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„Regulation of Translation during Cancer Progression“

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1. Abstract

Laminin B1 (LamB1) is a main component of the extracellular matrix and is involved in the regulation of tumor cell migration and invasion of during carcinogenesis. Metastasis of carcinoma cells is crucially linked to the process of epithelial to mesenchymal transition (EMT), which allows tumor cells to acquire a more motile phenotype and to dissociate from the epithelial cell cluster of the tumor. Expression profiling of polysome-associated mRNA revealed LamB1 to be translationally upregulated upon EMT of malignant hepatocytes. The enhanced translation of LamB1 in metastatic hepatocytes proved to be regulated by an internal ribosome entry site (IRES) located within the 5'-untranslated region (UTR) of the LamB1 transcript. IRES activity was detected by employing two independent reporter systems and verified by stringent assays for the presence of cryptic promoter or splice sites. The minimal cis-acting IRES sequence of 293 nucleotides that is required for cap-independent translation was localized directly upstream of the start codon. Notably, the IRES trans-acting factor (ITAF) La was identified by RNA affinity purification as regulatory factor that interacts with LamB1 5'-UTR. This interaction was verified by RNA-immunoprecipitation in vivo, which revealed enhanced binding of La to the minimal IRES motif of LamB1 after EMT. Consistently, cytoplasmic levels of La were elevated in EMT-transformed cells and correlated with increased LamB1 protein expression. Furthermore, IRES-driven translation of LamB1 was elevated in the presence of La *in vitro*. Importantly, the EMT-induced cytoplasmic translocation of La was found to be triggered by platelet derived growth factor (PDGF) that is downstream of transforming growth factor (TGF)-β signaling. Together, these data demonstrate that La interacts with the LamB1 IRES in the cytoplasm, resulting in enhanced cap-independent translation of LamB1 in malignant hepatocytes that have undergone EMT.

2. Zusammenfassung

Laminin B1 (LamB1) ist ein Bestandteil der extrazellulären Matrix und auf entscheidende Weise in der Regulation der Tumorzellmigration und –invasion während der Karzinogenese involviert. Ein essentieller Aspekt der Tumorprogression ist die Umwandlung epithelialer Tumorzellen zu einem mesenchymalen Phänotyp (EMT), der mit metastatischen Eigenschaften korreliert. Ein Expressionsprofil polysom-assozierter mRNA maligner Hepatozyten zeigte die Aktivierung der Translation von LamB1 in EMT-transformierten Zellen. Es stellte sich heraus, das die erhöhte Translation von LamB1 in metastasierenden Hepatozyten von einer internen Ribosomen-Bindungsstelle (IRES) reguliert wird, welche in der 5'-untranslatierten Region (UTR) des LamB1 Transkripts lokalisiert ist. Zwei unabhängige bicistronische Reportersysteme wurden für die Bestimmung der IRES-Aktivität von LamB1 verwendet. Zur Verifikation der IRES Aktivität wurde die Präsenz kryptischer Promotoren und Spleißstellen im 5'-UTR von LamB1 ausgeschlossen. Die minimale cis-agierende IRES Sequenz, welche für die „Cap"-unabhängige Translation notwendig ist, wurde mit einer Länge von 293 Nukleotiden direkt oberhalb des Startcodons lokalisiert. Mithilfe einer RNA Affinitätschromatography wurde der IRES trans-aktivierende Faktor (ITAF) La als Bindungspartner identifiziert, welcher mit der LamB1 5'-UTR in vitro interagiert. Diese Interaktion wurde durch eine RNA-Immunopräzipitation in vivo verifiziert. Weiters konnte die Bindung einer erhöhten Menge von La Protein an das minimale LamB1 IRES Motiv nachgewiesen werden. In Übereinstimmung mit dieser Beobachtung konnten erhöhte zytoplasmatische Mengen von La in EMT-transformierten Zellen detektiert werden. Überdies führte die Gegenwart von La zu einer gesteigerten IRES-meditierten Translation von LamB1 in vitro. Platelet derived growth factor (PDGF) wurde als auslösender Signaltransduktionsweg für die zytoplasmatische Translokation von La identifiziert. Zusammenfassend konnte damit nachgewiesen werden, dass der PDGF Signalweg für die zytoplasmatische Akkumulation von La und damit der Interaktion mit dem LamB1 IRES verantwortlich ist, wodurch die cap-unabhängige Translation von LamB1 in malignen Hepatozyten nach EMT aktiviert wird.

3. Introduction

3.1. Tumorigenesis of hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the third most common cause for cancer related mortality worldwide [1]. The vast majority of HCC develop as a consequence of chronic inflammatory liver disease and are preceded by liver fibrosis and cirrhosis [2]. Infection with hepatitis B and C virus is believed to be the main etiologic factor in over 80% of cases [3]. Other etiologies of HCC include dietary risk factors such as exposure to aflatoxin or chronic alcohol intoxication as well as hereditary hemochromatosis and non-alcoholic fatty liver disorders [3]. Reports demonstrate that the worldwide incidence of HCC increased in recent years [4]. Limited therapeutic options and late diagnosis due to the asymptomatic nature of early disease stages are the main reasons for the high mortality rate in patients with HCC [5]. Partial hepatectomy and orthotopic liver transplantation are considered the only effective treatments of HCC [6]. The option of partial hepatic resection is restricted to patients at an early disease stage with preserved liver function and resectable tumors [6]. Even after successful surgery the rate of tumor recurrence is high due to intrahepatic metastasis [7]. The development of new therapeutic approaches requires improved knowledge of tumorigenic processes and the signaling pathways that regulate relevant mechanisms such as tumor cell proliferation, angiogenesis, invasion and metastasis.

Angiogenesis and tumor vascularization

Intrahepatic metastasis critically depends on the ability of tumor cells to acquire migratory and invasive properties as well as on angiogenesis. The liver is a highly vascularized organ with an extraordinary regenerative potential [8]. Proliferative regeneration of hepatocytes in response to intoxication requires efficient neovascularization [9]. Angiogenesis relies on the recruitment and activation of pericytes and vascular endothelial cells [10]. The angiogenic process is induced by HIF1α in response to hypoxia, resulting in secretion of angiogenic factors including vascular endothelial growth factor (VEGF)-A, angiopoitin-2 and platelet derived growth factor (PDGF), which have been shown to be upregulated in HCC patients compared with cirrhotic patients [11]. These growth factors orchestrate the angiogenic switch in autocrine and paracrine signaling loops between endothelial cells, pericytes and tumor cells [12]. Neovascularization starts with destabilization of the existing vasculature leading to a high vascular permeability, which is thought to promote tumor metastasis [13]. Remodulation of the extracellular matrix (ECM) plays an important regulatory role throughout the whole angiogenic process (Figure 1). Endothelial cells are quiescent under normal conditions when they are bound to the capillary basement membrane [14]. Degradation of the basement membrane by metalloproteinases (MMPs) allows endothelial cells to dislodge from the existing vasculature and sequestered growth factors such as VEGF and basic

fibroblast growth factor (bFGF) are released [13]. When the basement membrane is disassembled the endothelial cells may interact with different domains of the same set of proteins [15]. This concept of regulation means that ECM signaling depends not only on composition but also on the structural configuration of the matrix. The provisional ECM regulates migration and proliferation of activated endothelial cells [16]. In tumors the ECM conformation reassembles the provisional matrix and therefore constantly stimulates the endothelial cells [16, 17]. As angiogenesis precedes changes in composition and arrangement of the ECM induce endothelial sprouting and tube formation.

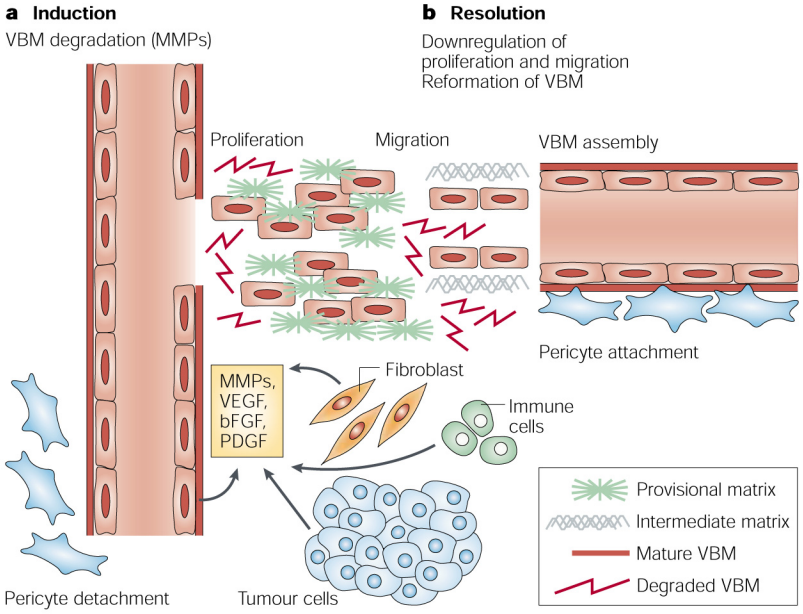

Figure 1: VEGF, PDGF and bFGF signaling coordinates the angiogenic process between pericytes, endothelial cells and tumor cells. The vascular basement membrane (VBM) is degraded by MMP into a provisional matrix. Growth factors are released from the provisional matrix or secreted by tumor and tumor stroma cells, leading to the generation of an intermediate matrix and the subsequent mature basement membrane [15].

Tumor cell dedifferentiation and metastasis

HCC arises from epithelial hepatocytes, which are highly differentiated cells with specialized metabolic function [8]. Dedifferentiation of malignant hepatocytes and dissemination from the primary tumor is a pivotal step towards vascular invasion and intrahepatic metastasis and therefore a central event in the pathogenesis of HCC. The underlying process, referred to as epithelial to mesenchymal transition (EMT), is characterized by loss of epithelial cell polarity and acquisition of a fibroblastoid phenotype that enables tumor cells to leave the epithelial cell cluster (Figure 2) [18-20]. Epithelial cells are arranged in monolayered sheets, in which cells exhibit a apical basolateral polarity [21]. While the apical side faces the lumen, the lateral side associates with the basement membrane. Collagen and laminins are the main ECM components of the basement membrane that bind lateral integrin receptors of the cell [15]. The integrin transmembrane receptors contain an extracellular domain that interacts with the ECM and an intracellular domain that is connected to the actin cytoskeleton.

Integrins therefore link the cellular cytoskeleton to the ECM and maintain the structural stability and polarity of the cell [22]. Furthermore, the cells are connected with each other by tight junctions, adherence junctions and desmosomes [19]. E-cadherin forms an intercellular belt of adhesion junctions through homotypic interaction of the extracellular domain that is characteristic for epithelial cells. The intercellular domain is connected through β- and α-catenin with actin filaments [23]. Loss of E-cadherin junctions, as well as tight junctions that are provided by occluding and claudins are central features of EMT [24]. Dissolution of cell-cell junctions leads to cytoskeletal rearrangements of the associated actin filaments into stress fibres and expression of the mesenchymal cytoskeletal protein vimentin resulting in a motile fibroblastoid phenotype. Detachment of cells from one another causes cell depolarization and changes of integrin receptor distribution and expression, facilitating cell motility and survival [22]. Concurrently with the attenuation of epithelial traits the cells undergoing EMT acquire a mesenchymal expression profile. Mesenchymal cell characteristics include an increased deposition of ECM components such as collagens, laminins and fibronection which promote cell migration through integrin mediated focal adhesion complex formation. Enhanced secretion of MMPs of EMT transformed cells facilitates matrix degradation and allows cell invasion [25].

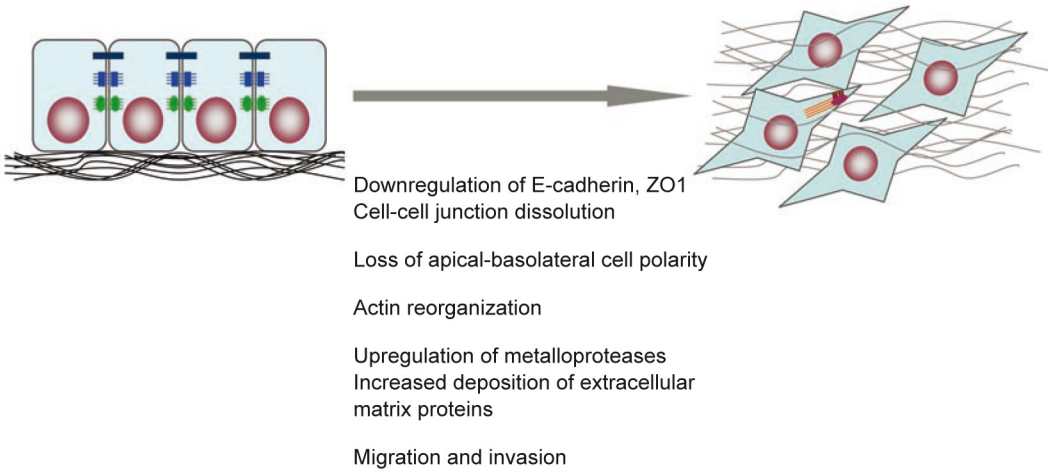

Figure 2: Epithelial to mesenchymal transition. Transition of epithelial to mesenchymal cells is characterized by the dissolution of cell-cell adherence junctions (blue), thigh junctions (black) and desmosomes (green). Cells loose the apical-basolateral polarity and aquire a mesenchymal phenotype by reorganisation of the actin cytoskeleton into stress fibres (red) [19].

EMT is a physiological event that takes place during gastrulation when epithelial cell sheets convert into mesenchymal cells to migrate and form the embryo and it is also involved in organogenesis [26]. EMT contributes to fibrotic, non-regenerative wound repair in the lung, kidneys and liver. It was shown that approximately 10% of fibroblasts in a fibrotic liver derive from hepatocytes via EMT [27]. However recent data contradict these findings leading to a controversal discussion wheter hepatic epithelial cell contribute to liver fibrosis [28]. Hepatocellular EMT is induced by the cooperation of TGF-β with active H-Ras signaling [29].

Ocogenic activation of Ras as well as enhanced secretion of TGF-β and establishment of autocrine TGF-β signaling loops are frequently observed in HCC patients and correlate with increased tumor aggressiveness and metastasis [30-33]. TGF-β signals through a complex of type I and type II serine threonine kinase receptors [34]. Type II phosphorylates the type I receptor, which in turn activates the associated transcription co-factors Smad2 and Smad3 by C-terminal phosphorylation. The activated Smads 2 and 3 are joined by Smad 4 and the Smad trimer then translocates into the nucleus where it regulates gene transcription. The Smad induced transcription response is mainly driven by three families of transcription factors including the Snail, ZEB and cHLH (Twist) family [35]. These transcription factors orchestrate the downregulation of epithelial genes such as proteins that comprise epithelial cell-cell junctions and activate the expression of mesenchymal genes including cytoskeletal proteins, ECM components and MMPs [36]. Besides carnonical Smad signaling, TGF-β also elicits Ras activated Erk MAP kinases, Rho GTPases and the PI3 kinase/Akt pathway [37]. These Smad independent pathways are important effectors that regulate cytoskeleton reorganization, cell growth and survival. TGF-β plays a dual role in tumor progression. In normal liver cells it acts as a tumor suppressor by inducing cell cycle arrest and apoptosis, whereas in malignant cells it controls EMT and dedifferentiation. The tumor promoting effect of TGF-β depends on the aberrant activation of oncogenic pathways that promote EMT and counteract the cytostatic cell response. Hyperactive Ras and downstream MAPK signaling is required for TGF-β mediated EMT [38]. MAPK signaling ehhances the TGF-β induced downregulation of E-cadherin and induction of matrix metalloproteinase expression [39, 40]. ERK kinases phosphorylate the linker region of receptor Smads and thereby modulate the TGF-ß induced canonical signaling [41]. Focal adhesion kinase (FAK) dependent activation of PI3K signaling on the other hand protects from TGF-β induced apoptosis [42, 43]. Studies of EMT kinetics in murine HCC models revealed the induction of EMT depends on MAPK signaling, whereas additional activation of PI3K signaling at a later time point is required for the maintenance of EMT [44]. The PI3 kinase mediated maintenance of EMT was found to depend on PDGF signaling [45]. Both PDGF receptors α and β were shown to be expressed upon EMT and PI3K signaling was driven by an autocrine loop of PDGF-A [46]. TGF-β and downstream PDGF signaling loops are regulated by autocrine secretion of malignant hepatocytes as well as by paracrine activation from myofibroblasts of the tumor stroma. Furthermore, PDGF signaling is essentially involved in regulating the nuclear accumulation of β-catenin [46]. In epithelial cells β-catenin is attached to the intracellular domain of the E-cadherin and links the adherence junctions to the cytoskeleton. Degradation of E-cadherin during EMT releases β-catenin into the cytoplasm. In absence of Wnt signaling cytoplasmic β-catenin is phosphorylated by GSK-β and marked for degradation. Active Wnt signaling inhibits GSK-3ß and allows the nuclear translocation of β-catenin and subsequent induction

of EMT target gene expression [12, 47]. The role of PDGF in β-catenin signaling remains to be clarified but interference with PDGF abolishes the nuclear accumulation of β-catenin [46]. Importantly, nuclear localization of β-catenin correlates with dedifferentiation of malignant hepatocytes to hepatocyte progenitors [48]. This data supports the connection between EMT induced dedifferentiation and the origin of tumor stem cells [18].

3.2. The role of Laminin B1 in tumorigenesis

Interaction of tumor cells with the ECM affects differentiation, angiogenesis, migration and cell differentiation and therefore plays an important role in tumorigenesis [15]. Laminins are the main non-collagenous component of the ECM. They assemble the basement membrane via formation of polymers and the nidogen mediated interaction with type IV collagen [49]. The heterotrimeric laminin glycoproteins are composed of several α-, β- and γ-subunits that give raise to fifthteen different laminin isoforms which are expressed in a tissue specific manner (Figure 3) [50, 51]. Six laminin isoforms contain laminin B1 (LamB1) as β-subunit, such as laminin 1 that is expressed in some adult epithelium including the liver and the laminin isoforms 8 and 10 that are expressed by endothelial cells [49].

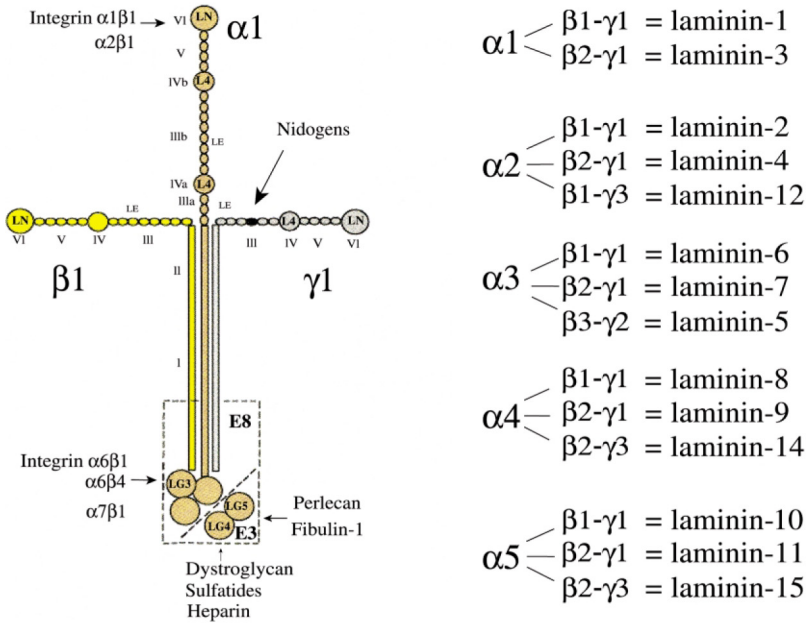

Figure 3: Composition of the heterotrimeric laminin isoforms. Diverse α-, ß- and γ- subunits compose fifthteen different heterotrimeric laminin isoforms [50]

LamB1 mediated signaling

Laminin signaling is implemented by heterodimeric integrins, α-dystroglycan, syndecans and the monomeric laminin receptor (LamR) [52, 53]. All of these receptors or coreceptors regulate signaling cascades that are essentially involved in tumorigenesis [54]. α-dystroglycan is thought to modulate Ras mediated MAPK signaling via interaction with

growth factor receptor-bound protein (Grb) 2 [55, 56]. Syndecans act as coreceptor and modulate signaling of a large variety of ligands such as VEGF, FGF and TGF-β [57-59]. The laminin isoforms that contain laminin B1 activate α1β1, α2β1, α3β1, α6β1, α7β1, α9β1and α6β4 integrin receptors [60]. Aberrant activation of integrin signaling is a frequent event during tumorigenesis and is involved in the regulation of tumor cell mitosis, migration, and proliferation and survival [61, 62]. Major integrin triggered signaling cascades that regulate cell motility include Ras/ERK, Src-like kinase/Cdc42, FAK/Rac-1and the RhoA pathway [61, 63]. Integrins enhance expression and activation of MMPs that facilitate cell migration by catalyzing ECM degradation. Interestingly, cell detachment from the ECM results in loss of integrin promoted, PI3K-, ILK-, ERK-, and JNK- mediated survival signals, leading to anoikis [64, 65]. Furthermore, cell proliferation essentially requires the combined activation of integrin and growth factor receptor mediated ERK signaling [66, 67].

The 67kDa LamR is specifically activated by interaction with LamB1 [53]. Overexpression of LamR is a frequent event in diverse carcinoma and correlates with enhanced metastatic potential and poor patient survival [68]. LamR drives laminin induced tumor cell attachment [69-71], tumor cell migration [72], invasion and agiogenesis [73] and was recently shown to be vital for tumor proliferation and survival [74]. Interestingly LamR was found to physically interact with α6ß4 integrin that is associated with carcinoma progression by contributing to apoptosis resistance, invasion and metastasis [75]. Furthermore LamR is able to remodel the structure of laminin-1 resulting in altered gene expression and increased tumor aggressiveness [76].

LamB1 in tumorigenesis

Tumor progression is characterized by deregulation of ECM-cell interactions through changes in laminin expression or their associated receptors [77]. Tumor neovascularisation, invasion and metastasis primarily depend on aberrant ECM-cell interactions [78]. For example, an angiogenic switch from laminin 9 and 11 to the LamB1 composing laminin 8 and 10 occurs in breast carcinomas [79]. Laminin 8 and 10 was found in vessel walls of carcinomas and their metastasis but not in normal tissue. Interestingly, expression of those laminins increased in a tumor progression dependent manner. Accordingly, LamB1 peptides were shown to stimulate endothelial cell adhesion, tube formation and sprouting [80]. An increasing number of studies implicate that LamB1 is essentially involved in the angiogenic process by stimulating the differentiation and vascular tube formation of endothelial cells. LamB1 peptides as well as laminin 1 were found to promote angiogenesis [81, 82]. Furthermore, laminin 1 was shown to enhance the metastatic potential of tumor cells by promoting tumor cell migration and proteinase secretion [72]. Carcinoma in situ are separated from surrounding tissue compartments by the basement membrane. Degradation and remodulation of the existing basement membrane by invading tumor cells is a

prerequisite for metastasis [83]. Dedifferentiated tumor cells as well as tumor stroma myofibroblasts secrete extracellular components and form their own matrix [83]. Tumor cell migration is regulated by the interplay of the remodulated ECM and changes in intergrin receptor expression. Acquisition of an invasive cell phenotype by EMT depends on the cooperation of integrin activated FAK signaling with receptor tyrosine kinase signaling, which together regulate Rac mediated restructuring of the cytoskeleton [54]. Besides its central role in tumor cell migration and metastasis, EMT mediated dedifferentiation is supposed to contribute to the acquisition of tumor stem cell traits [18]. Accordingly, integrins were found to drive self renewal and differentiation of stem cells [18, 84]. Thus the laminin composition of the ECM may also be involved in the regulation of stem cell differentiation. Laminin mediated ß1 integrin signaling in neuronal stem cell niches for example is required to maintain the stem cell phenotype [85]. Recently it was shown that human embryonic stem cells can be cultivated on laminin 10 without feeder cells or supplementation of any additional proteins [86]. Laminin isoforms therefore could also play a role in tumor cell dedifferentiation and formation of tumor stem cells.

LamB1 in HCC

Overexpression of LamB1 has been reported particularly in the context of HCC. Cirrhosis that frequently precedes HCC is characterized by increased ECM deposition as a result of chronic liver injury and scar formation [2, 4]. In transgenic mice, LamB1 expression was induced during transforming growth factor (TGF)-β driven liver fibrosis [87]. Proteome analysis of HCC patients revealed a rise in LamB1 levels in cirrhotic tissue compared with normal tissue and levels further significantly increase in carcinomas [88]. The elevation of LamB1 expression in HCC goes along with altered expression of beta1 integrins as well as LamR upregulation [89]. The combined deregulation of LamB1 and associated receptor expression in HCC affects multiple processes that are relevant for tumorigenesis, such as tumor cell survival, neovascularisation and migration.

