a consistent idea with available data supporting, at least in part through repressing let-7 biogenesis [51]. And *LIN28B* has also been proven as a specific substrate of *TRIM71*, its oncogenic activity is repressed post-transcriptionally by *TRIM71*. According

to the result in our study, that *TRIM71* inhibits cell proliferation only in HB could be due to its *TRIM71-LIN28B* mode of action. But the interplay loop is always more complex than other mechanisms, for instance, *HMGA2* is a gene that is essential in some tumor malignancies for metastasis [79, 80], but appeared not to be functionally linked in this study. On the other side, *LIN28B* suppresses *let-7*, which then down-regulates *TRIM71*, while *TRIM71* downregulates these two genes. This triple-negative feedback may be switched by several specific factors or signals that reinforces developmental steps. Certainly, in rare cases, this specific loop results in de-differentiation, proliferation or even other tumor formation. It seems that this switch may be responsible for HCC development where *LIN28B* displays heterochronic expression [81].

The interplay of Wnt/β-catenin signaling and *MYC* in hepatoblastoma has been described to occur predominantly in immature tumors and to activate a distinct transcriptional program that correlates with tumor aggressiveness [82]. *TRIM71* was proved a critical downstream effector for *MYC* enhancement of reprogramming [47]. As a target of *MYC*, it might be the reason why *TRIM71* is up-regulated in hepatoblastoma. We hypothesize that *TRIM71* causes β-catenin accumulation (Figure 17), although the analysis of the Wnt target genes in our study showed no significant upregulation of expression. It is still too early to perorate here due to the limitation of our data. Even though, *TRIM71* is known as a critical factor that cooperates with *AGO2* and binds to miRNA. This leads to post-transcriptional repression of *CDKN1A* for promoting G1-S cell phase, but our study demonstrates *TRIM71* inhibiting proliferation via *LIN28B* pathway overcomes its cell cycle enhancement and β-catenin tumor growth promotion activity in hepatoblastoma.

2. *TRIM71* might be an effector of stemness in pediatric hepatic tumor cells

In this study, we performed several cell based assays trying to provide a comprehensive function of *TRIM71* in childhood liver tumors. Scratch assay is a well-developed method for in vitro measuring cell migration. The results of our study suggest that aberrant *TRIM71* expression has no impact on migration in four cell lines. The absence of E-cadherin has been claimed to be related to nuclear accumulation of β-catenin in immature HBs [83],

and its interplay with growth-stimulating pathways evidenced in less differentiated cells. In addition, E-cadherin loss results in the induction of multiple transcription factors, as shown by gene expression analysis [84]. Absence of epithelial adhesion molecule E-cadherin was thought to enable metastasis through disrupting intercellular contacts, which is an early step in metastatic dissemination [85]. Although E-cadherin detection in this study revealed its stronger signal in HepT1 and HUH6 cell lines, there was no difference upon *TRIM71* overexpresses or knockdown. Moreover, *TRIM71* mRNA expression in HB tumors showed no difference between tumors with or without metastasis and invasion. In conclusion, *TRIM71* is no predictor of tumor metastasis.

A small proportion of tumor cells named cancer stem cells (CSCs) are responsible to sustain tumor formation and growth, which are critical for cell renewal and cell growth. CSCs have recently been identified in many solid tumors, such as breast, liver, and colon [86, 87]. They harbor characteristic stem cell properties like self-renewal and differentiation. Results from this study showed that *TRIM71* triggers the stimulation of colony formation capacity in both HBs and HCCs. This suggests that *TRIM71* is able to induce cancer cell self-renewal capacity, which is in line with the finding that *TRIM71* accumulates β-catenin in HBs. Independend of the regulatory function of *TRIM71*, being either RNA binding or ubiquitin ligation, it is safe to predict that *TRIM71* indirectly regulates stemness in HCC. *TRIM71* is known to post-transcriptionally downregulate *LIN28B*, which has an important role in the promotion of stemness [88]. *TRIM71* was proven to frequently associate with mRNA and immediately reduce their expression [89]. In 2014, *TRIM71* was first time published as an upstream regulator of the *LIN28B/let-7* pathway and negatively regulates miRNA *let-7*, which defined *let-7* as a general inhibitor of self-renewal as well. If we rely on this pathway, isn't it the opposite explanation of *TRIM71* inducing CSC self-renewal? So alternative mechanisms must be uncovered in this phenomenon. *TRIM71* was published to cooperate with *Ago2* to repress expression of the cell cycle regulator *CDKN1A*, which negatively regulates G1-S transition. Thus, they demonstrated *TRIM71* is a factor responsible to promote rapid embryonic stem cell self-renewal. *TRIM71* was identified not only to facilitate ESCs rapid proliferation, but also to balance stem cell self-renewal and differentiation [61]. *TRIM71* was reported to post-transcriptionally regulate the pro-differentiation transcription factor *EGR1*, which inhibits reprogramming [47].