3.3. Mechanisms of translation initiation

Translation initiation by ribosome scanning

Translation describes the production of proteins from mRNA matrices. The multistep process of translation is divided into initiation, elongation, termination and ribosome recycling [90, 91]. During translation initiation the AUG start codon is identified by a preinitiation complex and decoding of the open reading frame starts with a methionyl tRNA (Met tRNAi) that is specialized to read the initiation codon. Canonical initiation factors mediate the recruitment of the ribosomal subunits to the mRNA in order to form the preinitiation complex. At the start codon the preinitiation complex is joined by the 60S ribosomal subunit to generate the 80S ribosomal complex that drives protein synthesis.

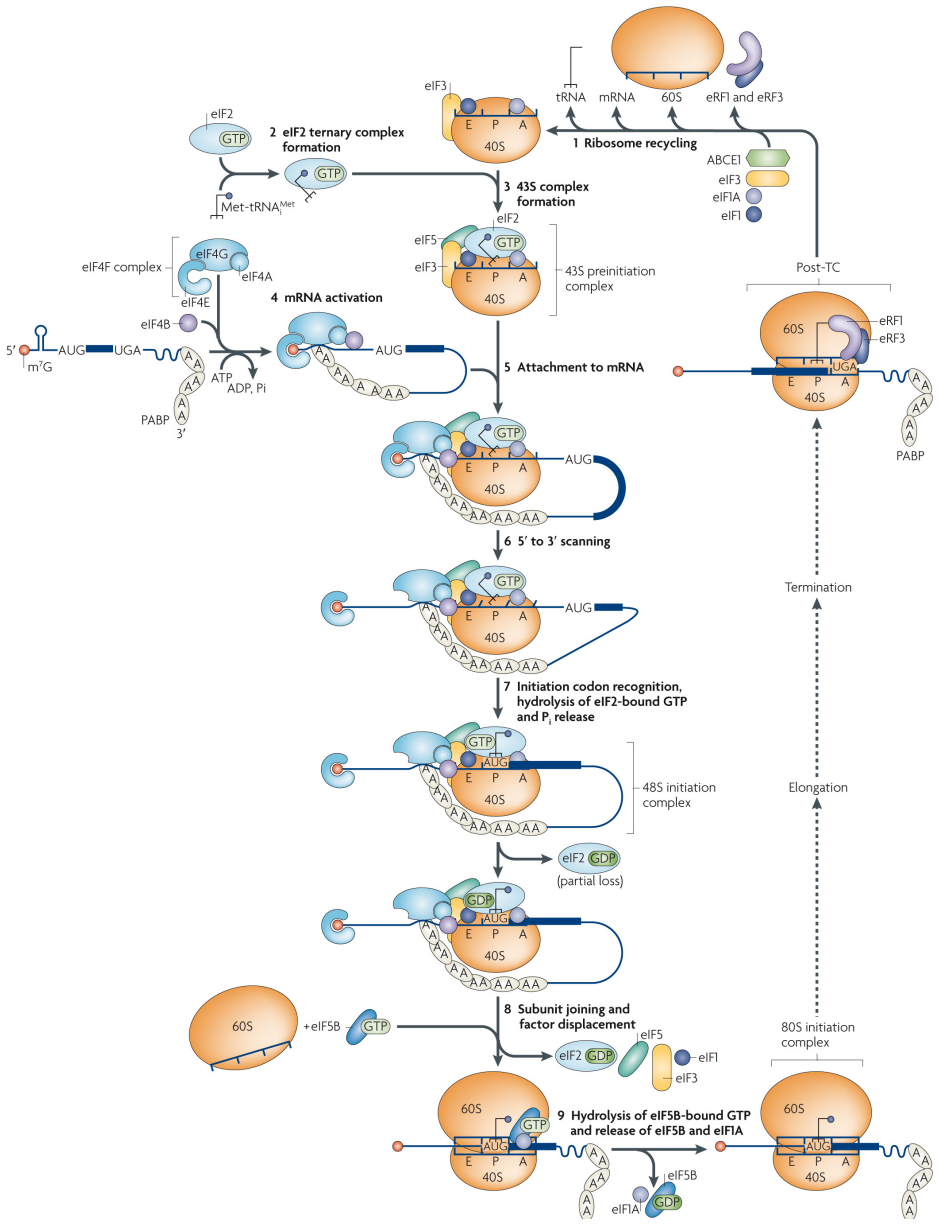

Figure 4: Translation initiation by cap dependent ribosome scanning [92].

Translation of the majority of transcripts is initiated by a mechanism referred to as ribosome scanning [92]. The preinitiation complex scans the untranslated region (UTR) until a suitable start codon is identified (Figure 4). This process starts with the recognition of the cap structure at the 5' terminus of the mRNA by eukaryotic initiation factor (eIF) 4E. eIF4E is part of the initiation factor complex eIF4F that also comprises eIF4A and the scaffolding protein eIF4G [93]. The RNA helicase eIF4A mediates the unfolding of the RNA secondary structure [94]. Unwinding of the secondary structure is assisted by eIF4H and eIF4B and promotes the formation of the initiation complex [94]. eIFs 1, 1A, 2, 3 and the Met tRNAi are preassembled to the 43S preinitation complex that is recruited to the 5' cap of the mRNA by the eIF4F complex [95]. The scaffolding protein eIF4G links the eIF4F complex to the ribosomal subunit by joining with eIF3, which is directly associated with the ribosome of the preinitation complex [96]. The whole preinitiation complex then scans downstream until the start codon is found, which is complementary to the Met tRNAi. A suitable match to the start codon triggers the arrest of the preinitation complex. Hydrolysis of GTP in the eIF2-GTP-Met tRNAi ternary complex results in release of eIF2-GDP and other eIFs and allows joining of the 60S ribosomal subunit [97]. eIF1 and eIF1A discriminate against initiation at AUG triplets in suboptimal context by blocking the full hydrolyzation of eIF2-GTP [97]. Codons that differ from the optimal context as described by Kozak et al. may be bypassed [98]. Finally, the 80S initiation complex that is competent of protein synthesis is formed.

The initation step is rate limiting to the whole translational process [99]. Translation efficiency of different mRNAs varies and depends to a great extent on features of 5'-UTR that influence the scanning process and start codon selection [100]. Certain RNA secondary structures constrain the interaction of the preinitiation complex with the mRNA or the scanning of the 5'-UTR [101]. Also decoy start codons and upstream open reading frames impede the recognition of the correct start codon. Some mechanisms to overcome these limitations of ribosome scanning have been described in viruses. For example, leaky scanning enables the preinitiation complex to skip start codons that differ from the optimal context [102]. Bypassing of secondary structures of the 5'-UTR via ribosome shunting was also observed in viruses [103]. In mammalians, translation of the stress response gene ATF4 is regulated by ribosome reinitiation [104, 105]. Reinitiation allows the preinitiation complex to resume translation at the start codon after reading of upstream ORFs without detachment. The mechanism is active during stress when eIF2 phosphorylation results in reduced rate of preinitiation complex formation and global downregulation of translation.

Besides the 5'-UTR also some features of the 3'-UTR were found to influence translation efficiency. The length of the poly(A) tail determines not only mRNA stability but regulates translation initiation as well. Poly(A) binding protein (PABP) associates with the preinitiation complex by interaction with eIF4G resulting in a loop conformation of the mRNA [106, 107].

Interaction with PABP is thought to stabilize translation initiation by enhancing the 5'cap binding of the preinitiation complex and by stimulating 60S ribosomal subunit joining.

Regulation of translation initiation

eIFs that recognize the 5' cap structure and activate the mRNA for the binding of the preinitiation complex are subject to many regulatory mechanisms (Figure 5). Protein synthesis is delimitated during stress conditions or starvation [108]. Deactivation of eIFs allows a global, fast and transient reduction of the translation rate. The main target of translation control is the cap binding protein eIF4E. Direction of the eIF4F complex to the mRNA 5'cap structure by eIF4E is required for subsequent joining of the ribosomal subunit and translation initiation by ribosomal scanning. A protein family of eIF4E binding proteins (4E-BPs) competes with eIF4G for the joined eIF4E binding site thereby inhibiting the arrangement of the eIF4F complex [109]. Interaction of 4E-BPs with eIF4E is regulated by phosphorylation. Hypophosphorylated 4E-BPs have a high binding affinity, whereas phosphorylated 4E-BPs interact only weakly with eIF4E [110]. 4E-BP phosphorylation is performed by the mammalian target of rapamycine (mTOR) kinase, which is a part of the PI3K/Akt signaling cascade [110]. The PI3K/Akt pathway is triggered by diverse tyrosine kinase receptors in response to growth factors or intergins. mTor has several phosphorylation targets that are involved in the cap recognition process including eIF4G and S6 kinases (S6K) [111]. S6K in turn activates eIF4B resulting in enhanced interaction with eIF3. Another substrate of S6K is Pdcd 4, which is ubiquitinylated and degraded upon phosphorylation [112]. Pdcd 4 acts as tumor suppressor by binding to and inactivating the RNA helicase eIF4A. Another pathway essential for translation control is MAPK signaling, which regulates phosphorylation of the cap binding protein eIF4E by Mnk 1, Mnk 2 and p38 kinases [113]. Mnk 1 and Mnk 2 are part of the MEK/ERK cascade, while p38 is downstream of a second independend MEK signaling cascade [114]. Both pathways are regulated by Ras in response to growth factor signaling. Deactivation of eIF4E by impaired phosphorylation or increased 4E-BP binding leads to reduced cap dependent phosphorylation [115]. Another mechanism that specifically regulates cap dependent translation is mediated by micro RNAs (miRs). miRNAs are part of a major post transcriptional control mechanism of gene expression. Estimated 10.000 mRNAs of the transcriptome are subject to miR regulation [116]. miRs are 22nt short double stranded RNAs that are integrated into a protein complex referred to as RNA induced silencing complex (RISC) [117]. The RISC complex recognizes complementary RNA sequences within the 3'-UTR and impairs expression of a specific mRNA subset [118]. Different mechanisms have been proposed for miR mediated regulation of gene expression. Recently, it was reported that the RISC complex competes with eIF4E for the cap structure and thereby inhibits cap-dependent but not IRES-mediated translation [119, 120]. Repressed mRNAs accumulate in P-bodies, which contain enzymes for RNA

degradation [121]. miRs and their target mRNAs were also found to accrue in stress granules indicating that miRs are involved in the regulation of the cellular stress response [122]. Another important stress induced regulatory mechanism affects not only the cap recognition but the whole translation initiation process. The PI3K/Akt signaling is regulated by amino acid availability and the cellular oxygen level. PI3k/Akt mediated stress response leads to phosphorylation of eIF2 at the α subunit. Phosphorylated eIF2α is a competitive inhibitor of eIF2B, which mediates the hydrolysis of eIF2-GDP during translation initiation [123]. Impaired release of eIF2-GTP from the initiation complex results in a reduction of the translation rate. Four different kinases that are triggered by different kinds of cellular stress phosphorylate eIF2α at the same residue leading to a similar stress response [124]. PKR is activated by double stranded RNA during viral infections, PERK reacts to unfolded proteins in the endoplasmatic reticulum, HRI is activated by heme deprivation and amino acid starvation triggers GCN2 [124].

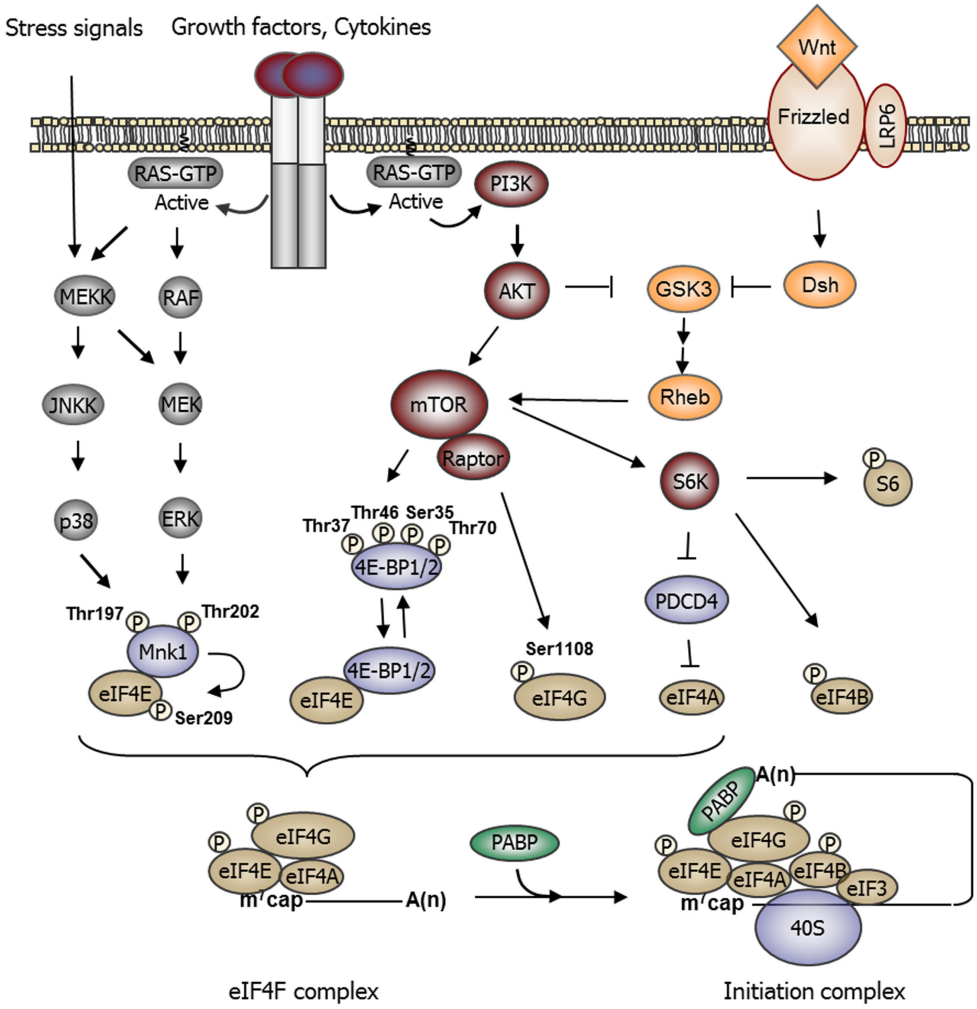

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Figure 5: Regulation of translation initiation. The activity of translation initiation factors is regulated by the MAPK and PI3K pathway in response to growth factor signaling or cellular stress. (adapted from [125])

Translation initiation by internal ribosome binding

Certain mRNAs are capable to circumvent the common translation initiation mechanism of ribosome scanning. A structural motif of the RNA referred to as internal ribosome entry site (IRES) that is located in the 5'-UTR directly upstream of the start codon allows straight binding of the preinitation complex [126]. Translation is initiated immediately without scanning. Therefore IRES mediated translation is independent of the cap structure and insensitive to regulatory mechanisms that reduce global protein synthesis by deactivation of the cap binding protein eIF4E [127]. This alternative form of translation initiation was initially described in poliovirus and encephalomyocarditis virus [128, 129]. Polioviruses shut off protein synthesis of infected host cells. The viral 2A protease was found to cleave the eIF4E binding site of the scaffolding protein eIF4G, preventing the assembling of the preinitation complex at the 5' cap structure of the mRNA [130]. Consequently, the first cellular IRES element from the immunoglobulin heavy chain binding protein (BIP) was found due to its continued translation in poliovirus infected cells [131, 132]. A small but growing number of cellular mRNAs that confer IRES mediated translation have been described since [133, 134]. They are expressed during physiological conditions that require a transient downregulation of translation such as cell cycle progression, differentiation, cellular stress and apoptosis [108, 135]. Cellular or viral IRESs elements generally display variations in their secondary structure, sequence composition and length [126, 136]. Viral IRESs are divided into subtypes by structural similarities [136]. Secondary structure and isolated motifs of the primary sequence are conserved among species and viral subtype. The set of initiation factors that are required for internal initiation depends on the IRES type. IRES of picorna viruses need the complete preinitiation complex except eIF4E, but they don't have to compete with the host cell for eIFs since cap dependend translation is shut off by 2A protease [130, 137]. Picornavirus IRESs are long and highly structured. Their initiation is stimulated by IRES transacting factors (ITAFs) that are thought to stabilize the complex conformation of the secondary structure [138]. In contrast, the IRES of hepatitis C virus don't require the eIF4F complex at all and internal initiation is mediated by eIF3 in cooperation with eIF2 or eIF4B [139, 140]. The ability to use eIF4B instead of eIF2 may enable translation when eIF2 is deactivated by the host cell. Notably, the cricket paralysis virus IRES initiates translation independent of all eIFs and does not even require tRNAi [141]. The IRES mimics the anticodon stem loop of the tRNAi and contains also a start codon. In contrast to relatively compact viral IRES structures, cellular IRES elements can be composed of multiple short motifs that are arranged over a longer stretch of the 5'-UTR [142]. Regions between these IRES motifs are not essential for IRES activity. Secondary structures of cellular IRES are less well studied and show a greater diversity than viral IRES [143]. A common IRES structure motif was not yet identified [144]. There seem to be several distinct mechanisms for

ribosome recruitment and translation initiation. Most cellular IRES do not require eIF4E or intact eIF4G but depend on eIF4A and eIF3, suggesting that the IRES element needs to be remodeled for initiation. Recently, the Src- kinase mRNA was reported to contain the first IRES element that seems to be able of direct binding to the 40S ribosomal subunit [145]. Several ITAFs were found to support cellular IRES activity including for example the multifunctional La protein, polypyrimidine tract binding protein (PTB), poly(A) binding protein (PABP) and different heterogeneous nuclear ribonucleoproteins (hnRNPs) [127]. ITAFs are believed to enhance the interaction between the IRES structure and components of the translation initiation complex. The exact mechanism is not known but ITAFs are thought to remodulate partial motifs of the IRES structure to promote ribosome binding or to function as initiation factors that mediate ribosome recruitment [146]. IRES mediated translation of Apaf-1 is enhanced by upstream of N-Ras (UNR), which promotes the binding of the second ITAF PTB [147]. Both ITAFs unfold the RNA secondary structure and these changes of RNA conformation allow the ribosome recruitment. Other IRES were found to be capable of direct base pairing with the rRNA by a Shine-Delgarno like mechanism [148]. A prominent example for well understood modular IRES is c-myc. The secondary structure of the c-myc IRES was predicted by enzyme mapping [149]. Mutation experiments revealed that pseudoknots in the first domain seem to attenuate IRES activity, suggesting that ITAFs which bind to this domain open this pseudoknot structures to enhance IRES translation [149]. The main IRES activity was tracked down to a small region of 50 nts [150]. Studies showed that the sequence and not the structure of this region is important for IRES activity [150]. Together these data indicate that cellular IRES are not compact motifs of the RNA secondary structure but rather involve a number of small regions with a specific primary or secondary structure that interact with ITAFs or components of the translation machinery.

3.4. Physiological significance of internal translation initiation

ITAFs regulate cell fate decisions

IRES mediated translation promotes initiation of cellular mRNAs with highly structured 5'-UTR, which are less efficiently translated under normal physiological conditions [151]. More importantly, IRES translation is enhanced when cap dependent translation is compromised, allowing the translation of important regulatory factors during cellular stress conditions, mitosis, hypoxia or nutrient deprivation [152]. It is believed that IRES translation becomes more competitive for available translation factors and ribosomes when cap dependent translation is impaired [108]. Many mRNAs that confer IRES translation encode factors that protect cells from stress or apoptosis and therefore play an important role in cell fate decisions [153]. It has been shown that different mRNA subsets are induced in response to diverse physiological stress conditions IRES translation. This differential activation of IRES translation is regulated by ITAFs, which bind selected IRES structures and positively modulate their activity (Table 1). Most ITAFs belong to the group of hnRNPs that are known to shuttle between the nucleus and cytoplasm [154]. The ITAF compartimentalization is regulated in response to cellular conditions that impair cap dependent translation. The exact mechanism how subcellular localization of ITAFs affects IRES translation is not known. One hypothesis proposes the translocation of ITAFs to the cytoplasm where they interact with target mRNAs and support translation [154]. Another theory suggests that ITAFs bind their responding mRNAs in the nucleus and thereby sequester it from the translation machinery until they translocate into the cytoplasm [155]. Signaling events that regulate the cytoplasmic translocation have not yet been identified for most ITAFs. In some cases such as for hnRNP A1, stress induced phosphorylation was shown to trigger cytoplasmic localization and enhanced binding affinity for IRESs [156, 157]. Despite the involvement of IRES translation in the regulation of various physiological and pathophysiological conditions there is only moderate progress in the clarification of underlying mechanisms. A major problem of characterizing signaling events that regulate IRES translation arises from the complexity of mechanisms that control internal initiation. Great variations in the requirement of eIFs and ITAFs for initiation of diverse IRES elements suggest different underlying mechanisms. Moreover, the majority of proteins that mediate internal initiation are master regulators of proliferation, survival or apoptosis [134]. Their expression is tightly regulated on multiple transcriptional and translational levels. Recent studies provide evidence for the significance of IRES translation in regulating angiogenesis, mitosis and cell survival.

Table 1: List of ITAFs and their mRNA targets [127]

ITAF/trans-*acting factor*	*IRESes interacts with*
PTB (hnRNP1)	EMCV, FMDV, TMEV, PV1, HRV, HCV, HAV, human T-lymphotrophic virus type 1, Apaf-1, IGF-IR, BAG-1
N-PTB (neuronal PTB)	TMEV, Apaf-1
La	EMCV, HCV, PV1, human T-lymphotrophic virus type 1, HIV-1 (gag RNA), XIAP, Bip/Grp78, coxsackievirus B3
Unr	HRV, Apaf-1
ITAF45	FMDV
HnRNPE2 (PCBP2)	PV1, HRV, Coxsackievirus B3, c-*myc*
HnRNPE1 (PCBP1)	PV1, c-*myc*
HnRNPC1/C2	PDGF2/c-sis, XIAP, c-*myc*
HnRNPL	HCV
HnRNPK	c-*myc*
DAP5	DAP5, c-*myc*, Apaf-1, XIAP
GAPDH	HAV
Nucleolin	HRV, PV1
ELAV/Hu	p27
Ribosomal protein S9	HCV, CSFV
Ribosomal protein S5	HCV

Data from the IRES database at http://ifr31w3.toulouse.inserm.fr/IRESdatabase/

IRES mediated translation during angiogenesis

Angiogenesis describes the formation of new or growth of preexisting vessels. During wound repair angiogenesis plays an important role in meeting the increased oxygen and nutrient supply of regenerating tissue [17]. The same mechanism is activated in tumor cells and plays an essential role in tumor growth and metastasis [17]. VEGF regulates proliferation, proteolytic activity and migration of endothelial cells and therefore is the key mediator of angiogenesis [158]. VEGF expression is tightly regulated at diverse levels including transcription and alternative splicing. Recent studies suggest a pivotal role for IRES regulated translation during angiogenesis. The VEGF-A mRNA harbours two IRESs. The upstream IRES-B translates a protein isoform (L-VEGF) that is 180 nts amino acids longer than the isoform translated by the downstream IRES-A [159]. L-VEGF is cleaved into a C-terminal fragment that is secreted and a intracellular N-terminal fragment. The proteolytic cleavage generates the VEGF121 isoform that is predominantly expressed during tumorigenesis and processes higher angiogenic activity than other VEGF isoforms [159]. IRES-A mediated translation generates secreted VEGF isoforms only [159]. Interestingly IRES-B but not IRES-A translation is impaired by microRNA-16 (miR-16) [160]. Translational repression by miR-16 does not affect mRNA stability or VEGF transcription. The miR-16 binding site is located within the 3'-UTR of VEGF-A mRNA near the ITAF binding site for hnRNP L, which might be involved in the miR-16 induced reduction of IRES translation [161]. miR-16 therefore might function as a repressor of hypoxia induced of VEGF IRES translation and subsequent angiogenesis.