Notably, cell self-renewal was known to be supported by a shortening of G1 phase in human embryonic stem cells [90]. This means *TRIM71* might contribute to promote reprogramming of the cell cycle, and finally, cancer cell self-renewal.

3. Clinical value of *TRIM71* in HB remains unclear

Based on the results presented here the clinical relevance of *TRIM71* in HB and HCC is not clear. The reason for this might be inferred from the multiple functions this protein has, which reach from its RNA binding activity to its ubiquitin ligase activity, and its multiple targets, containing several oncogenes and tumor-suppressors. Due to the limitation of our data available in this study, it is still not the time to conclude its precise role in hepatic liver tumors. Overall, our data clearly suggest that although *TRIM71* promotes "stemness" in HB cell lines, it also inhibits cell proliferation. But the deep mechanism of *TRIM71* remains unclear, that is worth to investigate in the future.

Outlook and perspectives

P53 is a well-investigated tumor suppressor, and its expression level and activation are regulated by various TRIM proteins carrying E3 ubiquitin ligase activity, such as *TRIM13, TRIM19, TRIM28* [91]. As a member of *TRIM* proteins, *TRIM71* is one of the most important genes. Thus, its influence on *p53* and further functional studies in hepatic childhood cancers should be discovered diligently. Moreover, better understanding of mechanisms of signaling pathways in regulating tumorigenicity, CSC self-renewal, proliferation, and differentiation always are critical for the development of effective anticancer therapies [92]. This study again provides evidence how important it is to integrate a gene into the pathway in research. *TRIM71,* involved in *LIN28B/let-7* pathway, is a better entry point in the study of pediatric liver tumors.

Summary

The *TRIM71* (*Tripartite Motif Containing 71*) gene encodes the E3 ubiquitin-protein ligase that is able to hamper key factors through its ubiquitinylation and miRNA activities, thereby regulating growth and maintenance of embryonic stem cells. Moreover, it has been proven to be involved in many essential signaling pathways. *TRIM71* was identified as a novel oncogenic protein in human liver cancer and as a strong predictor of poor prognosis. However, its role in pediatric liver tumors remains unknown.

In this study, we aimed to identify the impact of *TRIM71* in liver tumor behavior, to explore its clinical relevance for varying outcomes of childhood hepatoblastoma patients, to describe the regulatory network of candidate genes and to integrate it into a signaling pathway. We therefore made use of patient samples and four different liver tumor cell lines (derived from two hepatoblastomas and two hepatocellular carcinomas), in which expression analyses and *TRIM71*-modulating gain- and loss-of-function experiments were performed, respectively.

TRIM71 is significantly overexpressed in 31 hepatoblastoma tumor compared to normal liver samples. *TRIM71* induction inhibits cell proliferation, while its reduction stimulates cell proliferation, which is only observed in the ß-catenin mutated hepatoblastoma cells. However, no impacts could be found in apoptosis and migration due to *TRIM71* up or down-regulation. Clonogenic assays identified the capacity of *TRIM71* to raise self-renewal in hepatoblastoma and hepatocellular carcinoma cells. Association analyses show that *TRIM71* overexpression is a general phenomenon in hepatoblastoma, irrespective of their clinical characteristics. However, *TRIM71* overexpression more likely appeared in tumors with ß-catenin mutations. Besides, *TRIM71* overexpression drives β-catenin accumulation in hepatoblastoma cells.