IRES mediated translation during mitosis

IRES translation of CDK-1, CDK-11 and BCL-2 was found to be regulated during mitosis. Modulation of IRES mediated translation plays a critical role in cell cycle progression during the G2/M phase when cap dependent translation is compromised (Figure 6) [162, 163]. IRES translation of CDK-11/p58 that is involved in spindle formation was shown to be regulated by

an ITAF signaling cascade. The pathway starts with cytoplasmic accumulation of the ITAF hnRNP C1/C2, which enhances IRES driven translation of unr [164]. Unr is another ITAF that in turn positively modulates IRES translation of p58 [164]. Activation of unr is regulated by a negative feedback loop that is mediated by unr itself and is controlled by cytoplasmic accumulation of the PTB ITAF [164]. The whole signaling mechanism is based on IRES translation and therefore allows a precise modulation of p58 levels under conditions when cap dependent translation is severely repressed. Furthermore, IRES driven protein synthesis of CDK-1 and BCL-2 was found to prevent cell apoptosis during mitosis [165, 166]. Proteolytic cleavage of the eIF4G family member DAP5 during apoptosis results in a truncated protein that positively modulates IRES translation of CDK-1 and BCL-2 and promotes cell survival [166]. Additionally, CDK-1 expression during mitosis is regulated by AKT mediated phosphorylation of hnRNP A1 [157]. Phosphorylation of hnRNP A1 renders the ITAF inactive and prevents the enhancement of CDK1 IRES translation. Also cyclin D1 IRES translation is activated during mitosis by cytoplasmic translocation of the La ITAF [167].

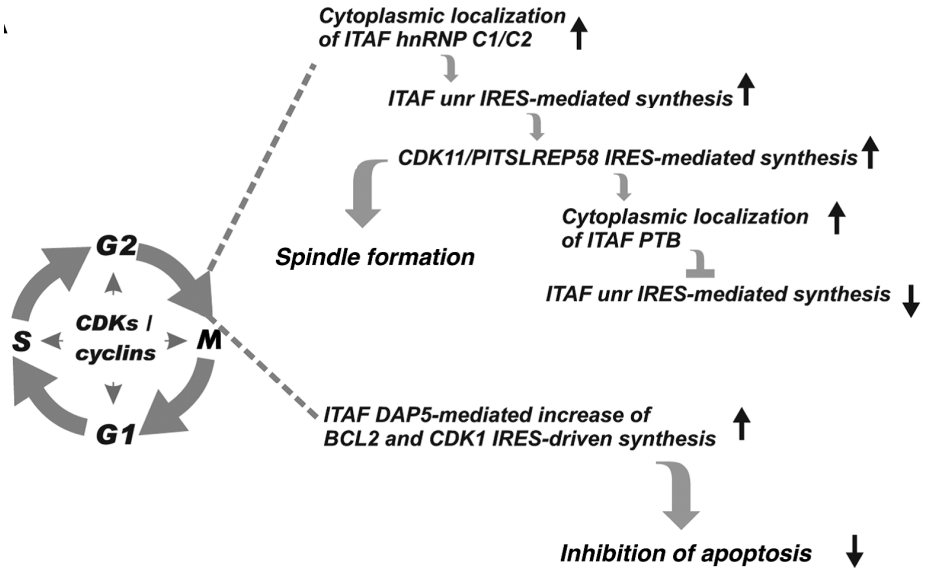

Figure 6: ITAF signaling during cell cycle progression. IRES translation of CDK-11 is during G2/M transition is regulated by an ITAF signaling cascade. IRES mediated synthesis of BCL-2 and CDK-1 prevents apoptosis during cell cycle progression [162].

3.5. Translation regulation during tumorigenesis

Dual translation initiation mechanism of oncogenes

The central pathways that are deregulated during tumor progression also control translation activity. MAPK and PI3K signaling cascades drive tumorigenesis and regulate translation in multiple ways [168]. Aberrant activation of PI3K Akt signaling in response to growth hormones or through PTEN mutations is frequently observed in human cancers resulting in phosphorylation of mTOR complex 1 (mTORC1) [169]. Active mTORC1 promotes cap dependent translation through phosphorylation of eIFG1 and inactivation of 4E-BPs [170]. Other downstream effectors of mTORC1 are the S6 ribosomal protein kinases S6K1 and S6K2 [170]. S6K1 facilitates tumor proliferation by promoting eIF4A helicase activity through degradation of PDCD4 and activation of eIF4B [111, 112]. mTORC1 is also activated by ERK, the effector kinase of the Ras MAPK pathway [170]. Moreover, the promotion of tumorigenesis by Ras MAPK signaling depends on stimulation of translation through phosphorylation of eIF4E by ERK regulated MNK1 and MNK2 [171]. Deregulation of translation during tumorigenesis primarily affects those mRNAs that contain an IRES element. Increased availability of initiation factors that are required for the ribosome scanning mechanism, such as the cap binding protein eIF4E, eIF4G or the RNA helicase eIF4A stimulates cap dependent translation of a small mRNA subset that are usually translated with a low efficiency due to the IRES secondary structure [172]. Physiological conditions such as amino acid starvation, growth arrest, apoptosis or hypoxia that occur during cancer progression result in transient downregulation of cap dependent translation [152]. Important key factors that are involved in the regulation of these conditions contain an IRES element that initiates translation when the cap binding complex is deactivated. Interestingly many of these key regulatory factors are considered oncogenes that drive tumorigenesis [134]. The dual mechanism of cap dependent and IRES mediated translation allows a permanent enhanced translation of important factors such as c-myc, VEGFA or BCL-2 [173].

Deregulation of the mRNA 5' cap-structure recognition

Tumors frequently develop an enhanced ability to confer cap dependent translation by upregulation of eIF4F activity through diverse mechanisms [174]. The cap binding protein eIF4E is overexpressed in various cancers and is strongly associated with decreased patient survival [175]. eIF4E is required for the oncogenic activity of H-Ras and for myc induced transformation of primary fibroblasts [176, 177]. Furthermore, NIH-3T3 cells can be transformed by ectopic eIF4E overexpression [178]. The tumor promoting activity of eIF4E is based on the increased preferential translation of specific mRNAs that have a highly structured 5'-UTR [172]. These mRNAs are thought to be less efficiently translated due to reduced competitiveness in the ability to recruit or retain the eIF4F complex or to unwind the secondary structure during ribosome scanning. mRNAs with highly structured 5'-UTRs that

translationally upregulated in response to increased eIF4E activity encode proteins involved in angiogenesis (VEGF A) and cell cycle progression (myc, cyclin D (CCND) 1, ornithine decarboxylase (ODC) 1, FGF2) [179, 180]. Recently, a study revealed increased phosphorylation of eIF4E in 67% of various human cancers compared to 30% in adjacent noncancerous tissue [181]. The phosphorylation grade correlated with tumor aggressiveness in many but not all cancer types. A possible reason for this could be the 4E-BP mediated counter regulation opposing eIF4E activity. Hypophosphorylated 4E-BP competitively binds eIF4E preventing the formation of the eIF4F cap binding complex. Deactivation of 4E-BP or decreased levels of 4E-BP 1 and 2 were shown to correlate with increased cancer progression and reduced survival [182, 183]. While increased availability of eIF4E leads to enhanced cap dependent translation of mRNAs that contain an IRES element, elevated abundance of eIF4G results in stronger IRES mediated translation [184]. Transcripts that contain an IRES element can utilize the eIF4G to drive IRES translation in absence of eIF4E. Cap dependent translation requires the cap binding protein eIF4E and does not profit from the sole elevation of eIF4G abundance. High levels of eIF4G 1 in the absence of deregulated eIF4E or 4E-BP were found in inflammatory breast cancer and drives translation of angiogenic and survival factors [185]. Hypoxia induced overexpression of eIF4G1 and 4E-BP1 functions as switch from cap dependent to IRES mediated translation in animal models of breast cancer.

Deregulation of the mRNA ribosome interaction

The eIF3 complex is composed of multible subunits, but only a few are essentially required for translation initiation [186]. They form a core complex that mediates the bridging function between eIF4F and the ribosome. Other subunits are thought to promote the interaction of eIF3 with specific mRNAs [186]. eIF3 subunits therefore play diverse roles in cancer. The oncogenes eIF3a, eIF3c and eIF3h are frequently overexpressed in human cancers and ectopic expression of these subunits was found to induce the transformation of NIH-3T3 cells [187]. Increased expression of eIF3a, eIF3c, and eIF3h leads to the activation of protein synthesis and to elevated translation rates of mRNAs that usually have a low translation efficiency including myc, FGF 2, Odc 1 and Ccnd1 [187]. Furthermore, eIF3a was found to downregulate the cell cycle inhibitor p27 (CDKN1B), thereby promoting cell proliferation [188]. In contrast, eIF3e might act as tumor suppressor by inhibiting cell growth and promoting apoptosis. eIF3e was found to be downregulated in breast and lung carcinomas [189].

Uncoupling of stress induced translation impairment

The role of eIF2 phosphorylation in cancer is complex and depends on the disease stage. At early disease stages phosphorylation of eIF2 reduces protein synthesis and allows cells to adapt to hypoxic situations [190, 191]. Phosphorylation of eIF2 during hypoxia is mediated by

PERK and counterregulated by the eIF2 phosphatase GADD34 [192]. Activation of PERK can cause tumor quiescence. Therefore, it is likely that PERK is uncoupled from translation regulation during tumor progression. An example for this mode of action was described in multiple myeloma cells that acquire quiescence by activation of PERK and encounter the downregulation of protein synthesis through activation of GADD34 [193]. PKR seems also to antagonize tumor progression. Elevated PKR levels were found to be associated with carcinoma cell differentiation [179, 194]. Concordantly, more aggressive types of brain cancer have elevated levels of eIF2, which might provide a way to escape the downregulation of protein synthesis [195].

3.6. References

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4. Aims

Interaction of tumor cells with the ECM plays an important role in cell invasion and metastasis. Especially LamB1 is involved in the regulation of tumor cell migration and angiogenesis. Recent results provide first evidence that the 5'-UTR of the LamB1 mRNA contains an IRES motif which directs translational upregulation of LamB1 expression during EMT and malignant progression of epithelial hepatocytes. Therefore IRES driven translation of LamB1 and its regulation by ITAFs might be a molecular mechanism of particular relevance for tumorigenesis.

Project aims are based on the hypothesis that the

(i) IRES element within the 5'-UTR of the LamB1 transcript is essential for cap-independent translation

(ii) increased expression of LamB1 in mesenchymal derivatives of carcinoma cells primarily results from enhanced IRES mediated translation of the LamB1 transcript

(iii) minimal IRES sequence of LamB1 interacts with ITAFs which allow efficient cap-independent translation of LamB1

(iv) interaction of ITAFs with the IRES of LamB1 is regulated during tumor progression

(v) translational upregulation of LamB1 expression during EMT depends on the cooperation of Ras and TGF-ß signaling

The aim of this work is the identification of the IRES motif within the 5'-UTR of the LamB1 transcript and its interacting ITAFs that regulate IRES-driven translation during tumor progression. Subsequent aims involve the dissection of signaling pathways that regulate ITAFs and control IRES-mediated translation of LamB1.

specific aims include to

(i) evaluate transcriptional mechanisms that might be additionally involved in the regulation of LamB1 protein expression during tumorigenesis

(ii) determine the minimal IRES element that is required for cap-independent translation of the LamB1 transcript

(iii) identify ITAFs that interact with the LamB1 5'-UTR and modulate IRES mediated translation efficiency

(iv) identify the signaling pathways that regulate ITAFs involved in IRES driven translation of LamB1 by interfering with TGF-ß and Ras signaling

(v) evaluate the ratio between cap-dependent and IRES-dependent translation of LamB1 during EMT

5. Experimental setup:

- Regulation of LamB1 translation during EMT was studied in a murine HCC model system based on immortalized hepatocytes that were isolated from a $p19^{ARF(-/-)}$ mouse (MIM1-4, Figure 7). These cells become tumorigenic after retroviral transmission with oncogenic Ha-Ras (MIM-R). Epithelial MIM-R cells undergo EMT in response to TGF-ß treatment by the cooperation of oncogenic Ras and TGF-ß signaling. They acquire a mesenchymal phenotype, establish an autocrine TGF-ß loop and become metastatic (MIM-RT).

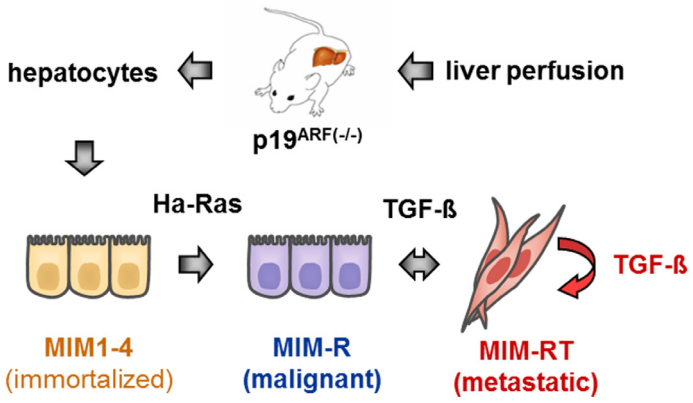

Figure 7: murine model of hepatocellular EMT.

- To investigate the involvement of transcriptional mechanisms in the regulation of LamB1 during tumor progression, the expression of mRNA isoforms in different human HCC cell lines was analyzed by Northern blotting and RACE-PCR of the 5'- and 3'-UTR.
- A bicistronic reporter system was employed to determine the IRES activity of LamB1 (Figure 8). The LamB1 5'-UTR was placed between two reporter into a bicistronic vector. Translation of the first reporter is cap-dependent while cap-independent translation of the second reporter is mediated by the IRES element within the 5'-UTR of LamB1. The minimal XIAP IRES element was used as positive control for IRES activity and the empty vector served as negative control.

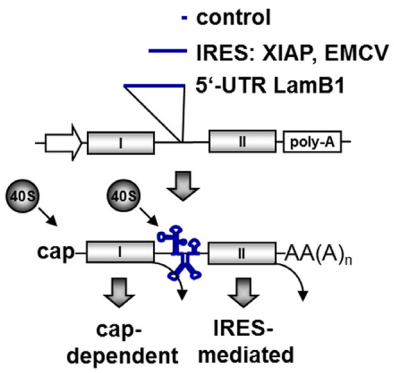

Figure 8: bicistronic reporter system

- The LamB1 5'-UTR was screened for cryptic promoter or splice sites in order to verify the IRES activity that was detected with the bicistronic reporter system. Cryptic promoter or splice sites may result in truncated transcripts of the second reporter that could be mistaken for IRES activity. The ratio of first and second reporter mRNA was quantified by qPCR to detect aberrant transcripts (Figure 9). The LamB1 5'-UTR was cloned upstream of a firefly luciferase reporter and the CMV promoter was removed from the vector in order to exclude the presence of a cryptic promoter within the 5'-UTR (Figure 10).

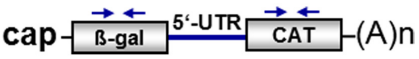

Figure 9: qPCR for truncated transcripts

Figure 10: cryptic promoter assay

- The minimal IRES element that is required for full IRES activity of LamB1 was determined by performing sequence deletions of the 5'-UTR in steps of 50 nts starting from either the 5' or 3' terminus. IRES activities of the truncated fragments were analyzed with bicistronic vectors.
- ITAFs that interact with the LamB1 IRES were identified by streptavidin-tethered RNA affinity purification (Figure 11). The LamB1 5'-UTR was cloned into the pTrap vector that contains a T7 promoter and two S1 aptameres which specifically bind steptavidin. *In vitro* transcription resulted in a fusion transcript of the 5'-UTR and S1 aptameres. The RNA transcript was applied to magnetic streptavidin beads and incubated with cell lysate. Beads were washed and the RNA protein complex was eluted with biotin. Proteins that specifically associated with the RNA were analyzed by SDS-PAGE and silver staining and further identified by Western blotting. Results were verified by performing a RNA protein immunoprecipitation with the identified ITAF (Figure 12). RNA interacting proteins were *in vivo* crosslinked by UV irradiation und precipitated with an antibody against the ITAF. The precipitated RNA protein complex was isolated, treated with DNase I and proteinase K and used for RT-PCR.

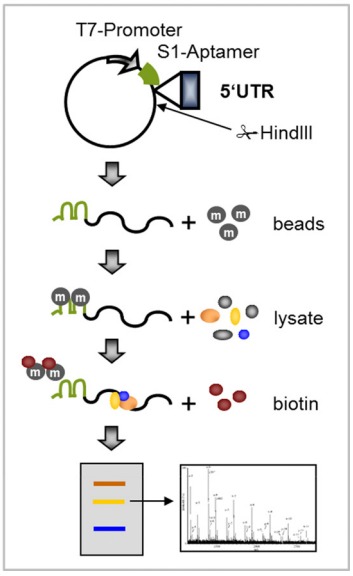

Figure 11: RNA affinity purification

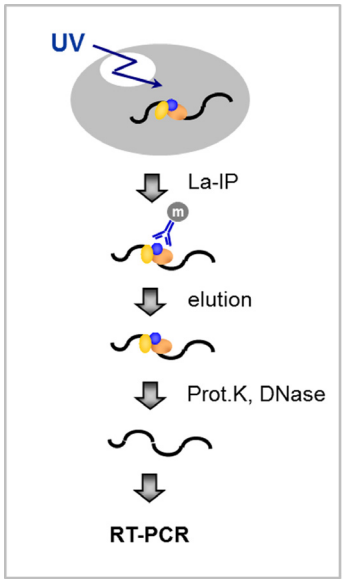

Figure 12: RNA Immunoprecipitation

- Genetically modified cell lines and pharmalogical inhibitors were used to study the signaling pathway involved in the regulation of the La ITAF that was found to be associated with the LamB1 IRES (Table 2). To impair PDGF signaling, a dominant negative (dn) version of the PDGF receptor α was introduced into MIM-R cells by retroviral transmission resulting in MIM-RdnP. Murine hepatocytes that express either oncogenic Ha-Ras (MIM-R), a S35-V12 Ras mutant that selectively activates MAPK (MIM-S35) or a C40-V12 Ras mutant that activates PI3K signaling (MIM-C40) were employed to further dissect the pathways involved in La regulation. These cell lines undergo EMT (MIM-RT, MIM-RTdnP, MIM-ST) or acquire a scattering phenotype (MIM-CT) in response to TGF-β. To verify and analyze the regulation of LamB1 IRES translation by La, cell lines with a stable knockdown of La were generated by retroviral transmission of shRNA. Pharmacological inhibitors against TGF-β, MAPK or PI3K signaling were used.

Table 2: Cell lines established to analyze the pathways involved in the regulation of La.

MIM-RdnP	MIM-RTdnP	dnPDGF receptor
MIM-RshLa	MIM-RTshLa	shRNA against La
MIM-C40	MIM-CT	PI3K activation
MM-S35	MIM-ST	MAPK activation

6. Conclusions

The data reveals that translational upregulation of LamB1 during hepatocellular tumor progression is directed by an IRES element located within the 5'-UTR of the transcript. IRES translation of LamB1 is enhanced by the ITAF La that accumulates in the cytoplasm upon EMT and binds the minimal IRES motif. Cytoplasmic accumulation of La is regulated by PDGF signaling via the cooperation of the MAPK and PI3K pathway. EMT transformed cells establish an autocrine TGF-β loop that triggers PDGF signaling leading to a persistent cytoplasmic localisation of La which results in enhanced IRES mediated translation of LamB1 (Figure 13).

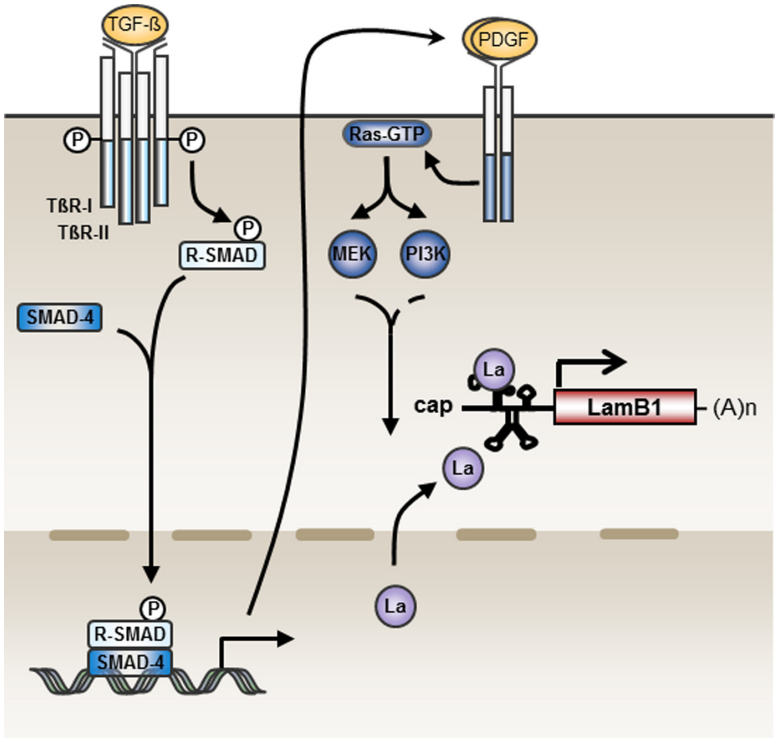

Figure 13: A model for the regulation of IRES mediated LamB1 translation upon hepatocellular EMT.

In detail the results allow following conclusions:

(i) LamB1 is not regulated by expression of alternative mRNA isoforms during hepatocellular tumorigenesis. Transcript variants that may arise from alternative splicing of the 5'-UTR or polyadenylation of the 3'-UTR could not be detected in human HCC cell lines.

(ii) The LamB1 5'-UTR drives cap-independent, IRES mediated translation. IRES activity was verified by excluding the presence of cryptic promoter or splice sites within the 5'-UTR.

(iii) The IRES structure within the LamB1 5-'UTR is localized between -293 and -1 nts upstream of the start codon. This is the minimal sequence that is required for full cap independent translation activity of the 5'-UTR.

(iv) The minimal IRES sequence contains a motif that regulates IRES translation in response to TGF-β and starts between -241 and -180 nts upstream of the start codon.

(v) LamB1 expression is translationally upregulated upon TGF-β induced EMT. Increased LamB1 levels result from enhanced IRES translation. LamB1 mRNA levels remain unchanged and cap-dependent translation rates are not affected by EMT.

(vi) The ITAF La interacts with the LamB1 5'-UTR *in vitro* and *in vivo* and binds to the minimal IRES sequence. Increased levels of La associate with the minimal IRES sequence in EMT transformed cells.

(vii) The presence of La enhances LamB1 IRES activity *in vitro*. Stable knockdown of La reduces bicistronic LamB1 IRES activity as well as endogenous LamB1 levels in EMT transformed cells.

(viii) La levels are not regulated upon EMT, but La accumulates in the cytoplasm upon short term TGF-β treatment as well as in EMT transformed cells.

(ix) PDGF signaling is activated by TGF-β upon EMT and regulates the subcellular localization of La.

(x) The synergistic MAPK and PI3K signaling downstream of PDGF might account for triggering the cytoplasmic accumulation of La.

(xi) Besides the persistent PDGF mediated enhancement of LamB1 IRES translation in EMT transformed cells, short-term treatment of epithelial cells with TGF-β additionally results in transient PDGF receptor expression and cytoplasmic localization of La and a slight upregulation of LamB1 IRES translation. This activation of cap-independent translation might be a result of cellular stress.

Concluding remarks:

Expression of LamB1 in malignant hepatocytes during stress conditions and the PDGF driven activation of IRES translation in metastatic tumor cells suggests an important role of LamB1 in tumorigenesis. LamB1 was shown to be involved in the regulation of tumor cell migration by integrin and LamR activation. Enhanced cap independent LamB1 translation during EMT might therefore be relevant for tumor metastasis. Furthermore, the role of IRES mediated LamB1 translation in angiogenesis remains to be investigated. Neovascularisation of tumors is induced by hypoxia and involves the IRES mediated translation of HIF-1α, VEGF and PDGF. Interestingly, LamB1 was found to regulate endothelial tube formation during vascularization. The PDGF triggered enhancement of IRES mediated LamB1 translation would allow its expression during stress conditions such as hypoxia. Thus, PDGF signaling would be a suitable regulatory mechanism for the activation LamB1 translation during tumor angiogenesis.

7. Manuscript I

The crosstalk of RAS with the TGF-β family during carcinoma progression and its implications for targeted cancer therapy

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7.1. Abstract

Both RAS and transforming growth factor (TGF)-β signaling cascades are central in tumorigenesis and show synergisms depending on tumor stage and tissue context. In this review we focus on the interaction of RAS subeffector proteins with signaling components of the TGF-β family including those of TGF-βs, activins and bone morphogenic proteins. Compelling evidence indicates that RAS signaling is essentially involved in the switch from tumor-suppressive to tumor-promoting functions of the TGF-β family leading to enhanced cancer growth and metastatic dissemination of primary tumors. Thus, the interface of these signaling cascades is considered as a promising target for the development of novel cancer therapeutics. The current pharmacological anti-cancer concepts combating the molecular cooperation between RAS and TGF-β family signaling during carcinoma progression are critically discussed.

7.2. Introduction

Carcinoma Progression

The malignant growth and aberrant differentiation of epithelial tissues, collectively designated as carcinoma, develop by a multistep process. Most frequently, carcinoma progression leads to metastatic spread of primary tumor cells and to colonization in distant organs [1,2]. Six or even seven properties have been defined which allow neoplastic cells to acquire and to increase malignancy [3-5]. Among these mechanisms are the (i) unlimited replicative potential, (ii) evasion of apoptosis, (iii) self-sufficiency in growth signals, (iv) ability to develop blood vessel for neoangiogenesis, (v) insensitivity against anti-growth factors such as transforming growth factor (TGF)-β, (vi) tissue invasion and metastasis, and (vii) cancer-related inflammation supported by the tumor-stroma. The sequence of genetic and epigenetic events resulting in the malignant progression of carcinoma cells appears to be random in a stochastic point of view, but is most frequently accompanied by the loss of functional differentiation and the acquisition of a migratory phenotype. The prototypical conversion of carcinoma cells to fibroblastoid descendants through transdifferentiation has been described as epithelial to mesenchymal transition (EMT), allowing carcinoma cells to infiltrate the tumor-stroma and to enter as well as to exit the circulatory system in order to generate macrometastasis [6-8].

Various stimuli generated by cell autonomous events and the tumor surrounding impinge on carcinoma cells to induce the crosstalk between rat sarcoma (RAS) signaling proteins and components of the TGF-β superfamily. Important downstream signaling events of RAS involved in the cooperation with the TGF-β/Smad family include the (i) RAS-activated factor (RAF)/mitogen activated protein kinase (MAPK) extracellular regulated kinase (ERK) kinase (MEK)/ERK pathway (RAS-RAF-MEK-ERK signaling), (ii) phosphatidylinositol- 3 kinase (PI3K) pathway activating the serine/threonine kinase AKT-protein kinase B (PKB) and the transcription factor nuclear factor (NF)-κB, and (iii) additional RAS effectors such as RAS-related RAL proteins, phospholipase C (PLC)ε, T cell lymphoma invasion and metastasis-1 (TIAM1), RAS interaction protein-1 (RIN1) and RAS association domain-containing family (RASSF) proteins [9,10]. The enormous complexity underlying the convergence of these signaling cascades will be summarized and discussed in the light of advanced and novel targeted anti-cancer therapies.

TGF-ß Super Family

With more than 30 members, the TGF-β super family is the largest human cytokine family including TGF-β1-3, activins, nodal, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs) and anti-Muellerian hormone (AMH). A phylogenetic tree of all family members and their receptors can be found in a recent review [11]. Structurally, TGF-β cytokines are characterized by cysteine knots which are based on six conserved cysteines

forming three intramolecular disulfide bonds. A seventh conserved cysteine is used by most ligands for dimerization *via* an intermolecular disulfide bond [12]. The canonical signaling cascade for cytokines of the TGF-β family is the Smad pathway, which can be divided into two major branches (Fig. 1). Which branch of the pathway is activated, depends largely on the binding and recruitment of specific ligands to different combinations of type I and II TGF-β family receptors [12,13]. In total, there are seven type I (activin receptor-like kinase, ALK1-7) and five type II (TGFBRII, ActRII, ActRIIB, BMPRII, AMHRII) receptors, together constituting the only mammalian family of transmembrane serine/threonine kinases [11]. TGF-β1 and activins first bind their type II receptors, which stabilizes interaction between type II and type I receptors and leads to phosphorylation of type I by type II receptors. Some BMPs in contrast bind to pre-formed tetramers of type I and type II receptors [13]. The type I receptors in turn are responsible for phosphorylation of receptor-regulated (R)-Smads at two C-terminal serine residues. ALK4, 5 and 7 phosphorylate R-Smads2 and 3, whereas ALK1, 2, 3, and 6 phosphorylate the R-Smads1, 5, and 8 (Fig. 1). In general, there is a considerable degree of promiscuity in the core Smad activation pathway as (i) several ligands can bind the same type II receptor, (ii) most type II receptors can pair with different type I receptors and (iii) each R-Smad can be phosphorylated by more than one type I receptor [11,13]. Nevertheless, most ligands activate exclusively either the Smad2/3 branch (activins, nodal, myostatin) or the Smad1/5/8 branch (most BMPs). The most notable exception is TGF-β1, which on the one hand activates Smad2/3 *via* TGFBRII and TGFBRI-ALK5 in epithelial cells and on the other hand has been shown to activate Smad1/5/8 either dependent on or independent of ALK1 in endothelial cells and additional cell types [14-16]. For relaying the signal into the nucleus, all R-Smads form a complex with the single common mediator (Co-)Smad4. The remaining two members of the Smad family (Smad6, Smad7) are classified as inhibitory (I)-Smads because they block signal propagation of the BMP branch (Smad6) or of all TGF-β family members (Smad7). However, a TGF-β and BMP-independent role of Smad7 has recently been suggested [17]. In the nucleus Smad complexes cooperate with a number of different transcription factors and co-activators or co-repressors to regulate the transcription of target genes [18]. Except for Smad2, all R-Smads as well as Smad4 have direct DNA binding activity. Phospho-Smad2/Smad4 complexes interact for instance with FOXH1 (forkhead box H1, formerly FAST1) and bind to ARE (activin response element) sequences in target gene promoters [19], whereas BMP-activated Smads regulate target genes *via* BRE (BMP response element) sequences in cooperation with Runx e.g. family transcription factors [20]. In addition to Smad-dependent signals, TGF-β can initiate a Smad-independent signaling pathway *via* the E3-ligase TRAF6 and subsequent activation of the MAP kinase kinase kinase TAK1 [21]. Activation of the MAPK cascade by TGF-β has also

been described to occur *via* tyrosine kinase activity of the TGF-β type I receptor resulting in receptor autophosphorylation and recruitment of the adaptor protein ShcA [22].

The TGF /Smad pathway cooperates with RAS signaling at three different levels (Fig. 1). First, a crosstalk between TGF-β family receptors and RAS has been demonstrated by phosphorylation of RAS pathway components through TGF-β family receptors. Secondly, Smad proteins are phosphorylated by RAS-regulated kinases (Fig. 2), which controls their nuclear/cytoplasmic distribution and activity. Thirdly, Smad- and RAS-dependent transcription factors interact and cooperatively regulate target gene transcription.

7.3. Cooperation of Ras with the TGF-ß superfamily

The Synergy of RAS and TGF-β Signaling

TGF-β acts as a potent inhibitor of epithelial cell proliferation through its capability to induce cytostasis by increasing expression of the cyclin-dependent kinase inhibitors (CDKIs) p15Ink4B and p21Cip1 and by repression of c-MYC as well as of inhibitors of differentiation (IDs). These modulations (i) cause differentiation and a block of cell cycle progression, (ii) stimulate cell death, and (iii) repress cytokine and chemokine expression [23]. The tumor-suppressive functions of TGF-β signaling are observed in epithelial tissues under physiological conditions and are mostly active during early stages of tumorigenesis. Importantly, loss of sensitivity to the effects of TGF-β through bypassing the pathway *via* selective mutations of TGFBRs or Smad4 has a pivotal role in the progression of a variety of carcinomas [24]. In contrast to the tumor suppressor activities of TGF-β, a multitude of studies demonstrated pro-oncogenic functions of TGF-β signaling by dysregulation of CDKIs, altered production of extracellular matrix (ECM) constituents *via* mobilization of myofibroblasts, autocrine regulation of mitogens, induction of cell motility and EMT, suppression of immunosurveillance, stimulation of angiogenesis and priming of tumor cells towards metastasis [25-27]. Most remarkably, increased expression and secretion of TGF-β1 in hepatocellular carcinoma (HCC), non-small cell lung cancer (NSCLC), gastric, colorectal, breast, and prostate carcinoma correlates with poor prognosis, indicating TGF-β as a tumor promoter which facilitates aggressiveness of carcinoma [25,28]. The mechanisms of the dichotomous role of the TGF-β pathway and how TGF-β signaling is switched from being tumor-suppressive to tumor-promoting is just beginning to be resolved.

A large body of literature indicates a prominent role of RAS signaling in the conversion from anti- to pro-oncogenic TGF-β signaling, however, the mechanisms of the synergy between oncogenic RAS and TGF-β signaling is still not fully understood [29]. Almost all of these investigations revealed that cancer cells undergo an increase in malignancy and retain a functional TGF-β/Smad core signaling, while tumor-suppressive functions of the canonical TGF-β/Smad pathway are concomitantly inhibited. RAS has been shown to mainly interfere with TGF-β/Smad signaling at the level of Smad2/3 activation and the composition of Smad-dependent transcription factor complexes (Fig. 1).

Involvement of the RAS Subeffector Pathways MAPK, PI3K and RhoA

Previous studies on keratinocyte, mammary, prostate and hepatocyte carcinogenesis models showed a synergistic cooperation of TGF-β and RAS to induce progression to undifferentiated, invasive tumors [30-34]. TGF-β and RAS signaling also cooperates in the intestinal epithelium where overexpression of K-RAS in combination with deletion of TGFRII generated metastatic adenocarcinomas with activated epidermal growth factor (EGF) signaling independent of Wnt/β-catenin [35]. Neither oncogenic K-RAS nor inactivation of

TGFRII on its own was able to induce colorectal tumors. In MDCK cells, sustained activation of RAF-MAPK induced EMT and cell invasion dependent on the RAFmediated autocrine loop of TGF-β [36]. Activation of RAF caused endocytosis and ubiquitin-dependent lysosomal degradation of E-cadherin [37], and inhibited the ability of TGF-β to induce apoptosis without affecting growth retardation. These data demonstrate that the RAF-MAPK pathway synergizes with TGF-β/Smad signaling in promoting malignancy [36]. In particular, hyperactivation of MEK-ERK signaling was responsible for the escape from TGF-β-induced apoptosis in HCC cells by impairing the upregulation of the NADPH oxidase NOX4, which is essential for mitochondrial-dependent apoptosis [38,39].

Cooperation of hyperactive RAF-MAPK and TGF-β/Smad signaling was required for EMT and metastasis of mammary epithelial cells *in vivo*, whereas activation of PI3K caused protection against TGF-β-induced apoptosis and scattering of cells leading to tumorigenesis in the absence of metastatic colonization [29,40]. In malignant hepatocytes, MAPK signaling in collaboration with TGF-β signaling was necessary and sufficient to induce EMT and to promote resistance to TGF-β-mediated cell cycle arrest as well as induction of apoptosis, whereas PI3K activation and TGF-β failed to form tumors [41]. Interestingly, the synergy of RAS and TGF-β signaling induced the secretion and autocrine regulation of platelet-derived growth factor (PDGF) by upregulation of PDGF receptors upon EMT, which resulted in PI3K activation and nuclear β-catenin accumulation [42-44].

Oncogenic K-RAS-ERK signaling in pancreatic adenocarcinoma promoted TGF-β-induced transcriptional downregulation of the tumor suppressor PTEN (phosphatase and tensin homolog) by simultaneous activation of PKB-AKT in a Smad4-independent manner which might provide a switch from growth suppression to growth promotion in pancreatic cancer [45]. Similarly, TGF-β-induced cell migration and EMT of mammary epithelial cells depended on the PI3K-AKT pathway and required Rho GTPase function. This was shown by the pharmacological and genetic interference with the PI3K-AKT pathway which blocked Smad2 phosphorylation and transcriptional responses induced by TGF-β [46]. TGFBRI-ALK5-dependent activation of the p38MAPK pathway was required for TGF-β- mediated EMT of breast carcinoma cells which associated with Rac1 activation and upregulation of tropomyosins (TPM1) necessary for TGF-β-induced stress fiber formation during EMT [47,48]. Interestingly, the upregulation of TPM1 is delayed by several hours as compared to immediate-early and intermediate response genes of TGF-β [49,50]. RAS appears to inhibit late-response but not immediate-early response TGF-β target genes as it did not affect the immediate phosphorylation of R-Smads and their nuclear translocation, but it reduced nuclear presence of Smad4 at later times [49]. Noteworthy, RAS-ERK signaling suppressed Smad-dependent expression of TPM1 and inhibited stress fibers in metastatic breast cancer cells which allowed invasion of EMT transformed cells. H-RAS-V12 prevented binding of

Smads to the TPM1 promoter by forcing CRM1-dependent nuclear export of Smad4 [51]. Animal studies demonstrated that H-RAS-V12 confers the metastatic potential in epithelial cells, whereas tropomyosin suppresses tumor growth and metastases. Thus, these data suggest that (i) TPM1 represents a convergent target of the synergism of RAS and TGF-β, and (ii) TGF-β-induced EMT is not sufficient for acquisition of invasive features, while activated RAS alters the TGF-β response and confers metastatic potential [52]. In this line, activation of RhoA through continuous stimulation of TGFBRI-ALK5 was required for efficient H-RAS-V12, V-RAF and V600E-BRAF mediated malignant transformation [53]. Smad3 was found to be critical for v-H-RAS-mediated transformation in murine embryonic fibroblasts through activation of Jun N-terminal kinase (JNK) and MAPK-ERK signaling [54]. In addition, Smad3 both suppressed and promoted a RASdriven cancer phenotype. Whereas expression of Smad3-dependent cytostatic genes of TGF-β1 were not altered by v-H-RAS along with an intact TGF-β1 growth arrest, expression of proteases and migration of keratinocytes was induced [55].

Smad Activation by Linker Phosphorylation

Kretzschmar *et al*. described that the linker region of Smad2 and Smad3 is phosphorylated at several serine/threonine sites through growth factor-mediated MAPK-ERK activation which results in cytoplasmic retention of Smad2/Smad3 and attenuation of TGF-β signaling [56]. In contrast, Smad2/Smad3 with phosphorylated linker regions have been reported to be predominantly localized in the nucleus in invasive late-stage colorectal carcinoma [57,58]. Mutations of the Smad3 linker region preventing JNK-dependent phosphorylation resulted in a preserved tumor-suppressive function of TGF-β and inhibited tumor cell invasion. The distinct phosphorylation patterns of the Smad2/Smad3 linker region or C-terminal region were characterized using selective antibodies. TGFBRI-ALK5 and hepatocyte growth factor (HGF) induced JNK-dependent phosphorylation of Smad2 and Smad3 at the same sites in the linker region allowing their translocation into the nucleus together with Smad4 [59]. JNK inhibition reduced TGF-β or HGF-mediated cell invasion. However, TGF-β but not HGF treatment induced C-terminal Smad2/Smad3 phosphorylation, whereas HGF treatment reduced TGF-β-dependent elevation of p15Ink4B, which is mediated by Smad3 phosphorylation at the C-terminal region. Other studies, however, have reported C-terminal phosphorylation of Smad2/Smad3 proteins in response to HGF and nocodazole, the latter mediated by the mitotic checkpoint kinase Mps1 in a TGF-β-independent fashion [60,61]. In colorectal cancer, Smad2/Smad3 is phosphorylated at C-terminal serine residues by TGFBRI-ALK5 while JNK and CDK4 differentially phosphorylated linker regions and Ctermini [62]. Dependent on CDK4, TGF-β generated pSmad2C/L (C/L, phosphorylation at C-terminus and linker region) and pSmad3C/L, which enhanced cell growth by upregulation of c-MYC. TGFBRI-ALK5 signaling together with JNK stimulated cell invasion through

pSmad2L/C mediated matrix metalloproteinase (MMP)-9 expression. Clinical samples confirmed nuclear localization of pSmad2L/C and pSmad3L/C at the invasion front of TGF-β-producing, metastatic colon carcinomas. These data suggest that CDK4 together with JNK alters TGF-β signaling from tumor-suppressive to tumor-promoting at late stages of colorectal cancer.

In the liver, tumor suppressive functions of TGF-β were mediated by pSmad3C (phosphorylation at C-terminus), while oncogenic activities such as cell proliferation and invasion were promoted by pSmad3L (phosphorylation at linker region) [63]. pSmad3Lmediated signaling induced ECM deposition by mesenchymal liver cells, and hepatitis B virus infected hepatocytes showed a transition from the anti-oncogenic pSmad3C to the fibrogenic/oncogenic pSmad3L pathway, accelerating liver fibrosis and increasing risk of HCC. Importantly, RAS-associated activation of JNK through pro-inflammatory cytokines such as interleukin-1β mediated this perturbed hepatocytic TGF-β signaling [64,65].

K-RAS and TGF-β signaling cooperated in the induction of SNAI1 (Snail), a transcriptional repressor of E-cadherin, in pancreatic cancer cells. This occured in a Smad-dependent manner independently of MAPK-ERK and JNK activation as well as in the absence of phosphorylation at the linker region of R-Smads [66]. SNAI1 also acts as a transcriptional repressor of RIN1, a RAB5 guanine nucleotide exchange factor (GEF), which downregulates receptor tyrosine kinases (RTKs) and promotes TGFBR signaling through enhanced endocytosis [67]. RIN1 integrates RAS and TGF-β signaling since persistent RTK-RAS activation stabilized TGF β/Smad induced SNAI1 protein by decreased glycogen synthase kinase (GSK)-3β, resulting in silencing of RIN1 and stabilization of RTKs by the concomitant reduction in RAB5-mediated TGFBR internalization.

Smad-Independent Mechanisms

R-RAS transformed EpH4 mammary epithelial cells were insensitive to TGF-β-mediated growth inhibition along with increased proliferation and malignancy in response to exogenous TGF-β. The effects of TGF-β were mediated through Smad-independent mechanisms and required the activation of TGF-β-associated kinase 1 (TAK1) and its downstream effectors JNK/p38MAPK/PI3K/AKT and mTOR pathways [68]. Independent of Co-Smad4, TGF-β signaling resulted in PI3K-mediated tyrosine phosphorylation of α-and β-catenin leading to the dissociation of the E-cadherin/β-catenin complexes from actin cytoskeleton and reduced cell adhesion. Both PI3K and PTEN were associated with Ecadherin/ β-catenin complexes and TGF-β decreased the level of PTEN enhanced β-catenin phosphorylation [69].

RAS Interferes with TGF-β/Smad at the Level of Transcription

A mechanistic link between RAS, p53 and TGF-β has been demonstrated, as RAS-MAPK activity induced p53 N-terminal phosphorylation through CK1ε/δ enabling the interaction of

p53 with TGF-β-activated Smads and promoting TGF-β-dependent cytostasis [70]. Interestingly, the growth-promoting effects of activated RAS are balanced by the wild type p53/Smad cooperation that sustains TGF-β growth control and thus limits neoplastic transformation. A more recent study in breast carcinoma showed insights into the enigma of the pro-metastatic switches of mutant-p53 and TGF-β by demonstrating that RAS-activated mutant-p53 and TGF-β cooperate to counteract the activity of the p53 family member p63. The p63 protein is a master regulator for maintaining normal epithelial stem cells by protecting them from apoptosis and coordinating their differentiation [71]. Noteworthy, oncogenic H-RAS/CK1ε/δ induced mutant-p53 phosphorylation and subsequent assembly of a mutant-p53/p63 protein complex in which Smads serve as essential platforms. Normal p63 functions are antagonized within this ternary complex of mutant-p53/p63 and TGF-β activated Smads, allowing the gain of metastatic properties. In addition, Sharp1 and Cyclin G2 have been identified as metastasis suppressors that mediate p63 effects. Downregulation of Sharp1 and Cyclin G2 in response to the TGF-β/mutant-p53/p63 pathway promoted the pro-oncogenic and invasive TGF-β responses. These data provide novel insights into the mechanism of the interplay between RAS and TGF-β, since RAS signaling promotes mutant-p53 phosphorylation and is required for the formation of the mutant-p53/Smad complex. RAS signaling is therefore suggested to play a significant regulatory role on the composition of co-activators and co-repressors of Smad transcriptional complexes.

RAS Interacts with the Activin and BMP Branches of the TGF-β Family

A role in tumor development and progression has also been demonstrated for several other TGF-β family members including activin A, several BMPs, GDF15/MIC-1, and nodal, to name just some prominent examples. As described for most TGF-β family members, promotion or suppression of tumorigenesis mostly depends on the stage and type of tumor. For activin, an anti-oncogenic activity has been described in breast [72,73], colon [74] and HCC [75], whereas pro-oncogenic effects have been demonstrated in lung [76], prostate [77], esophageal cancer [78,79] and oral cancer [80].

Activin A uses a different set of receptors than TGF-β but depends on the same Smad proteins (Smad2/3/4) for most of its reported activities and relies on similar mechanisms for tumor suppression namely induction of apoptosis and cell cycle arrest via p15Ink4B [81,82]. Specific loss of activin signals in the course of tumor development has been connected to inactivating mutations of activin receptors in colon, pancreas, and prostate cancer [74,83,84] as well as overexpression of antagonistic proteins such as follistatin and follistatin-related gene (FLRG) in breast cancer and HCC [73,75]. In those malignancies with pro-tumorigenic roles of activin A, the signaling pathways are only beginning to be explored. Recently it has been shown that activin A, which inhibits growth and induces apoptosis in prostate cancer cell lines, is overexpressed and enhances cell migration in bone metastatis [77]. This

oncogenic effect was dependent on the interaction of Smad3 with androgen receptor (AR), demonstrating that activin A can switch to a tumor-promoting function similar to TGF-β depending on the stage of cancer progression. The activin antagonist follistatin suppressed metastasis in small cell lung cancer indicating a contribution of activin A to tumor dissemination [85]. In addition, ERK and p38MAPK-dependent signal transduction by activin A has been shown to be involved in the transcriptional control of Pit-1 and tyrosine hydroxylase [86,87]. In keratinocytes, activin induced stress fiber formation and migration in a Smad-independent fashion that includes RhoA and MEKK1 signaling resulting in phosphorylation of JNK and c-Jun [88]. This pathway was suggested to regulate epithelial morphogenesis and might contribute to pro-tumorigenic mechanisms of activin A. The RAS-GAP binding protein Dok-1, which signals downstream of tyrosine kinase pathways, was found to act as an adaptor linking activin receptors with Smad3 and Smad4 activation [89]. Overexpression of Dok-1 enhanced activin-induced apoptosis and inhibited the RASERK pathway in a mouse B-cell line. As reported for TGF-β signal transduction, an impact of RAS-MAPK-dependent phosphorylation in the Smad 2/3 linker region might also affect activin signaling, however, clear evidence needs to be demonstrated. A recent study on transcriptional cooperation showed that activin A increases vascular endothelial growth factor expression via a physical interaction of Smad2 with the MAPK-regulated transcription factor SP1 in HCC cell lines [90]. The synergistic cooperation of activin A with fibroblast growth factor (FGF)2, which mediates tube formation of bovine aortic endothelial cells, could be inhibited by follistatin, Smad7 or inhibition of ERK [91].

The cooperation of the Smad2/3-dependent activin/nodal pathway and RAS-MAPKactivating FGF2 signals including expression of Nanog was also required for maintaining pluripotency of human embryonic stem cells [92,93]. Since both the activin/nodal and the FGF/FGFR axis are hyperactivated in lung cancer and melanoma [76,94-96], it will be interesting to find possible implications of this cooperation on the stem cell-like properties of tumor cells.

In contrast to TGF-β and activins, the BMP subfamily signals via the Smad1/5/8 of the canonical TGF-β pathway. Beside phosphorlylation at the C-terminus by ALK1/2/3/6, the linker region of Smad1 is phosphorylated by MAPKs (ERK, p38, JNK) as well as by GSK-3β [97-99], which has been connected to the inhibition of nuclear translocation and ubiquitin-mediated degradation, thus reducing Smad transcriptional activity. In other studies, however, RAS-ERK signals enhanced the transcriptional activity of Smad1 in response to BMP [100]. Smad-independent activities of BMPs include signaling via direct interaction of the cytoplasmic tail of BMPRII with LIM kinase 1 (LIMK1), a regulator of actin dynamics, and suppression of PTEN via RAS-ERK signaling in Smad4-negative colon cancer cells [101,102]. With respect to the role of BMPs in tumor progression, the picture is diverse. Frequent inactivation of the BMP signaling pathway by mutation of BMP receptors or Smad

proteins was found in colon cancer [103,104]. In lung cancer, an association between RAS mutations and silencing of BMP expression has been demonstrated. NSCLC patients with K-RAS codon 12 mutations were six times more likely to have epigenetically silenced BMP3b/GDF10 or BMP6 than those with wild type K-RAS [105]. The molecular mechanism behind this crosstalk, however, remains to be elucidated. BMP2 and BMP4 have been implicated in enhanced invasion and bone metastasis of breast cancer cells as well as in EMT of ovarian cancer cells [106,107]. BMP7 on the other hand has been demonstrated to counteract TGF-β1 induced EMT in renal tubular epithelial cells [108]. While induction of EMT by BMP4 was associated with Rho GTPase activation, inhibition of EMT by BMP7 involved Smad1-mediated signal transduction. Thus, context-dependent modulation of BMP signals by the cell is crucial for functional outcomes. Future studies need to work out the mechanisms how RAS-dependent signals contribute to pro- versus anti-tumorigenic effects of BMP-activated Smad signals.