In conclusion, we found that *TRIM71* is frequently overexpressed in pediatric liver tumors, and that it negatively regulates cell proliferation, while it positively regulates cancer cell self-renewal. This might be triggered at least in part by the accumulation of mutant ß-catenin, but its precise role in these molecular mechanisms has to be further investigated

Zusammenfassung

Das Gen *TRIM71* (*Tripatite Motf Containing 71*) kodiert eine E3 Ubiquitinligase, welche in einer Vielzahl essentieller zellulärer Signalwege involviert ist. Es hat sowohl durch die Ubiquitinylierung von Proteinen als auch durch Kooperation mit miRNAs eine wichtige Rolle für das Wachstum und den Erhalt embryonaler Stammzellen. Kürzlich konnte *TRIM71* als onkogenes Protein in menschlichen Lebertumorzellen identifiziert werden und korreliert hier mit einem schlechteren Outcome. Ob *TRIM71* auch bei der Entstehung pädiatrischer Lebertumoren involviert ist, ist bisher unbekannt.

Ziel dieser Studie war es, den Einfluss von *TRIM71* auf die Entwicklung pädiatrischer Lebertumoren und seine mögliche klinische Relevanz für das unterschiedliche Outcome von Patienten mit Hepatoblastom zu erkunden. Weiterhin sollten verschiedene potentielle Zielgene identifiziert und *TRIM71* dadurch in einen Signalweg intergiert werden. Dafür führten wir Expressionsanalysen an Patientenproben, sowie Experimente nach Überexpression oder Knockdown von *TRIM71* in vier verschiedenen Lebertumorzelllinien durch (zwei Hepatoblastom- und zwei Hepatozelluläres Karzinom Zelllinien).

Es zeigte sich, dass *TRIM71* in den 31 getesteten Hepatoblastom-Patientenproben signifikant überexprimiert ist. Die Induktion von *TRIM71* in den Zelllinien inhibiert ihre Proliferation, während verminderte Expression zu einer Wachstumsstimulation führte. Dies konnte jedoch nur in den Zelllinien gezeigt werden, die eine Mutation von ß-catenin vorweisen. Hoch- oder Runterregulation von *TRIM71* hatte keinen Effekt auf Apoptose und das Migationsverhalten der Zellen. In klonogenen Assays konnte beobachtet werden, dass *TRIM71* einen Einfluss auf die Fähigkeit zur Selbsterneuerung der Hepatoblastom- und Hepatozellüräres Karzinom-Zellen hat. Bei der Assoziation mit klinischen Parametern der getesteten Patientenproben zeigte sich, dass die Überexpression von *TRIM71* ein generelles Phänomen des Hepatoblastoms ist, welches nicht mit bestimmten klinischen Parametern korreliert. Es zeigte sich jedoch, dass eine Überexpression häufiger bei Tumoren mit einer ß-catenin Mutation auftritt. Weiterhin führt die Überexpression zu einer Akkumulation von ß-catenin in Hepatoblastom-Zellen.

Zusammenfassend zeigte sich, dass *TRIM71* häufig in pädiatrischen Lebertumoren überexprimiert ist. Es hemmt die die Zellproliferation, verstärkt jedoch die Fähigkeit zur Selbsterneuerung von Tumorzellen. Dies könnte möglicherweise zum Teil durch die Akkumulation von mutiertem ß-catenin im Zellkern beeinflusst sein, sein genauer Einfluss auf die molekularen Mechanismen ist jedoch nicht abschließend geklärt.

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Abbreviations

HB	Hepatoblastoma
HCC	Hepatocellular carcinoma
RPMI	Roswell Park Memorial Institute Medium
PS	Penicillin-Streptomycin
DPBS	Dulbecco's Phosphate-Buffered Saline
FCS	Fetal Calf Serum
dNTPs	deoxynucleotides triphosphate
DMEM	Dulbecco`s Medium
EDTA	Ethylenediaminetetraacetic acid
PBS-T	Phosphate buffered saline and 0.1% Teen-20
β-actin	Beta actin
β-catenin	Beta catenin
PCR	Polymerase chain reaction
RT-PCR	Real time-polymerase chain reaction
PRETEXT	Pretreatment extent of tumor
TRIM71	Tripartite Motif Containing 71
CSC	Cancer stem cell

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Affidavit

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I hereby declare, that the submitted thesis entitled

The functional role of TRIM71 in childhood liver cancer

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