7.4. Implications for targeted cancer therapy

Pathways of the RAS and TGF-β family are attractive targets for cancer therapy since they are activated in numerous carcinomas. Yet, the success of specifically targeting either RAS or TGF-β signaling has been limited due to the toxicity of drugs or to vast pleiotropic effects leading to physiological imbalances. As comprehensively outlined, experimental cancer research revealed that both RAS and TGF-β signaling pathways are promising targets to combat tumorigenesis, however, much remains to be investigated to understand the dual anti- and pro-oncogenic roles of TGF-β signaling and to develop proper anti-cancer concepts targeting TGF-β. Determination of deleterious or beneficial TGF-β signaling in cancer development and the complexity of the interrelationship between RAS and TGF-β signaling, which depends on the cell type, tumor stage and tissue context, complicates therapeutic interference with TGF-β signaling [109]. The aim of targeted therapy must be directed against the double-edge sword of TGF-β through inhibition of the pro-oncogenic aspects at advanced stages of cancer progression and the proper restoration of anti-oncogenic effects that are observed during early stages of tumor development.

Various strategies have been pursued to accomplish inhibition of TGF-β signaling including (i) sequence-specific anti-sense oligonucleotides for degradation of TGF-β mRNA, (ii) isoform selective, neutralizing antibodies or soluble TGFBRII fragments blocking the binding of TGF-β ligands to the heteromeric receptor complex, and (iii) low molecular weight inhibitors antagonizing the intracellular kinase activity of TGFBRs [110-112]. For instance, the small molecule inhibitor LY2109761 targeting TGFBRI-ALK5 and TGFBRII induced a complete abrogation of Smad-dependent and - independent signaling in human colon carcinoma cells harboring activated K-RAS, resulting in reduced tumor cell invasion and liver metastasis [113]. Remarkably, TGF-β antisense approaches showed reactivation of tumor-specific immune responses, rendering patients more susceptible to a number of different therapeutic measures [110]. Inhibitors in clinical settings such as human monoclonal antibodies against TGF-β2 (CAT-152) and TGF-β1 (CAT-192) successfully completed phase I/II trials. A TGF-β2 antisense oligonucleotide (AP 12009) has begun phase II/III testing to treat high grade gliomas and phase I/II trials against pancreatic cancer. However, these approaches in clinical trials focus on the inhibition of non-canonical and canonical TGF-β signaling rather than on the molecular crosstalk between TGF-β signaling and the RAS pathway. Recent strategies further involve targeting of TGF-β signaling in the tumor microenvironment, and in particular, the immune system which is critical for carcinoma progression. Importantly, TGF-β provided by bone marrow-derived Gr-1+CD11b+ immature myeloid cells interfered with the immune system by suppression of natural killer cells, cytotoxic T cell and B cell function [28,114]. Infiltration of Gr-1+CD11b+ into breast carcinoma resulted in increased MMP and TGF-β production enforcing tumor cell invasion

and metastasis [114]. Thus, Gr-1+CD11b+ cells act as an additional component causing the switch from tumor-suppressive to tumor-promoting functions which is of paramount importance for the timing of therapeutic TGF-β intervention. Recent studies suggested Gr-1+CD11b+ cells as biomarkers for patient selection in ongoing phase I/II clinical trials of TGF-β therapy which is supported by recent findings in HCC and colorectal cancer [26,115,116].

Efforts to target hyperactive RAS (H-RAS, N-RAS or K-RAS) signaling emanating from mutations by employing GTP analogues or inhibitors of post-translational modifications such as farnesylation failed to prove successful so far [117]. However, inhibition of RTKs upstream of RAS like EGF-R or ErbB2/Her2 is successfully used in the clinic in selected malignancies and patient collectives [118]. Moreover, inhibition of downstream effectors of RAS like RAF and MEK is currently being tested in clinical trials [119]. Recent studies showed that NF-κB, which is a master regulator of the inflammatory response, might be a more promising target for interfering with cancer development than RAS itself [120]. TBK1, activated by the RAS-RAL pathway and inducing NF-κB by phosphorylation of IκB (an inhibitor of NF-κB), has been described to be essential for mutated RAS signaling [121,122]. Targeting of NF-κB might be particularly promising, since RAS and TGF-β together induce NF-κB signaling associated with EMT and metastasis of mammary epithelial cells in vivo, which is reversible by NF-κB inhibition [123]. This therapeutic approach would favor the interference with the deleterious consequences of the RAS-TGF-β cooperation by the concomitant maintenance of tumor-suppressive TGF-β functions.

7.5. Concluding remarks

TGF-β family, which is fundamentally involved in tumor progression. It seems, however, that most carcinomas are not addicted to the oncogenic activity of RAS as its abatement by pharmacological intervention is insufficient to combat the disease. Yet, RAS activation with respect to its crosstalk with TGF-β and the concomitant onset of the pro-oncogenic aspects of the TGF-β family appears to comprehensively overcome physiological programs and to induce a state of cellular amnesia [124]. This state of cellular amnesia rather than addiction to a single oncogene like RAS results in bypassing checkpoint mechanisms which is essential for (i) EMT, cell invasion and metastasis (ii) immune suppression, (iii) neoangiogenesis, (iv) multiple drug resistance, and (v) self-renewal and cancer stemness during carcinoma progression. Pharmacological interference with TGF-β signaling and its modulation by RAS activation could be most effective, when it succeeds in targeting the pro-oncogenic aspects by simultaneously restoring the anti-oncogenic functions of TGF-β. Thus, future studies should focus on targeting the ambiguous role of TGF-β signaling in carcinoma progression, and should take combination therapy with inhibition of RTK-RAS signaling into consideration.

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7.7. Abbreviations

ActRII	activin type II receptor
ActRIIB	activin type IIB receptor
ALK	activin receptor-like kinase
AMH	anti-Muellerian hormone
AMHRII	anti-Mullerian hormone type II receptor
AR	androgen recptor
ARE	activin response element
BMP	bone morphogenic protein
BMPRII	BMP type II receptor
BRE	BMP response element
CDK	cyclin-dependet kinase
CDKI	cyclin-dependet kinase CDK inhibitor
Co-Smad	common mediator Smad
Dok-1	docking protein 1
ECM	extracellular matrix
EGF	epidermal growth factor
EGF-R	epidermal growth factor receptor
EMT	epithelial to mesenchymal transition
ERK	extracellular regulated kinase
FGF	fibroblast growth factor
FLRG	follistatin-related gene
FOXH1	forkhead box H1
GDF	growth and differentiation factor
GEF	guanine nucleotide exchange factor
GSK-3β	glycogen synthase kinase 3β
HCC	hepatocellular carcinoma
HGF	hepatocyte growth factor
H-RAS	Harvey rat sarcoma viral oncogene homolog
ID	inhibitor of differentiation
I-Smad	inhibitory Smad
JNK	c-Jun N-terminal kinase
K-RAS	Kirsten rat sarcoma viral oncogene homolog
LIMK1	LIM kinase 1
MAPK	mitogen activated protein kinase
MDCK	Madin-Darby canine kidney cells
MEK	mitogen activated protein kinase kinase

MH	Mad homology
MMP	matrix metalloprotease
Mps1	monopolar spindle 1
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor κB
NLS	nuclear localization signal
N-RAS	neuroblastoma RAS viral oncogene homolog
NSCLC	non-small lung cell carcinoma
PDGF	platelet-derived growth factor
PI3K	phosphatidyl-inositol-3 kinase
PKB	protein kinase B
PLCε	phosholipase Cε
PTEN	phosphatase and tensin homolog
RAF	rat sarcoma activated factor
RAS	rat sarcoma
RASSF	RAS association domain containing family
RIN1	RAS interaction protein 1
R-Ras	related RAS viral oncogene
R-Smad	receptor-regulated Smad
RTK	receptor tyrosine kinase
TAK1	TGF-β associated kinase 1
TGFBRI	TGF-β type I receptor
TGFBRII	TGF-β type II receptor
TGF-β	transforming growth factor β
TIAM1	T cell lymphoma invasion and metastasis 1
TPM1	tropomyosin 1
TRAF6	tumor necrosis factor receptor-associated factor 6
v-RAS	rat sarcoma viral oncogene

7.8. References

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7.9. Figures

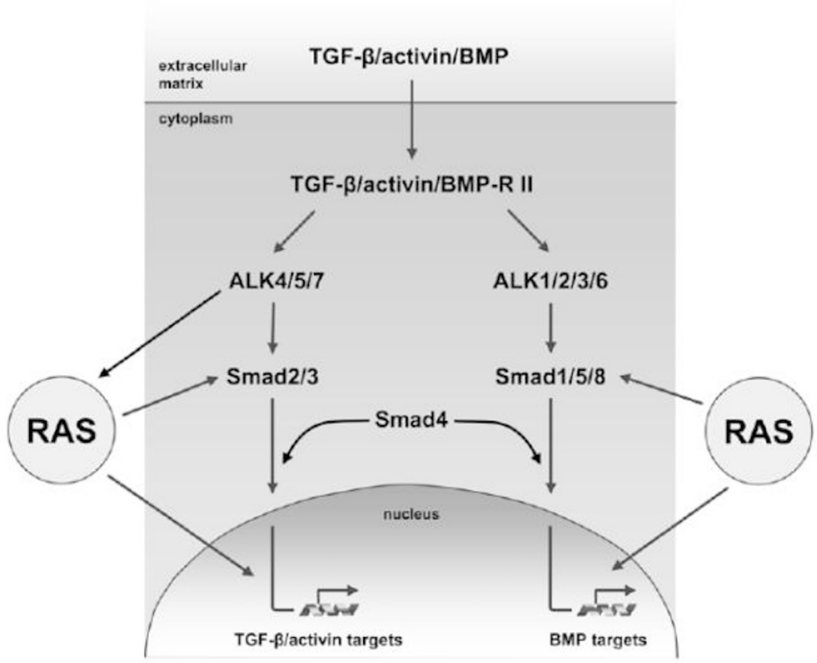

Figure 1

Collaboration between RAS signaling and the TGF-β/activin/BMP pathways. Upon binding of cytokines of the TGF-β/activin/BMP family to TGF-β/activin/BMP-specific type II receptors, heterodimerization with type I receptors of activin receptor-like kinases (ALKs) occurs. Type I receptors further phosphorylate receptor-regulated (R)-Smads (Smad1, Smad2, Smad3, Smad5 and Smad8) and cause their nuclear translocation together with the common-partner (Co-) Smad4. In the nucleus, Smad complexes bind to their respective response elements in target gene promoters and act as transcription factors which modulate gene expression. TGF-β/activin/BMP signaling is controlled at the level of (i) ligand mobilization and binding, (ii) receptor heterodimerization and activation, (iii) phosphorylation of R-Smads and nuclear shuttling of R/Co-Smad as well as (iv) Smaddependent transcription complexes. The crosstalk of RAS and TGF-β family proteins involves (i) modulation of RAS subeffector proteins by TGF-β family receptors, and (ii) the interference of RAS signaling components either with the activation and nuclear translocation of Smad effectors or with Smad-dependent regulation of transcription.

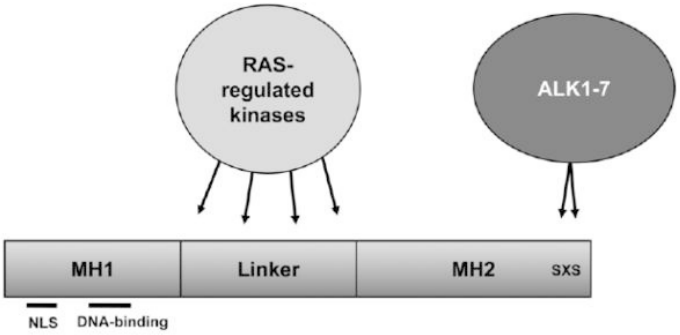

Figure 2

Schematic domain structure and phosphorylation pattern of Smad proteins. Smads consist of the N-terminal Mad-homology (MH) 1 region and the C-terminal MH2 domain which is joined by the linker region. The MH1 region is required for DNA-binding and contains a nuclear localization signal (NLS), whereas the MH2 region is involved in protein-protein interactions required for regulation of transcription. In Smad2, direct binding of DNA is blocked by an additional sequence in the MH1 domain. ALK1-7 phosphorylate R-Smads at the two serine residues of the C-terminal SXS motif, which generally leads to heterooligomerization with Smad4 and nuclear translocation. Several RAS-regulated kinases such as ERK, JNK and p38MAPK phosphorylate Smad proteins predominantly in the linker region and modulate their activity.

8. Manuscript II

La enhances IRES-mediated translation of Laminin B1 during malignant epithelial to mesenchymal transition

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8.1. Abstract

The majority of transcripts that harbor an internal ribosome entry site (IRES) are involved in cancer development via corresponding proteins. A crucial event in tumor progression referred to as epithelial to mesenchymal transition (EMT) allows carcinoma cells to acquire invasive properties. The translational activation of the extracellular matrix component laminin B1 (LamB1) during EMT has been recently reported suggesting an IRES-mediated mechanism. In this study, the IRES activity of LamB1 was determined by independent bicistronic reporter assays. Strong evidences exclude an impact of cryptic promoter or splice sites on IRES-driven translation of LamB1. Furthermore, no other LamB1 mRNA species arising from alternative transcription start sites or polyadenylation signals were detected that account for its translational control. Mapping of the LamB1 5'-untranslated region (UTR) revealed the minimal LamB1 IRES motif between -293 to -1 upstream of the start codon. Notably, RNA affinity purification showed that the La protein interacts with the LamB1 IRES. This interaction and its regulation during EMT were confirmed by ribonucleoprotein immunoprecipitation. In addition, La was able to positively modulate LamB1 IRES translation. In summary, these data indicate that the LamB1 IRES is activated by binding to La which leads to translational upregulation during hepatocellular EMT.

8.2. Introduction

The interaction of tumor cells with the extracellular matrix (ECM) is essential for cancer invasion and metastasis. Laminins constitute the ECM as the main non-collagenous glycoproteins of the basement membrane and affect the migratory behavior of a variety of malignant cell types [4]. Laminin B1 (LamB1) represents one moiety of the three β-subunits that assemble with α- and γ-chains to form a multitude of heterotrimeric laminin isoforms [5]. LamB1 modulates laminin-mediated integrin signaling that promotes cell adhesion, motility and differentiation [6]. In addition, LamB1 is the ligand of the monomeric 67 kDa laminin receptor (LamR; [7-9]) that drives tumor cell invasion as well as angiogenesis and is strongly expressed in many metastatic cancers [10-12]. The overexpression of LamB1 has been particularly reported in hepatocellular carcinoma (HCC), which is frequently preceded by fibrosis and cirrhosis and thus accompanied by increased ECM deposition. Accordingly, LamB1 expression is augmented during transforming growth factor (TGF)-β induced liver fibrosis of transgenic mice [13]. Proteome analysis of HCC patients revealed a rise of LamB1 levels in cirrhotic tissue and a further increase in carcinoma cells [14]. The elevated LamB1 expression in HCC associates with upregulation of β1 integrin and LamR [15-17].

A crucial event in tumor progression is the gain of invasive properties of carcinoma cells by epithelial to mesenchymal transition (EMT). This process is characterized by the loss of epithelial cell polarity and acquisition of a fibroblastoid phenotype enabling tumor cells to leave epithelial cell organisation [18]. In HCC cells, EMT can be induced by the synergy of TGF-β and oncogenic Ras signaling, which are frequently activated in HCC patients [18-21]. Recently, we showed that LamB1 is translationally upregulated in HCC cells that have undergone EMT through the cooperation of TGF-β with Ras [22]. The translational activation of LamB1 was proposed to be regulated by an internal ribosome entry site (IRES).

The IRES secondary structure located within the 5'-untranslated region (UTR) of the mRNA allows direct ribosome binding and translation initiation independently of the cap structure. Translation of key regulatory proteins that are involved in cancer- relevant processes such as angiogenesis, mitosis and apoptosis is frequently driven by internal initiation [23]. IRES-translated transcripts escape from downregulation of cap-dependent protein synthesis and foster tumor cells to overcome stress conditions induced by hypoxia or nutrient deprivation [24]. Deregulation of translation control affects IRES-mediated translation in a dual way. Cap-dependent translation of IRES-competent transcripts is increased in the presence of high levels of activated translation initiation factor eIF4E, while same transcripts are translated by IRES-dependent mechanism when eIF4E is downregulated in response to cellular stress or other stimuli [25, 26]. Furthermore, IRES-mediated translation is regulated by IRES transacting factors (ITAFs) which mainly comprise heterogeneous nuclear ribonucleoproteins (hnRNPs) that shuttle between cytoplasm and nucleus [27]. ITAFs selectively bind IRES

structures and modulate their activity, allowing a distinguished response of IRES transcripts to cellular conditions that affect translation. A role of ITAFs in regulating cell survival and angiogenesis has been described although little is known about tumor-associated alterations of ITAFs [28].

Here we demonstrate that the 5'-UTR of LamB1 harbors a *bona fide* IRES structure localized between -293 to -1 upstream of the start codon. RNA affinity purification as well as *in vivo* UV crosslinking combined with RNP immunoprecipitation allowed the identification of La protein as an ITAF that binds to the LamB1 IRES. Moreover, La was found to positively regulate the LamB1 IRES activity which might account for the increased IRES mediated LamB1 translation of EMT-transformed hepatocytes.

8.3. Material and Methods

Construction of the plasmids

The LamB1 5'-UTR was cloned downstream of the Firefly luciferase open reading frame (ORF) into p-F resulting in pLam-F[22]. Primer were designed according to GenBank sequence NM_002291. Plasmids for the cryptic promoter assay were generated by removal of the CMV promoter from pF, pLam-F and pEMCV-F [22]. Bicistronic plasmids were constructed based on the pβgal/5'(-162)/CAT vector (a kind gift from Dr. Martin Holcik) [29]. The XIAP IRES sequence was removed from pβgal/5'(-162)/CAT to generate the empty control vector pβgal/CAT, or replaced by the LamB1 5'-UTR resulting in pβgal/Lam/CAT. Deletion fragments for the mapping of the 5'-UTR were amplified by PCR and cloned into pβgal/CAT. Primer sequences are shown in Supplementary Table S1. Plasmids for the streptavidin-tethered RNA affinity purification were generated by inserting the LamB1 5'-UTR or the minimal XIAP IRES sequence into the pTrap vector (kindly provided by Dr. Martin Dienstbier) [30]. Primer sequences are shown in Supplementary Table S2.

Cell culture

Immortalized murine MIM-1-4 hepatocytes were grown on collagen-coated culture dishes in RPMI 1640 plus 10% fetal calf serum (FCS), 40 ng/ml human TGF-α (Sigma, St.Louis, USA), 30 ng/ml human insulin-like growth factor II (IGF-II, Sigma, St.Louis, USA), 1.4 nM insulin (Sigma, St.Louis, USA) and antibiotics, as described previously [31]. Malignant epithelial MIM-R hepatocytes are derived from MIM-1-4 cells by expressing oncogenic Ha-Ras and green fluorescent protein as outlined recently [32]. TGF-β1 (R&D Systems, Minneapolis, USA) was used at a concentration of 2.5 ng/ml for the first 72 hours of EMT induction. For long-term treatment of MIM-R hepatocytes, TGF-β1 was supplemented to the medium at a concentration of 1 ng/ml, resulting in fibroblastoid MIM-RT cells after more than two weeks. Human SW480 and HEK293 cells were cultured in DMEM and HepG2 cells in MNP each plus 10% FCS. The human HCC cell lines Hep3B and AKH3p were grown in RPMI 1640 medium supplemented with 10% FCS. All cells were kept at 37°C and 5% CO2 and routinely screened for the absence of mycoplasma.

Western blot analysis

The preparation of cellular extracts, separation of proteins by SDS polyacrylamide gel electrophoresis and immunoblotting were performed as described recently [21]. Cytoplasmic and nuclear cell fractions were generated using a Proteo-JET® kit (Fermentas, St.Leon-Rot, Germany) according to the manufacturers description. 30 µg protein extract was loaded onto gels and immunological detection of proteins was performed with the Super Signal detection system (Pierce Chemical Company, Rockford, USA). The following primary antibodies were used: anti-laminin B1 (Neo Markers, Fremont, USA), 1:1.000; anti-hnRNP A1 (Santa Cruz Biotechnology, California, USA), 1:1.000; anti-actin (Sigma, St. Louis, USA), 1:2.000; anti-La

(Santa Cruz Biotechnology, California, USA) and (Cell Signaling, MA, USA), 1:1.000; anti-tubulin (Sigma, St. Louis, USA), 1:1.000; anti-nucleoporin (BD Biosciences, NJ, USA), 1:1.000. Secondary antibodies (Calbiochem, LaJolla, USA) were used at dilutions of 1:10.000.

Reverse transcriptase (RT)-polymerase chain reaction (PCR), quantitative (q)PCR and RACE-PCR

RNA was extracted, DNaseI treated and reverse transcribed using a RNA isolation and cDNA synthesis kit (Quiagen, Hilden, Germany) as recommended by the manufacturer. Aliquotes of the cDNA were employed for RT-PCR using Ready-To-Go PCR beads (Amersham, Uppsala, Sweden). Amplification products of semiquantitative PCR were visualized by ethidium bromide staining on a 1% agarose gel. Primer sequences are shown in Supplementary Table S3. qPCR was performed with Fast SYBR green (Applied Biosystems, CA, USA) according to the recommendations of the manufacturer and quantified with the 7500 Fast Real Time PCR System (Applied Biosystems). Primer sequences are shown in Supplementary Table S4. 5' and 3' RACE-PCR was performed using a First Choice® RLM-RACE Kit (Applied Biosystems, CA, USA) according to the manufacturer's recommendation. Primer sequences are shown in Supplementary Table S5.

Transient transfection and reporter assays

Cells were plated on 6-well plates and transiently transfected after 24 hours with Lipofectamine Plus as recommended by the manufacturer (Invitrogen, Carlsbad, USA). Cells were lysed 48 hours post transfection and enzymatic reporter activities were determined. Luciferase activity was measured with a Luminoskan microplate reader (Labsystems, Farnborough, UK) as recently described [22]. β-galactosidase (β-gal) activity was photometrically determined using o-nitrophenyl-β-d-galactopyranoside and chloramphenicol acetyltransferase (CAT) activity was measured by ELISA (Roche, Mannheim, Germany) as recommended by the manufacturer. In assays analyzing the interference of cap-dependent translation, reporter activities were normalized to mRNA levels as quantified by qPCR. Otherwise, relative IRES activity of bicistronic assays was calculated as the ratio of CAT/β-gal. Luciferase activities of monocistonic vectors were normalized to β-gal activities of a co-transfected β-gal reporter [22]. Assays were performed in triplicate and results represent the average of three independent experiments.

RNA-protein immunoprecipitation

Cells were transfected with a bicistronic vector as described above or directly UV crosslinked with 400 mJ/cm2 before cell lysis. Lysates were centrifuged at 1200 g and supernatants were aliquoted corresponding to 3x106 cells. 5 µg La antibody (Santa Cruz Biotechnology, California, USA) were crosslinked to 50 µl Dynabeads® Protein A (Invitogen, California, USA) using Bis(sulfosuccinimidyl)suberate (BS3; Thermo Fisher Scientific, Massachusetts,

USA) as described by the manufacturer. Crosslinked Dynabeads® were incubated with aliquots of precleared cell lysates at room temperature, washed and the immunoprecipitated RNA-protein complexes were eluted at 95°C. Eluates were treated with proteinase K (Fermentas, St.Leon-Rot, Germany) and the RNA was ethanol-precipitated after extraction with phenol-chloroform. After treatment with DNaseI (Fermentas), mRNA was purified using a mRNA isolation kit (Roche, Mannheim, Germany) and subjected to cDNA synthesis. Subsequent RT-PCR or qPCR was performed as described above. To determine the amount of input RNA that was used for immunoprecipitation, RNA was isolated from aliquots of cell lysates using a RNA isolation kit (Quiagen, Hilden, Germany), treated with proteinase K and processed as described above. The buffer for cell lysis and immunoprecipitation consisted of 30 mM HEPES pH 7.3, 160 mM KCl, 2.5 mM MgCl2, 1 mM DTT, 0.1% NP-40, 0.5% Triton X-100 and 10% glycerol. The washing buffer included PBS pH 7.4 and 0.02% Tween. The elution buffer was composed of 100 mM Tris/HCl pH 7.4, 5% SDS, 70 mM β-mercaptoethanol and 5 mM DTT.

Northern blot analysis

Cells were grown on a 6 cm culture dish and transfected with bicistronic vectors using Lipofectamine Plus as recommended by the manufacturer (Invitrogen, Carlsbad, USA). mRNA was isolated with a mRNA isolation kit (Roche, Mannheim, Germany). RNA samples were heated at 65°C for 15 minutes in RNA sample buffer, cooled on ice and separated on a MOPS-formaldehyde agarose gel. RNA was transferred to a membrane (NEN life science products, MA, USA) and UV crosslinked. Probes were amplified by PCR from the pβgal/Lam/CAT vector in the presence of 32P-labeled dCTP. Primer sequences are described in Supplementary Table S6. Unincorporated nucleotides were removed using a Gelextraction Kit (Quiagen, Hilden, Germany). Membranes were prehybridized for 4 hours and then hybridized over night at 42°C with 5x107 cpm of radiolabelled probe. Membranes were washed at 65°C and exposed to x-ray films at -80°C.

***In vitro* translation**

Bicistronic vectors containing the LamB1 5'-UTR or XIAP IRES were in vitro transcribed using a MEGAshortscript® kit (Applied Biosystems, CA, USA). Rabbit reticulocyte lysate (Promega, Madison, USA) was programmed with the transcripts and in vitro translation was performed according to the guidelines of the manufacturer. CAT activities were determined by ELISA (Roche, Mannheim, Germany) and normalized to transcript levels quantified by CAT RT-PCR. In order to preclear the reticulocyte lysate from La, 5 µg La antibody was crosslinked to 50 µl Dynabeads® Protein A (Invitogen, California, USA) by using BS3 as described by the manufacturer (Thermo Fisher Scientific, Massachusetts, USA). Reticulocyte lysate was incubated with crosslinked Dynabeads® for 30 min at room temperature followed

by removal of Dynabeads®. 1 µg of recombinant La (Prospec, Rehovot, Israel) was added to reconstitute La activity. Results show the average of three independent experiments.

Streptavidin-tethered RNA affinity purification

2x107 cells were lysed and precleared with 50 µl streptavidin-linked magnetic beads (Sigma, St. Louis, USA). Another 50 µl of magnetic beads were washed and resuspended in 200 µl buffer. 5 µg of in vitro transcribed S1-aptamer fused RNA and 5 µl RNasin (Promega, Madison, USA) were added and the beads were incubated on a thermomixer for 90 minutes at 4°C with moderate shaking. RNA-associated beads were washed with 200 µl buffer and further incubated with the precleared protein extract on a thermomixer for 90 min at 4°C whilst shaking. Beads were then washed 5 times with buffer. RNA-protein complexes were eluted by incubating with buffer containing biotin at a final concentration of 10 mM at 4°C for 160 minutes. Eluted proteins were analyzed by SDS polyacrylamide gel electrophoresis followed by silver staining or Western blot. The buffer used for cell lysis, affinity chromatography, washing and elution contained 30 mM HEPES pH 7.3, 160 mM KCl, 2.5 mM MgCl2, 1 mM DTT, 0.1% NP-40, 0.5% Triton X-100 and 10% glycerol.

Stable knock-down of La

MISSION® shRNA lentiviral transduction particles (Sigma, St. Louis, USA) were used to knock-down La (NM_009278) in MIM-R and MIM-RT hepatocytes. Cells were transduced with viral particles containing shRNA against La (TRCN0000071284, TRCN0000071287) or non-target shRNA (SHC002V) in a pLKO.1 vector and treated with puromycine (5 µg/ml) for selection.

Statistical analysis

Data were expressed as means ± standard deviation (SD). The statistical significance of differences was evaluated using a paired, non-parametric Student’s t-test. Significant differences between experimental groups were * $p<0,05$, ** $p<0,01$ or *** $p<0,005$.

8.4. Results

LamB1 mRNA in cancer cells

Recently we identified the cap-independent translational upregulation of LamB1 during TGF-β-induced EMT of epithelial MIM-R hepatocytes to invasive, fibroblastoid MIM-RT cells [22]. To further assess translational control of LamB1 during EMT, we studied a conceivable impact of LamB1 mRNA isoforms. Differential utilization of transcripts generated by e.g. alternative splicing is known to cause aberrant gene expression during oncogenesis [33]. Notably, the LamB1 5'-UTR sequence published in NCBI (NM_002291.2) is not entirely supported by expressed sequence tags (ESTs) or full length cDNA. Rather, the EST database dbEST contains transcripts that start at -147 nts or further downstream and fail to confirm the region from -335 to -154 in the 5'-UTR. *In silico* analysis of the 5'-UTR using Zuckers mfold version 4.11 predicted a strong secondary structure with a minimal free energy of -154 kcal/mol which could account for insufficient amplification and could explain missing ESTs [34, 35]. Therefore, we tested whether the first 1-181 nts of the published sequence were amplified from DNA contaminations. Interestingly, RT-PCR analysis of different human cell lines after DNase treatment showed amplification of the entire 5'-UTR sequence comprising 335 nts as published in NCBI (Figure 1A). Combined DNase and RNase treatment failed to show amplicons, excluding the presence of DNA. Thus, our data confirm the expression of the published human LamB1 5'-UTR sequence.

Furthermore, we performed RACE-PCR of the 5'- and 3'-UTR to identify any transcript variants that may be involved in translational regulation of LamB1 (Figure 1B). In accordance, we were able to amplify a single 335 nts long 5'-UTR sequence in various human cancer cell lines. No other transcripts were detected suggesting the absence of alternative splice products of the LamB1 5'-UTR. In addition, we analyzed variations in the LamB1 3'-UTR since shortening of the 3'-UTR by alternative cleavage or polyadenylation was demonstrated to be involved in translational activation of oncogenes [36]. RACE-PCR of the 3'-UTR resulted in a single amplicon of 135 nts, indicating that the LamB1 3'-UTR is not altered in tumorigenesis. Consistently, LamB1 Northern blot analysis of two human tumor cell lines displayed single transcripts (Figure 1C). Together, these data suggest that translation of LamB1 is not regulated by alternative 5'-UTR species or shortening of the 3'-UTR.

Translational activation of LamB1 is IRES mediated in EMT-transformed cells

We recently showed that the LamB1 5'-UTR directs translation of a bicistronic transcript when inserted in the intercistronic region of a vector containing Renilla and Firefly luciferase reporters [22]. Although this bicistronic vector is widely used there have been issues with false positive signals derived by alternative splicing [37, 38]. Furthermore, it has been described that intensities of IRES activities can strongly vary depending on the type of bicistronic reporters [39, 40]. Therefore we employed an alternative bicistronic vector

encoding β-galactosidase (β-gal) as upstream and chloramphenicol acetyltransferase (CAT) as downstream reporter [37]. The LamB1 5'-UTR was inserted into the intercistronic region and the empty vector served as negative control (Figure 2A). Since the cellular IRES of X linked inhibitor of apoptosis (XIAP) and its ITAFs are well documented, the minimal XIAP IRES sequence was utilized as positive control [29, 41, 42]. Human SW480 colon carcinoma cells were transfected with bicistronic vectors and β-gal and CAT activities were determined (Figure 2B). Reporter activities were normalized to mRNA levels quantified by qPCR, and thus display the ratio of active protein to bicistronic transcript levels allowing conclusions on translation efficiencies. These data show an about 7-fold increase of LamB1 IRES-mediated CAT translation as compared to control. Figure 2C normalizes CAT to cap-dependent β-gal translation rates of the bicistronic transcripts, indicating a more than 6-fold upregulation of LamB1 IRES activity. These data corroborate our recent findings and demonstrate that the LamB1 5'-UTR mediates cap-independent translation in two independent bicistronic reporter systems [22].

We next analyzed whether the IRES activity of LamB1 is regulated upon hepatocellular EMT by employing bicistronic experiments in immortalized MIM-1-4 hepatocytes, malignant epithelial MIM-R hepatocytes that were transformed with oncogenic Ras as well as in those that have undergone EMT upon TGF-β treatment (MIM-RT). All cells were transfected with bicistronic constructs containing either the LamB1 5'-UTR, the minimal XIAP IRES or the empty vector. β-gal (Figure 2D) or CAT activities (Figure 2E) were normalized to transcript levels and displayed as the ratio of IRES- to cap-dependent translation (Figure 2F). As expected, β-gal levels displayed no significant variations of cap-dependent translation rates in the cell lines (Figure 2D). CAT activities were 2.7-fold elevated in EMT-transformed MIM-RT hepatocytes as compared to epithelial MIM-R cells (Figure 2E), suggesting activation of LamB1 IRES translation upon hepatocellular EMT. Normalization of β-gal to CAT activities as shown in Figure 2F revealed comparable data as depicted in Figure 2E. These results are consistent with recently published data that were obtained using Renilla and Firefly luciferase reporters [22]. Notably, LamB1 IRES translation showed higher levels than XIAP in the malignant progression of hepatocytes (Figure 2E and 2F). This effect might be cell type specific since LamB1 and XIAP exhibited similar activities in SW480 colon cancer cells (Figure 2C). Together, these data show that the LamB1 5'-UTR confers translation of the downstream cistron in the β-gal/CAT constructs, indicating IRES-mediated translation. Furthermore, LamB1-driven IRES translation in this assay is activated upon hepatocellular EMT which corroborates recent data [22].

The LamB1 5'-UTR does not direct aberrant transcription or cryptic splicing

Bicistronic assays to determine IRES activity have been criticized since reporter activity of the downstream cistron may arise from the presence of cryptic promoter or splice sites within

the 5'-UTR rather than from the IRES. In order to detect any aberrant bicistronic transcripts, we performed Northern blot analysis of SW480 and MIM-R cells transfected with the bicistronic vector containing LamB1 5'-UTR or empty control. Probes for the β-gal and CAT reporter hybridized to the bicistronic mRNA revealed single transcripts of equal size (Figure 3A). Truncated mRNAs generated from cryptic promoter or splice sites could not be detected. As Northern blot analysis could fail to detect smaller transcripts due to limited sensitivity, we determined the ratio of CAT to β-gal cistrons by qPCR. This analysis revealed that ratios were comparable between bicistronic vectors containing either XIAP IRES, LamB1 5'-UTR or empty control (Figure 3B). These data indicate that the LamB1 5'-UTR does not direct aberrant transcription or splicing.

To further analyze cryptic promoter activity, we constructed vectors containing the LamB1 5'-UTR, the EMCV IRES or the empty control upstream of a Firefly luciferase reporter, each in presence or absence of a CMV promoter. These vectors were transfected into MIM-R hepatocytes together with a β-gal reporter plasmid and Firefly activities were normalized to β-gal levels (Figure 3C). Interestingly, promoterless plasmids showed residual luciferase activities of 6-8%. However, these remaining activities are considered to be independent of the sequence upstream of the luciferase reporter, since they were almost equally detected in vectors under investigation. These results suggest that cryptic transcription occurs within the Firefly luciferase, which has already been reported [43]. To identify the origin of the aberrant transcription, we performed RT-PCR using primer that bind within the LamB1 5'-UTR (A,B,C) or within the Firefly luciferase open reading frame (D,E,F; Figure 3D). To exclude DNA contaminations RNA extracts were treated with RNase resulting in no amplicon. Only primer located within the Firefly luciferase (E,F) amplified transcripts of promoterless vectors in presence or absence of the LamB1 5'-UTR, suggesting a cryptic promoter activity within the Firefly luciferase rather than in the LamB1 5'-UTR. These data provide strong evidence that the LamB1 5'-UTR does not drive aberrant transcription and that translation of the bicistronic vector containing the LamB1 5'-UTR was indeed mediated by an IRES.

Determination of the minimal LamB1 IRES motif

To determine the minimal *cis*-acting IRES motif of the LamB1 5'-UTR, we generated bicistronic constructs containing deleted fragments of the 5'-UTR (Figure 4). The sequence was truncated in steps of 50 nts starting from the 5' or 3'-end. MIM-R and MIM-RT hepatocytes were transfected with the constructs and CAT activities were normalized to β-gal levels. The segment between -293 to -1 upstream of the start codon retained full IRES activity, while the region between -241 to -1 showed reduced IRES activity indicating that the minimal IRES motif starts within -293 and -241. A sequence upstream of the start codon containing 82 nts (-82 to -1) displayed 31% activity of the full length 5'-UTR and therefore might have still some ability to attract the translation initiation complex. Deletion of this region

caused a reduced activity of the remaining fragment (-335 to -82) to 64% of the full length 5'-UTR, suggesting that it is not essential for internal ribosome binding but may work as an enhancer of IRES activity. Furthermore, a motif that regulates IRES activity in response to TGF-β induced EMT might start within -241 to -180, since the -241 to -1 fragment is still regulated between MIM-R and MIM-RT cells while 5'-deletion to -180 nts results in similar IRES activities in both cell lines. Taken together these data reveal a minimal IRES motif required for translational activity that starts between -293 to -241 and ends between -82 and -1, and in addition, suggest an IRES domain between -241 to -180 that responds to TGF-β.

La interacts with the LamB1 5'-UTR *in vitro* and *in vivo*

IRES-driven translation has been shown to be regulated by ITAFs [27]. Therefore, we aimed to identify RNA binding proteins that interact with the LamB1 5'-UTR by streptavidin-tethered RNA affinity purification. Constructs containing the LamB1 5'-UTR or the minimal XIAP IRES downstream of two S1 aptamers were generated. The minimal XIAP IRES was applied as control for selectivity since several interacting ITAFs have already been described [41, 42, 44]. The constructs were *in vitro* transcribed and RNA was conjugated to magnetic streptavidin beads. Whole protein extracts of HEK293 cells were incubated with either streptavidin beads alone, streptavidin beads conjugated to the transcript of the LamB1 5'-UTR or streptavidin beads conjugated to the XIAP transcript. Elution with biotin allowed a high selectivity for RNA associated proteins. Analysis by SDS electrophoresis followed by silver staining revealed a 47 kDa protein that interacts with the LamB1 5'-UTR (Figure 5A). This protein was identified as La/SSB by Western blot analysis (Figure 5B). The La protein as well as the 37 kDa protein hnRNP A1 bound to the XIAP control RNA. Both ITAFs have been described as regulators of XIAP IRES activity and therefore demonstrate the selectivity of this experimental approach [41, 42]. The control reaction using streptavidin beads alone did not yield any proteins (Figure 5A and 5B). From these data we concluded that La selectively binds the LamB1 5'-UTR *in vitro*.

This interaction was verified *in vivo* by anti-La RNA immunoprecipitation (IP) (Figure 5C). Lysates were generated from SW480 or HEK293 cells after UV crosslinking of RNA-protein complexes. Cell lysates were immunoprecipitated with magnetic beads conjugated to La antibody or unconjugated beads as control. RNA was isolated from either cell lysates (Input), IP with anti-La conjugated beads (La) or unconjugated beads (beads) and RT-PCR was performed. To exclude the presence of DNA contaminations, a control reaction without reverse transcription (r.t.) was included. The ability of the antibody to precipitate the La protein was verified by Western blot (Supplementary Figure S1). As shown in Figure 5C, LamB1 and XIAP but not Actin transcripts could be amplified from the immunoprecipitated mRNA in both cell lines. While XIAP serves as a positive control, actin mRNA is known to lack interaction with La, showing the selectivity of the IP [45]. Thus, these data indicate the

physical interaction of La with the LamB1 mRNA in two different human cell lines. To further investigate this interaction and analyze whether La binds the LamB1 5'-UTR *in vivo*, the La-IP was performed in HEK293 cells transfected with the bicistronic construct containing either the LamB1 5'-UTR, the XIAP IRES or the empty control. RT-PCR for the CAT reporter revealed that La selectively binds the LamB1 5'-UTR and XIAP IRES but not the empty vector (Figure 5D). Taken together, these data show that La associates with the LamB1 5'-UTR *in vitro* and *in vivo.*

La binding to the LamB1 IRES is regulated upon EMT

Since La represents an ITAF that binds to the LamB1 5'-UTR, we next investigated whether the interaction between La and the LamB1 IRES is regulated during EMT. Therefore we performed La RNA-IP employing epithelial MIM-R and fibroblastoid MIM-RT hepatocytes. Cells were transfected with bicistronic vectors containing the minimal LamB1 IRES, the XIAP IRES or the empty control. RT-PCR for the CAT reporter revealed that La binds to the LamB1 and XIAP IRES in MIM-R as well as MIM-RT cells (Figure 6A). qPCR for the CAT reporter was performed to reliably quantify the amount of LamB1 bicistronic transcripts that interact with La in each cell line (Figure 6B). Quantified levels of immunoprecipitated bicistronic transcripts were normalized to the amount of bicistronic transcript that was used for the IP. Interestingly, higher levels of La protein associated with the minimal LamB1 IRES in MIM-RT cells than in MIM-R cells suggesting that the interaction of La with the LamB1 5'-UTR is upregulated after EMT. This observation is consistent with the bicistronic assay that revealed a significant increase of IRES activity upon EMT (Figure 2F) and with recent data showing that La is an activator of several cellular IRESs [42, 46, 47]. We further investigated the regulation of La during EMT in our cell model. Western blot analysis revealed comparable levels of La protein in MIM-R and MIM-RT cells, while LamB1 expression was clearly elevated in EMT-transformed cells (Figure 6C). Thus, La is not regulated during EMT at the level of total protein expression. As recent reports described that La can shuttle between nucleus and cytoplasm, we studied this possible mode of regulation by subcellular fractionation. Interestingly, Western blot analysis showed that MIM-RT cells display increased cytoplasmic La levels compared to MIM-R cells (Figure 6D). In addition, treatment of MIM-R cells with TGF-β resulted in slightly enhanced cytoplasmic levels of La protein. Notably, elevated levels of cytoplasmic La correlated with LamB1 upregulation. In summary, these data suggest that La protein accumulates in the cytoplasm during TGF-β induced EMT, where it binds to the minimal LamB1 IRES motif to increase translation.

LamB1 IRES activity is enhanced by La

As the interaction of La with the LamB1 IRES is regulated upon EMT, we investigated whether La enhances IRES-mediated translation of LamB1. Therefore, we studied the effect of La on LamB1 IRES activity using an *in vitro* translation assay (Figure 7A). Rabbit

reticulocyte lysate (RRL) was programmed with *in vitro* transcribed bicistronic RNA containing the LamB1 5'-UTR and the IRES-dependent CAT activities were determined. Prior to *in vitro* translation, RRL was precleared from La with magnetic beads conjugated to La antibody or incubated with unconjugated beads as control. The preclearing of lysates was verified by Western blot analysis (Supplementary Figure S2). Treatment with unconjungated beads resulted in a slight decrease of CAT activity suggesting that some factors required for LamB1 IRES translation unspecifically bind to the beads. When RRL was precleared from La, the CAT activity was further significantly decreased (Figure 7A). Notably, the reduced LamB1 5'-UTR mediated translation could be restored by addition of recombinant La. These results suggest that La positively affects IRES-driven translation of LamB1. Since La has been described as activator of XIAP IRES translation, we employed the minimal XIAP IRES in the same experimental setting, which led to similar results (Supplementary Figure S3; [42]). These data suggest that La works as an ITAF that binds to the minimal LamB1 IRES and enhances its translational activity during hepatocellular EMT.

To study the effect of La on LamB1 IRES translation *in vivo*, we generated MIM-R and MIM-RT cells with a stable knock-down of La expression. Western blot analysis revealed a strong knock-down of La using two different shRNA constructs (shLa 84, shLa 87) as compared to non-target control (sh-NT) or untreated cells (ctrl) (Figure 7B). Notably, MIM-RT but not MIM-R cells showed reduced levels of LamB1 after knock-down of La. These data suggest that La mediated stimulation of IRES-driven LamB1 translation after EMT is diminished by silencing of La. To further study the effect of impaired La expression on LamB1 IRES activity, bicistronic assays were performed (Figure 7C). MIM-R and MIM-RT cells with a stable knock-down of La (shLa 84, shLa 87), non-target shRNA (sh-NT) or the untreated control (ctrl) were transfected with a bicistronic vector containing the LamB1 5'-UTR. Remarkably, LamB1 IRES activity was significantly reduced in EMT-transformed MIM-RT cells harboring a La knock-down. In contrast, the knock-down of La had no significant effect on LamB1 IRES activity in MIM-R cells. These data concurs with the regulation of LamB1 protein expression (Figure 7B), indicating that La enhances IRES-mediated translation during EMT, which is impaired after knock-down of La.

8.5. Discussion

LamB1 is the β-subunit of laminin isoforms that constitute the ECM and contribute to tumor invasion and metastasis. The control of LamB1 expression at the transcriptional level by retinoic acid is well described, while the regulation at the level of translation is poorly understood [48]. We recently provided first evidence for IRES-mediated translation of LamB1 5'-UTR by using bicistronic Renilla-Firefly luciferase assays [22]. However, it has been reported that the level of IRES activity can strongly vary depending on the type of bicistronic reporters [39, 40]. In addition, suspicious splicing has been detected with a Renilla-Firefly luciferase vector [37, 38]. To verify our recent results, we therefore utilized an alternative bicistronic system encoding β-gal as upstream and CAT as downstream reporter [37]. Consistently, the LamB1 5'-UTR was able to mediate cap-independent translation of the downstream cistron in murine and human cell lines (Figure 2). Interestingly, the bicistronic activity of LamB1 and minimal XIAP IRES was similar in human SW480 colon cancer cells while it was strongly increased in murine MIM hepatocytes. These data indicate that the LamB1 IRES activity depends on the cellular context rather than on the bicistronic vector used for the determination of IRES activity.

Since bicistronic activity of the downstream reporter may result from cryptic promoter or splice sites within the 5'-UTR, we performed various assays to reliably exclude aberrant transcripts. Northern blot analysis as well as qPCR for β-gal and CAT reporters did not reveal any truncated bicistronic mRNAs (Figure 3). Noteworthy, the accurate determination of cryptic promoter activity by employing a monocistronic Firefly luciferase vector resulted in residual reporter activities after removal of the CMV promoter (Figure 3C). These enzymatic activities were detected independently of the presence or absence of LamB1 5'-UTR. RT-PCR revealed short transcripts generated by the Firefly luciferase reporter of the promoterless vector indicating a cryptic activity of the reporter itself as already described (Figure 3D, [43]). Thus, stringent assays to detect aberrant transcripts corroborate a *bona fide* IRES activity of the LamB1 5'-UTR [22], which requires a minimal *cis*-acting IRES motif localized between -293 and -1 nts upstream of the start codon (Figure 4)

IRES-mediated *de novo* synthesis of proteins involved in angiogenesis, apoptosis or mitosis plays an important role during tumorigenesis. We initially found by expression profiling that LamB1 is translationally upregulated in Ras-transformed hepatocytes (MIM-R) that undergo EMT in response to TGF-β (MIM-RT) [22]. In this study, the bicistronic assay showed a 2.7-fold increase of IRES activity in EMT-transformed cells suggesting that elevated LamB1 levels result from activated IRES translation (Figure 2E). However, diverse mechanisms are known to account for selective translational control in cancer cells including alternative splicing of the 5'-UTR or 3'-UTR shortening through alternative cleavage or polyadenylation [33, 36]. To test a possible involvement of transcript species in the regulation of LamB1

expression during tumorigenesis, we performed RACE-PCR of the 5'- and 3'-UTR. No alternative transcript variants could be detected in different human carcinoma cell lines (Figure 1B). These results are consistent with Northern blot analysis suggesting that LamB1 translation is not regulated by alternative splicing or 3'-UTR shortening (Figure 1C). Therefore, the elevated translation of LamB1 upon EMT is caused by IRES activation and does not involve alternative splicing or 3'-UTR shortening.

Hence, we next aimed to identify ITAFs that interact with the LamB1 IRES and activate its translation upon EMT. Streptavidin-tethered RNA affinity purification with a transcript containing the LamB1 5'-UTR downstream of two S1 aptamers revealed that La selectively binds the 5'-UTR *in vitro* (Figure 5A). This finding was verified *in vivo* by an anti-La RNA-IP of UV crosslinked cells in two human cell lines (Figure 5C). Consistent with our data, a recently published RNA-IP of BCR/ABL-transformed myeloid cells revealed LamB1 mRNA associated to La but not to hnRNP A1, hnRNP E2 or hnRNP K [45]. Furthermore, La binds the LamB1 5'-UTR as well as the minimal LamB1 IRES within the bicistronic transcript after transient transfection of cells (Figure 5D, 6A). The amount of La that is associated with the minimal IRES is significantly elevated in EMT-transformed MIM-RT cells as compared to MIM-R hepatocytes (Figure 6B). Accordingly, Western blot analysis of cytoplasmic and nuclear cell fractions revealed the cytoplasmic translocation of La in response to TGF-β induced EMT (Figure 6D). Furthermore, La enhanced LamB1 IRES activity in an *in vitro* translation assay (Figure 7A), suggesting that the elevated levels of LamB1 in EMT-transformed cells results from increased IRES-mediated translation in the presence of cytoplasmic La. In line with these data, the stable knock-down of La significantly reduced LamB1 IRES activity of a bicistronic construct in EMT-transformed cells (Figure 7C). In addition, endogenous LamB1 protein levels were decreased in La-silenced cell lines (Figure 7B). In contrast, La knock-down in epithelial cells did not affect LamB1 IRES translation and endogenous LamB1 protein levels. In summary, these results suggest that La accumulates in the cytoplasm during hepatocellular EMT, where it binds the minimal LamB1 IRES motif to enhance IRES-mediated translation.

A role of La in the LamB1 regulation during EMT is of particular interest as recent studies suggest an important role of La in carcinogenesis. La is a RNA binding protein involved in RNA processing and translation that predominantly localizes in the nucleus [49]. Elevated La levels have been reported in chronic myeloid leukaemia cells, cervical cancer and several types of cancer cell lines [22, 50, 51]. La was shown to activate IRES-dependent translation of XIAP which supports tumorigenesis by preventing apoptosis [42]. In addition, La regulates the IRES-mediated expression of cyclin D1 that stimulates cell proliferation [22]. Accordingly, we found cyclin D1 translationally upregulated in MIM-RT hepatocytes that have undergone EMT by employing polysome-bound versus total RNA (data not shown). Furthermore, AKT-

dependent shuttling of La to the cytoplasm leads to translational activation of a specific mRNA subset in glial cells suggesting that La could be involved in oncogenic effects of aberrant AKT activation in cancer cells [52]. In this respect, it will be of relevance to dissect the molecular mechanisms of La activated LamB1 IRES translation in the context of EMT that is induced by the synergy of Ras and TGF-β signaling and involves the activation of AKT and MAPK pathways [21].

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8.8. References

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Figures

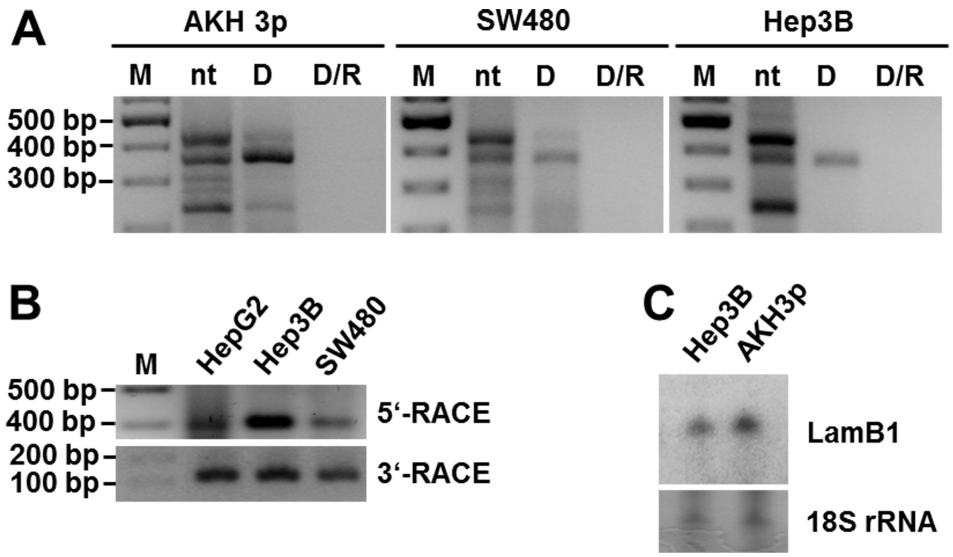


Figure 1. LamB1 UTRs in cancer cells. **(A)** The entire LamB1 5'-UTR containing 335 nts was amplified from various human cancer cells. Non-treated (nt), DNase- (D) or DNase plus RNase-treated (D/R) mRNA populations were subjected to RT-PCR (M, marker). **(B)** RACE-PCR of the LamB1 5'- and 3'-UTR in human cancer cell lines resulted in single amplicons of expected size. **(C)** Northern blot analysis revealed a single LamB1 transcript.

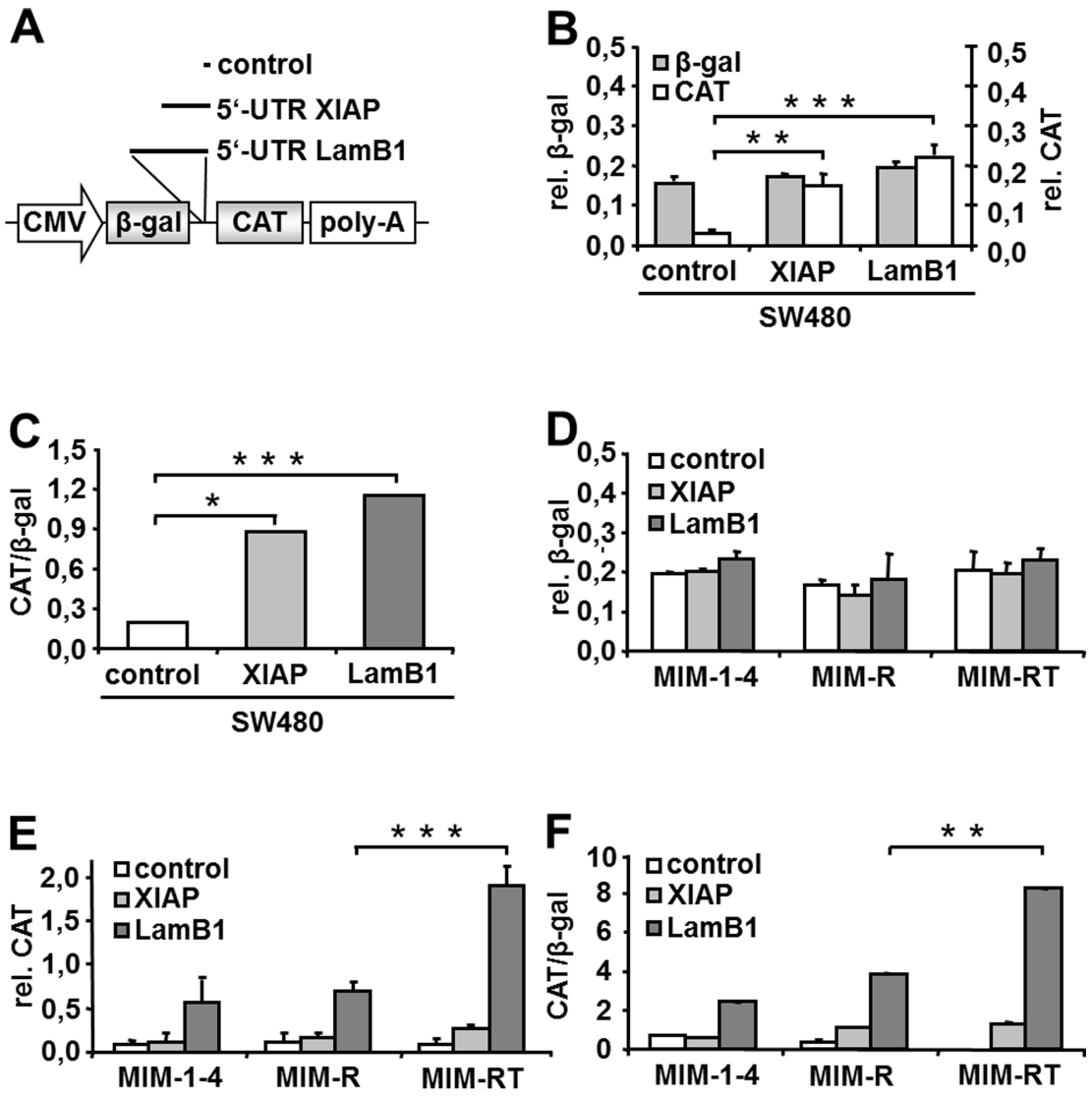


Figure 2. The LamB1 5'-UTR shows IRES activity that is activated during EMT. **(A)** Vectors employed for the bicistronic assay contain the full length LamB1 5'-UTR or the minimal XIAP IRES within the linker region of the β-galactosidase (β-gal) and chloramphenicol transferase (CAT) reporter. The empty vector is taken as negative control. **(B)** SW480 colon carcinoma cells were transfected with bicistronic vector and β-gal and CAT activities were determined after 48 hours. β-gal and CAT activities were normalized to transcript levels as quantified by qPCR. **(C)** The LamB1 5'-UTR as well as the XIAP display IRES activity in human SW480 cells. Shown is the ratio of IRES-mediated CAT to cap-dependent β-gal translation. **(D)** β-gal and **(E)** CAT activities of MIM-1-4, MIM-R and MIM-RT cells each transfected with bicistronic vectors. Cells were lysed after 48 hours and reporter activities were normalized to mRNA levels as quantified by qPCR. **(F)** As depicted by the ratio of CAT to β-gal activity, MIM-RT cells that have undergone EMT by TGF-β show significant upregulation of LamB1 IRES activity as compared to Ras-transformed malignant MIM-R hepatocytes. * $p<0,05$, ** $p<0,01$ or *** $p<0,005$.

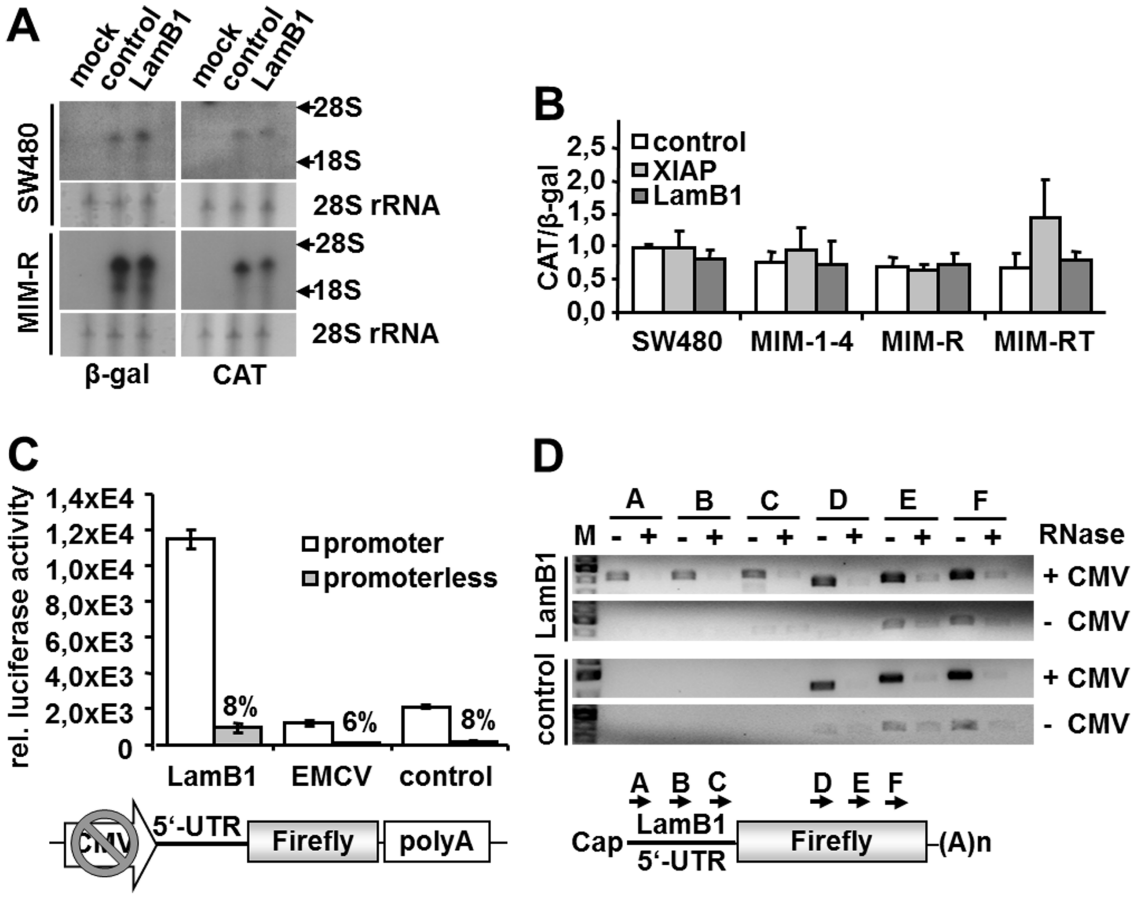


Figure 3. LamB1 IRES activity is not affected by cryptic promoter or splice sites. **(A)** Northern blot analysis of murine MIM-R hepatocytes and human SW480 colon cancer cells transfected with bicistronic constructs. Hybridization with probes for the β-gal and CAT reporter revealed single transcripts of equal size. Arrows indicate the position of 28S and 18S rRNA on the blot. 28S rRNA is shown as a loading control. **(B)** qPCR for β-gal and CAT in cells transfected with bicistronic vectors were normalized to RhoA levels, resulting in equal CAT to β-gal ratios for the vector containing LamB1 5'-UTR and empty control. **(C)** To test cryptic promoter activity in the LamB1 5'-UTR, the sequence was cloned upstream of a Firefly luciferase reporter and the CMV promoter was removed. The viral EMCV IRES and the empty vector were used as negative controls. Vectors were co-transfected with a ß-gal expressing plasmid into MIM-R cells. Firefly luciferase activites were measured after 48 hours and normalized to β-gal activities. **(D)** The cryptic promoter activity of the Firefly reporter was analyzed by linear semiquantitative RT-PCR using primer localized within the LamB1 5'-UTR (A,B,C) or the Firefly luciferase ORF (D,E,F). Arrows mark the position of forward primer each giving rise to an amplicon with 400-500 nts. Samples were treated with RNase to detect DNA contaminations.

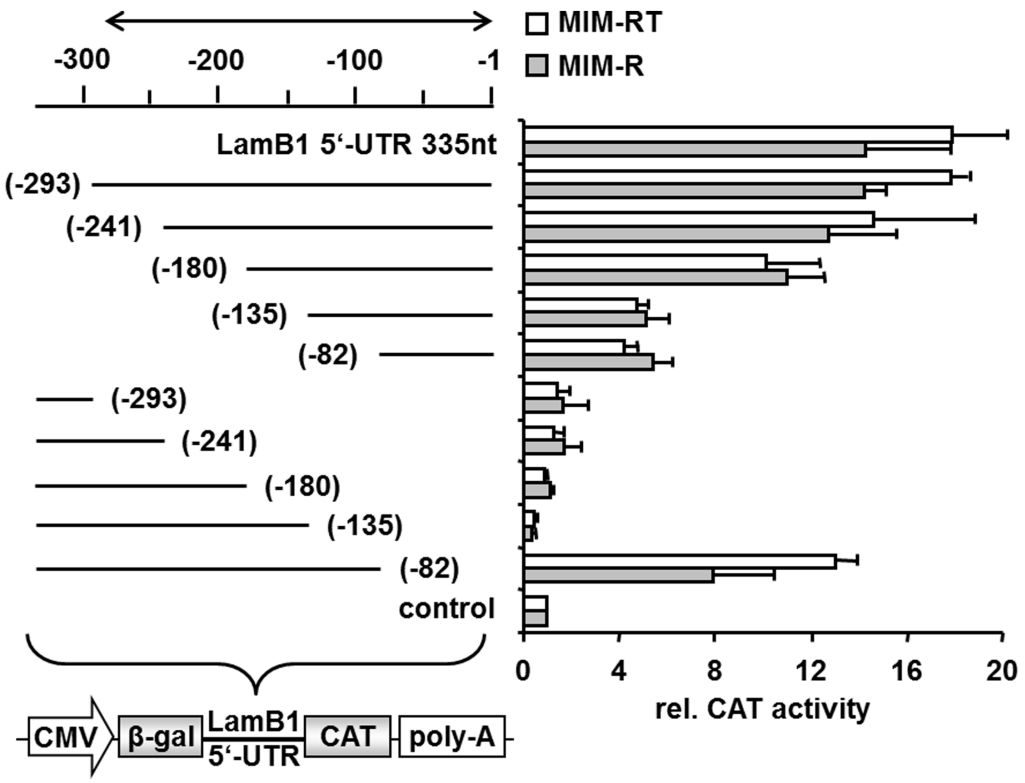


Figure 4. *Cis*-acting sequence requirements for LamB1 IRES activity. The sequence of the LamB1 5'-UTR was deleted in steps of approximately 50 nts starting from the 5'-UTR or 3'-UTR and cloned into the bicistronic β-gal/CAT reporter vector. IRES activities of bicistronic vectors were determined after transfection into MIM-R and MIM-RT cells. Lysates were analyzed after 48 hours post transfection and CAT activities were normalized to β-gal activities.

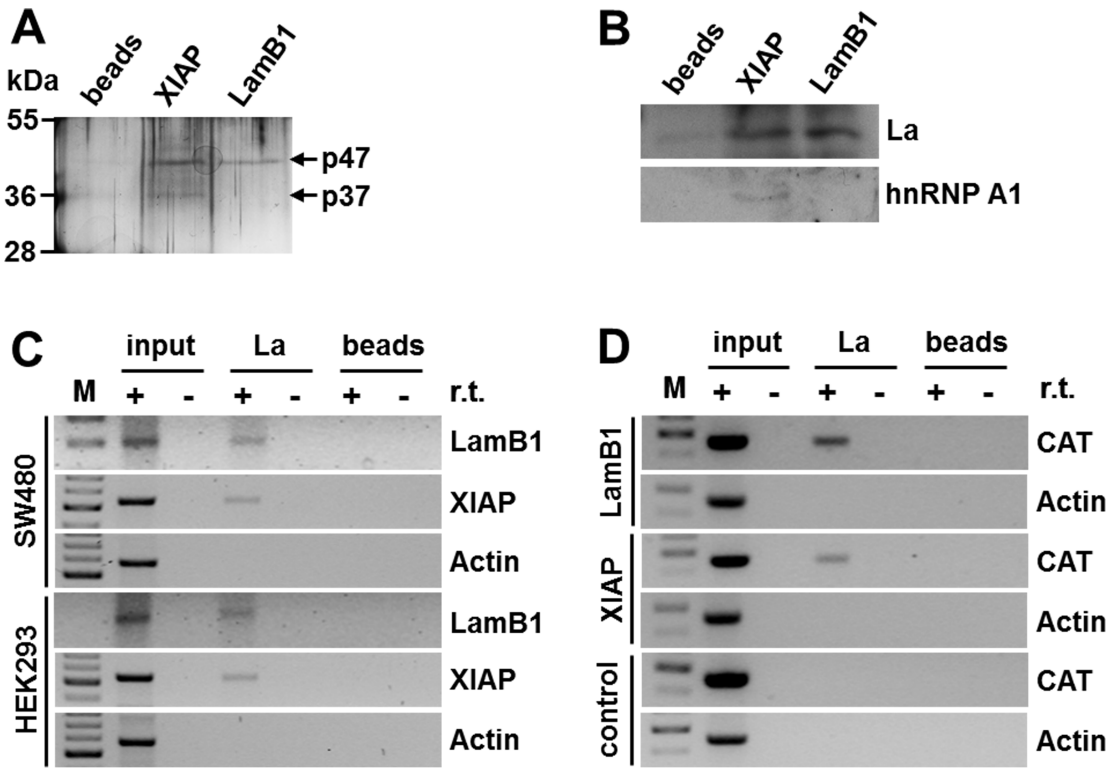


Figure 5. Identification of La interacting with the LamB1 IRES. **(A)** The LamB1 5'-UTR or XIAP minimal IRES were cloned into the pTrap vector. *In vitro* transcription of the linearized vector resulted in transcripts containing two S1 aptamers that bind streptavidin followed by the sequence of interest. The transcripts were linked to streptavidin beads and employed to pull down proteins from HEK293 cell extract. RNA-protein complexes were subjected to SDS electrophoresis. Silver staining shows a 47 kD protein interacting with both the LamB1 and XIAP IRES and a 37 kD protein interacting with the XIAP IRES only. **(B)** Proteins were identified by Western blotting as La (47 kD) and hnRNPA1 (37 kD). **(C)** Lysates of UV crosslinked SW480 and HEK293 cells were subjected to RNA-IP using the La antibody followed by RT-PCR for LamB1. cDNA was generated with or without reverse transcriptase (r.t.) to detect DNA contaminations. **(D)** HEK293 cells were transfected with bicistronic vectors, UV crosslinked after 48 hours and subjected to RNA-IP employing the La antibody followed by RT-PCR for CAT.

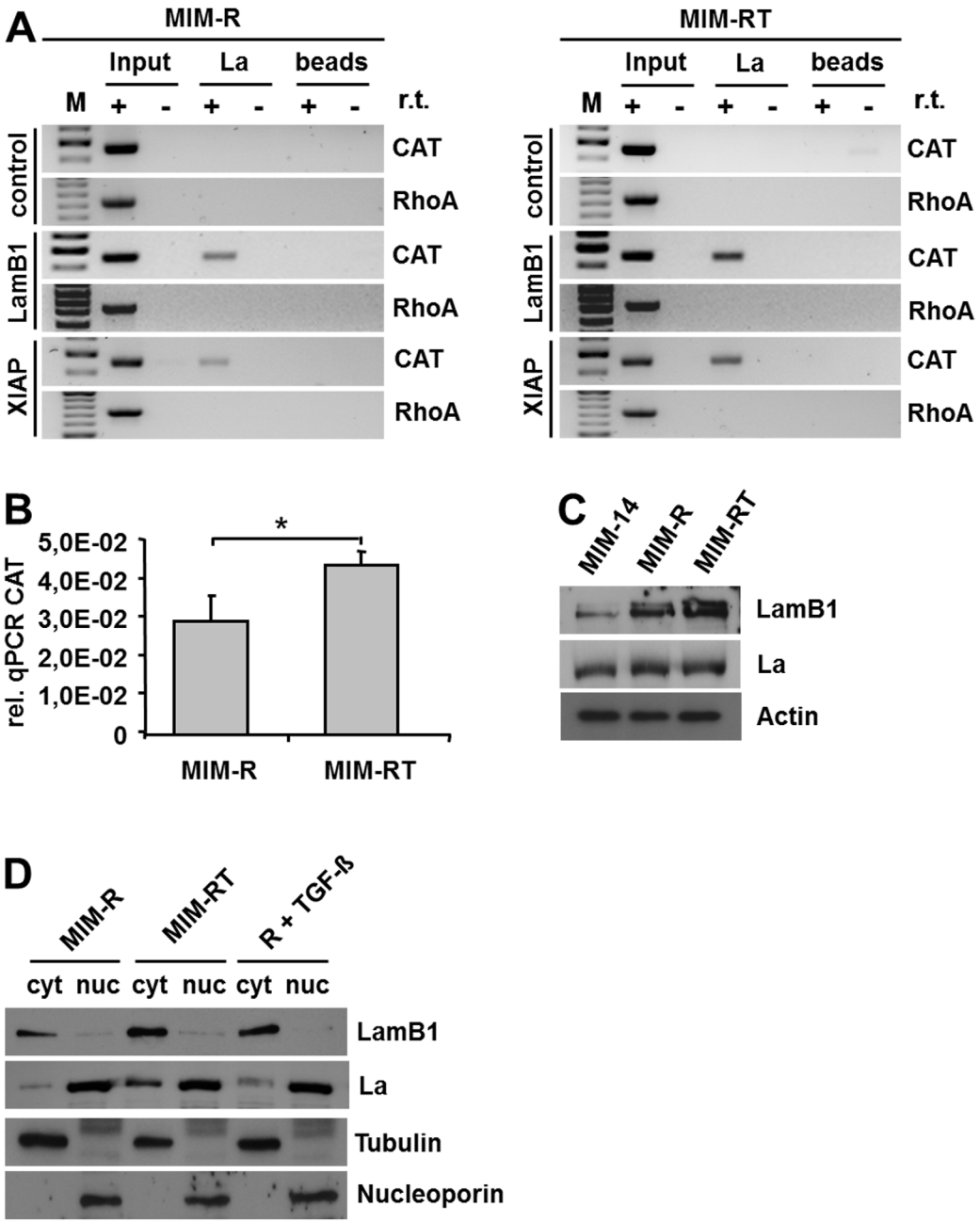


Figure 6. The binding of La to the LamB1 IRES is regulated upon EMT. **(A)** MIM-R and MIM-RT cells were transfected with bicistronic vectors containing the minimal LamB1 IRES, the minimal XIAP IRES or the empty control and after 48 hours UV crosslinked prior to cell lysis. Cell extracts were immunoprecipitated with La antibody followed by RT-PCR for the CAT reporter. cDNA was generated with or without reverse transcriptase (r.t.) to detect DNA contaminations. **(B)** Levels of La bound to LamB1 bicistronic transcripts were quantified by qPCR and normalized to LamB1 RNA input levels. **(C)** Total protein levels of La and LamB1 in MIM1-4, MIM-Ras and MIM-RT cells were analyzed by Western blotting. **(D)** Nuclear and cytoplasmic fractions of MIM-R and MIM-RT cells as well as MIM-R hepatocytes treated for 24 hours with 2,5 ng/ml TGF-β analyzed by Western blotting. The purity of cytoplasmic and nuclear cell fractions was assessed by tubulin and nucleoporin, respectively.

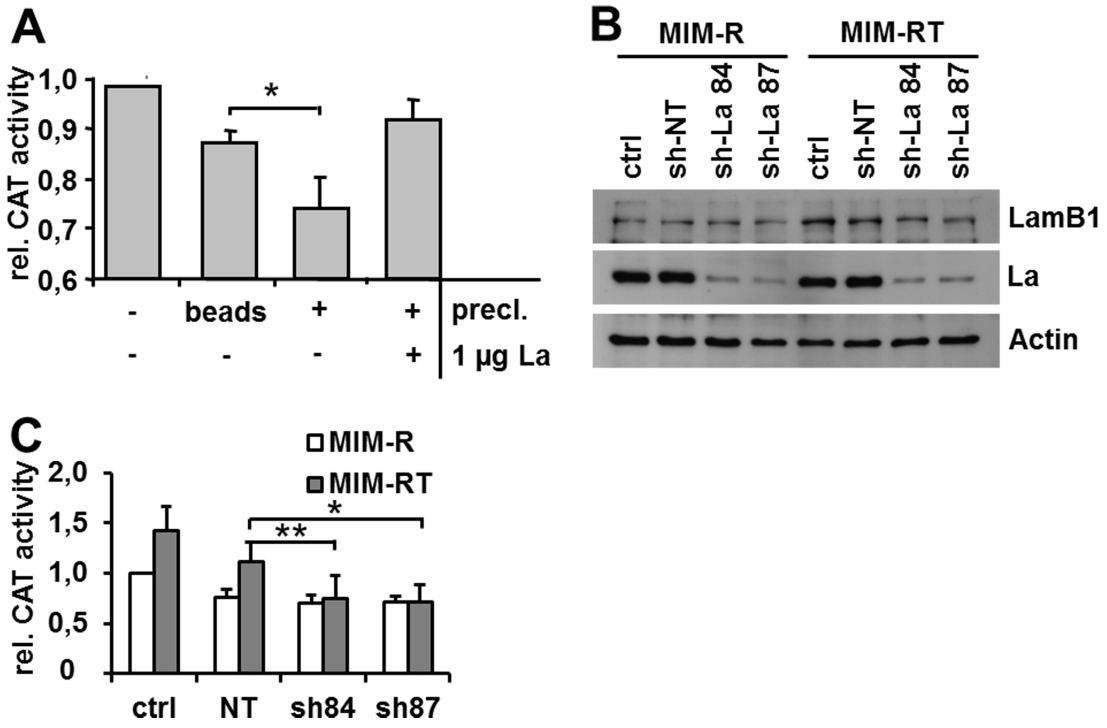


Figure 7. La stimulates IRES-driven LamB1 translation. **(A)** Rabbit reticulocyte lysate was programmed with the bicistronic vector containing the LamB1 5'-UTR. Lysates were precleared (precl.) with beads crosslinked to La antibody (+) or with nonconjugated beads (beads). Depletion of La from lysates reduced LamB1 IRES-mediated translation of the CAT reporter and addition of 1 µg recombinant La restored LamB1 IRES activity. * $p<0,05$. **(B)** MIM-R and MIM-RT cells were lentivirally infected with two different shRNA contructs against La (shLa 84, shLa 87) or non-target shRNA (sh-NT). The knock-down of La compared to the untreated control (ctrl) as detected by Western blotting. **(C)** The generated cell lines were transfected with a bicistronic vector containing the LamB1 5'-UTR. Lysates were analyzed 48 hours post transfection and CAT activities were normalized to β-gal activities. * $p<0,05$ or ** $p<0,01$.

8.9. Supplementary Data

Supplementary Table S1. Primer containing NheI and XhoI restriction sites. Used to amplify fragments of the LamB1 5'-UTR, which were cloned into the bicistronic vector pβgal/CAT for mapping of the minimal IRES motif.

description	forward	reverse
pβgal(42-335)CAT	gctagcggctttcaaacaaaagag	gcgctcgaggccggctccct
pβgal(94-335)CAT	gctagctcctcgcagagttg	gcgctcgaggccggctccct
pβgal(155-335)CAT	cctagctagcaggcgcctccct	gcgctcgaggccggctccct
pβgal(200-335)CAT	cctagctagcttctttgggctcggg	gcgctcgaggccggctccct
pβgal(253-335)CAT	cctagctagcggaagacgggaag	gcgctcgaggccggctccct
pβgal(1-42)CAT	gctagcgggacctggaagcgccccag	ctcgagcgaatctgcgatcgctgcg
pβgal(1-94)CAT	gctagcgggacctggaagcgccccag	ctcgagaccgggtgaggtcccg
pβgal(1-155)CAT	gctagcgggacctggaagcgccccag	ctcgagtctgctcatgcgcatacgg
pβgal(1-200)CAT	gctagcgggacctggaagcgccccag	ctcgagagggaattagaaagtttagccttggg
pβgal(1-253)CAT	gctagcgggacctggaagcgccccag	ctcgagctttccggcgacgcgagct
pβgal(42-253)CAT	gctagcggctttcaaacaaaagag	ctcgagctttccggcgacgcgagct

Supplementary Table S2. Primer containing KpnI and BamHI restriction sites. Used for amplification of the LamB1 5'-UTR or minimal XIAP IRES sequence, which were cloned into the pTrap vector.

description	forward	reverse
LamB1 5'-UTR	gcgcggtaccgggacctggaagcgccccag	gcgcggatccgccggctccctgcagccacg
XIAP IRES	gcgcggtaccctcgagattagaatgtttc	gcgcggatccgcttctcttgaaaaragga

Supplementary Table S3. Primer used for RT-PCR.

description	forward	reverse
human LamB1	ggcgtcttctccactcctct	gcatagcagctggacggaat
human XIAP	cttgtgtacctgcagacatc	agagtccagcacttgctaac
RhoA	ggaagaaactggtgattgttggtg	tcgtggttggcttctaaatactgg
Actin	agcacggcatcgtcaccaac	ggcgacgtagcacagcttct
CAT reporter	accagaccgttcagctggat	ccttgtcgccttgcgtataa

Supplementary Table S4. Primer for SYBR green quantitative RT-PCR.

description	forward	reverse
β-gal reporter	actatcccgaccgccttact	ctgtagcgctgatgttgaa
CAT reporter	gcgtgttacggtgaaaacct	gggcgaagaacttgtccata
RhoA	ggaagaaactggtgattgttggtg	tcgtggttggcttctaaatactgg
murine LamB1	caggaaccggagttcagcta	gcaggtggctaacaatacag

Supplementary Table S5. Primer used for the RACE-PCR of the human LamB1 5'-UTR and 3'-UTR .

description	forward	reverse
3' outer primer	ctgacattgccagagctgagat	
3' inner primer	ggatccagataaagctcaagaattagcaagac	
5' outer primer		tgcaagtggctgacgatacagtag
5' inner primer		ggatcccgggctctgcacagggctaagaaa

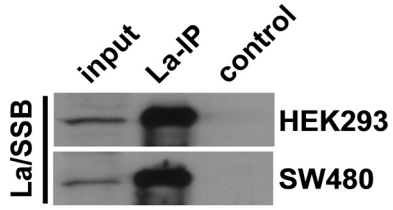
Supplementary Table S6. Primer used to generate Northern blot probes specific for transcripts of the bicistronic vector containing the LamB1 5'-UTR (pβgal/Lam/CAT).

description	forward	reverse
β-gal probe	aagcagaagcctgcgatgtc	gccgtggcctgattcattcc
CAT probe	accagaccgttcagctggat	ccttgtcgccttgcgtataa
5'UTR probe	ccccgcagcgatcgcagatt	gcggcagaggagtggagaag

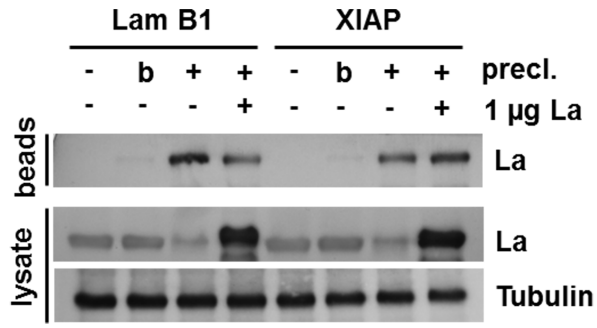
Supplementary Table S7. Primer used to detect cryptic Firefly luciferase activity of the pLam-F vector, which contains the LamB1 5'-UTR upstream of the Firefly luciferase reporter.

description	forward	reverse
A	gggacctggaagcgccccag	ggcgtcttccatggtggctt
B	tctgttcctcccaaggctaa	ccgaacggacatttcgaagt
C	ggagccggcctcgagatctg	acaccacggtaggctgcgaa
D	ctcccggttttaatgaatac	tgtcaatcagagtgcttttg
E	ttgaagaagagctgtttctg	gcttccggattgtttacata
F	gcgcggtcggtaaagttgtt	gcgacgtaatccacgatctc

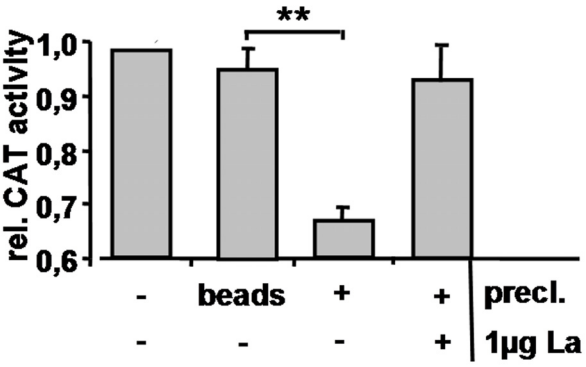
Supplementary Figure S1. Western blot analysis of La-RNA immunoprecipitation. RNA-protein complexes of UV crosslinked HEK293 or SW480 cells were immunoprecipitated with La antibody. Aliquots of cell lysates (input) and immunoprecipitations performed with anti-La conjungated (La-IP) or unconjugated (control) beads were treated with RNase A and subjected to Western blot analysis.



Supplementary Figure S2. Levels of La protein in rabbit reticulocyte lysates used for *in vitro* translation assays of LamB1 and XIAP. The preclearing of La in rabbit reticulocyte lysates was analyzed by Western blotting. La levels were determined in lysates precleared (precl.) with unlinked beads (b) or beads crosslinked to La antibody (+) and after addition of 1 µg recombinant La (lower panel). SDS-buffer eluates of beads used for preclearing show La that was immunoprecipitated from the lysates (upper panel).



Supplementary Figure S3. XIAP IRES activity depends on La. Rabbit reticulocyte lysate was programmed with the bicistronic vector containing the minimal XIAP IRES. Lysates were precleared (precl.) with unlinked beads (beads) or beads crosslinked to La antibody (+). Removal of La from lysates reduced XIAP IRES-mediated translation of the CAT reporter, whereas addition of 1 µg recombinant La restored IRES activity.



9. Manuscript III

PDGF enhances IRES mediated translation of Laminin B1 by cytoplasmic accumulation of La during epithelial to mesenchymal transition

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Key words: PDGF, IRES, Laminin B1, epithelial to mesenchymal transition, La

9.1. Abstract

The extracellular matrix protein Laminin B1 (LamB1) regulates tumor cell migration and invasion. Carcinoma cells acquire invasive properties by epithelial to mesenchymal transition (EMT) which is a fundamental step in dissemination of metastatic cells from the primary tumor. Recently, we showed that enhanced translation of LamB1 upon EMT of malignant hepatocytes is mediated by an internal ribosome entry site (IRES). We demonstrated that the IRES transacting factor La binds the minimal IRES motif and positively modulates IRES activity of LamB1. Here we show that platelet-derived growth factor (PDGF) enhances IRES activity of LamB1 by the increasing cytoplasmic localization of La during EMT. Accordingly, cells expressing dominant negative PDGF receptor display reduced cytoplasmic accumulation of La and show no elevation of IRES activity or endogenous LamB1 levels after stimulation with PDGF. Furthermore, La mediated regulation of LamB1 IRES activity predominantly depends on MAPK/ERK signaling downstream of PDGF. Notably, LamB1 expression is not significantly downregulated by the impairment of the translation initiation factor eIF4E. *In vivo*, knock-down of La associated with decreased LamB1 expression and reduced tumor growth. Together, these data suggest that PDGF is required for the cytoplasmic accumulation of La that triggers IRES-dependent translation of LamB1 during EMT.

9.2. Introduction

Hepatocellular carcinoma (HCC) is a leading cause of cancer death worldwide with increasing incidence over recent years [1 , 2]. The majority of HCC develops as a consequence of chronic liver inflammation and is preceded by liver fibrosis and cirrhosis [3]. Main reasons for the high mortality rate of patients with HCC are the late diagnosis at an advanced stage of disease and intrahepatic metastasis [4]. HCC derives from the neoplasia of hepatocytes which are highly differentiated and metabolically specialized epithelial cells [5]. During HCC progression, trans-differentiation of hepatocytes frequently occurs through epithelial to mesenchymal transition (EMT), which is characterized by abrogation of cell-cell contacts and acquisition of a fibroblastoid, highly motile phenotype [6]. EMT-transformed malignant hepatocytes show metastatic abilities by disseminating from the primary tumor and invading the surrounding tissue [7]. Dissociation of adherence junctions is accompanied by loss of cell polarity and cytoskeletal rearrangements [8]. It involves changes in integrin expression and distribution [9]. Integrins are a family of heterodimeric transmembrane receptors that connect the extracellular matrix (ECM) to the cytoskeleton of the cell. In particular, integrin β1 complexes are upregulated in Ras-transformed cells that have undergone EMT in response to transforming growth factor (TGF)-β [9-11]. They potentiate growth factor receptor driven MAPK/ERK and PI3K/AKT signaling and activate latent TGF-β [12, 13]. Moreover, β1 integrin mediated MAPK/p38 activation was found to be essential for EMT [14]. The extracellular interaction partners of integrins are laminins which are the main non-collagenous component of the ECM [15]. Laminins are heterotrimeric glycoproteins composed of several different α, β, and γ subunits which assemble in fifteen laminin isoforms that are expressed in a tissue specific manner [16]. Laminin B1 (LamB1) is the β1 subunit of six laminin isoforms including laminin 1 that is expressed in hepatocytes [16, 17]. LamB1 mediates β1 integrin signaling and activates a specific p67kDa laminin receptor (LamR) that regulates cell migration, proliferation and survival [18, 19]. Proteome profiling of HCC patients showed elevated LamB1 levels in cirrhotic liver tissues and a further increased expression in HCC cells [20]. Concomitantly, β1 integrin receptors and LamR were found to be upregulated in HCC correlating with increased tumor aggressiveness as well as poor patient survival [21, 22]. EMT-transformed cells rearrange their extracellular surrounding by enhanced secretion of ECM components as well as by cleavage through matrix metalloproteinases [23, 24]. This ECM remodeling facilitates tumor cell migration and invasion, thus essentially contributing to metastasis [24].

To study EMT of HCC cells, we previously established p19ARF deficient immortalized hepatocytes that become tumorigenic upon expression of oncogenic H-Ras and further undergo EMT in response to TGF-β treatment [25]. EMT-transformed hepatocytes establish an autocrine TGF-β signaling loop that upregulates PDGF signaling components including

PDGF receptor α (PDGF-Rα) [26]. LamB1 is translationally upregulated in hepatocytes that have undergone EMT [27] through an internal ribosome entry site (IRES) located in the 5'-untranslated region (UTR) of LamB1 mRNA. IRES-mediated translation of LamB1 is enhanced upon EMT by the cytoplasmic accumulation of the IRES transacting factor (ITAF) La that binds the minimal IRES motif [28]. In this study we show that the cytoplasmic accumulation of La is regulated by PDGF and downstream MAPK/ERK signaling in EMT-transformed cells. Cytoplasmic levels of La are essentially required for the enhanced IRES translation of LamB1 in mesenchymal hepatocytes. In vivo, stable knock-down of La results in reduced LamB1 expression and impaired tumor formation.

9.3. Material and Methods

Construction of plasmids

The bicistronic plasmid containing the 5'-UTR of Laminin B1 (LamB1) between β-galactosidase (β-gal) and chloramphenicol acetyltransferase (CAT) reporters was constructed as described recently [28]. Primers were designed according to GenBank sequence NM_002291. The monocistronic vector contains the LamB1 5'-UTR upstream of a Firefly luciferase reporter as described recently [27]. Vectors expressing 2A protease of rhinovirus serotype 2 (2Awt) or the inactive mutant C106A (2Amut) were generated as outlined [27].

Cell culture

Immortalized p19ARF deficient MIM-1-4 hepatocytes were grown on collagen-coated culture dishes in RPMI 1640 plus 10% fetal calf serum (FCS), 40 ng/ml human TGF-α (Sigma, St. Louis, USA), 30 ng/ml human insulin-like growth factor II (IGF-II, Sigma, St. Louis, USA), 1.4 nM insulin (Sigma, St. Louis, USA) and antibiotics, as described previously [29]. Epithelial MIM-R, MIM-S35 or MIM-C40 hepatocytes were derived from MIM-1-4 cells by stable retroviral transmission with a bicistronic construct expressing either oncogenic v-Ha-Ras and green fluorescent protein (GFP) or the Ras mutants S35-V12-Ras or C40-V12-Ras, respectively [25]. For interference with PDGF signaling, MIM-R cells were retrovirally transmitted with a bicistronic vector expressing the dominant negative (dn)PDGF receptor α and red fluorescent protein (RFP), resulting in MIM-RdnP [26, 30]. Treatment of the epithelial cell lines MIM-R, MIM-S35, MIM-C40 and MIM-RdnP with TGF-β1 (R&D Systems, Minneapolis, USA) for more than two weeks resulted in cells with a stable mesenchymal phenotype, namely MIM-RT, MIM-ST, MIM-CT and MIM-RTdnP, respectively. TGF-β1 was used at a concentration of 2.5 ng/ml for the first 72 hours of EMT induction. For long-term treatment of epithelial hepatocytes, 1 ng/ml TGF-β1 was supplemented to the medium [25]. Recombinant human PDGF-A/B (PeproTech, Rocky Hill, USA) was used at a concentration of 10 ng/ml. The pharmacological inhibitors LY294002 (Cell Signaling, MA, USA) against PI3K and UO126 (Cell Signaling, MA, USA) against MEK1/2 were used at a concentration of 10 μM. The inhibitor against TGF-β-RI/II (Lilly, Indianapolis, USA) was used at a concentration of 20 μM. All cells were kept at 37°C and 5% CO2 and routinely screened for the absence of mycoplasma.

RNA interference

MISSION® shRNA lentiviral transduction particles (Sigma, St. Louis, USA) were used to stably knock-down La (NM_009278) in MIM-R and MIM-RT cells as outlined recently [28].

Western blot analysis

The preparation of cellular extracts, separation of proteins by SDS-PAGE and immunoblotting were performed as described [28]. Cytoplasmic and nuclear cell fractions were generated using a Proteo-JET® kit (Fermentas, St.Leon-Rot, Germany) according to the manufacturer's description. 30 µg protein extract was loaded onto gels and immunological detection of proteins was performed with the Super Signal detection system (Pierce Chemical Company, Rockford, USA). The following primary antibodies were used: anti-laminin B1 (Neo Markers, Fremont, USA), 1:1000; anti-actin (Sigma, St. Louis, USA), 1:2000; anti-La (Cell Signaling, MA, USA), 1:1000; anti-tubulin (Sigma, St. Louis, USA), 1:1000; anti-nucleoporin (BD Biosciences, NJ, USA), 1:1000; anti-phospho-ERK (Cell Signaling, MA, USA), 1:1000; anti-ERK (Cell Signaling, MA, USA), 1:1000; anti-phospho-AKT (Cell Signaling, MA, USA), 1:1000; anti-AKT (BD Biosciences, NJ, USA), 1:1000; anti-phospho-eIF2 (Cell Signaling, MA, USA), 1:1000; anti-eIF2 (Cell Signaling, MA, USA), 1:1000; anti-phospho-p38 (Cell Signaling, MA, USA), 1:1000; anti-p38 (Santa Cruz Biotechnology, California, USA), 1:1000; anti-PDGF-receptor α (Cell Signaling, MA, USA), 1:1000. Secondary antibodies (Calbiochem, LaJolla, USA) were used at dilutions of 1:10 000. Signals were scanned and quantified with ImageQuant 5.0 (Amersham Biosciences, Little Chalfont, UK).

Quantitative polymerase chain reaction (qPCR)

RNA was extracted, DNaseI treated and reverse transcribed using a RNA isolation and cDNA synthesis kit (Quiagen, Hilden, Germany) as recommended by the manufacturer. Aliquots of cDNA were employed for Fast SYBR green qPCR (Applied Biosystems, CA, USA) and quantified with the 7500 Fast Real Time PCR System (Applied Biosystems, CA, USA). Primer sequences are shown in Supplementary Table S1.

Transient transfection and reporter assays

Cells were seeded on 6-well plates and transfected after 24 hours with Lipofectamine Plus as recommended by the manufacturer (Invitrogen, Carlsbad, USA). Cells were lysed 48 hours post transfection and reporter activities were determined. Luciferase activity was measured with a Luminoskan microplate reader (Labsystems, Farnborough, UK) as described recently [27]. β-gal activity was photometrically determined using o-nitrophenyl-β-d-galactopyranoside and CAT activity was measured by ELISA (Roche, Mannheim, Germany) as recommended by the manufacturer. In assays analyzing the interference of cap-dependent translation, reporter activities were normalized to mRNA levels as quantified by qPCR. Otherwise, relative IRES activity of the bicistronic assay was calculated as the ratio of CAT/β-gal. Assays were performed in triplicate and results represent the average of three independent experiments.

Subcutaneous tumor formation

Cells were detached from tissue culture plates and washed with PBS. 1x106 cells in 100 µl Ringer solution were each subcutaneously injected into immunodeficient C.B-17 SCID mice. Each cell line was injected four times. Experiments were performed according to the Austrian guidelines for animal care and protection.

Immunohistochemistry

Mice were sacrificed and tumors were fixed in paraformaldehyde as previously described [30]. Paraffin embedded tumor sections of 4 µm were stained with hematoxylin and eosin (H&E). Primary antibodies against LamB1 (Neo Markers, Fremont, USA) and La (Cell Signaling, MA, USA) were used at a dilution of 1:100. Biotinylated secondary antibodies were diluted 1:200. The immunoperoxidase procedure was performed using a Vectastain Elite ABC kit (Vector Laboratories Inc., CA, USA) as described by the manufacturer. DAB-Ni substrate (Vector Laboratories Inc., CA, USA) was employed for color development. Sections were counterstained with hematoxylin and mounted in Entellan®.

Immunocytochemistry

Cells were grown on collagen coated Superfrost® plus glass slides (Thermo Scientific, MA, USA) and fixed with paraformaldehyde. Primary antibodies against LamB1 (Neo Markers, Fremont, USA) and La (Cell Signaling, MA, USA) were used at a dilution of 1:100. Immunological stainings were performed as described for immunohistochemistry.

Statistical analysis

Data were expressed as means ± standard deviation. The statistical significance of differences was evaluated using a paired, non-parametric Student’s t-test. Significant differences between experimental groups were *P<0.05, **P<0.01 or ***P<0.005.

9.4. Results

IRES mediated translation of LamB1 during EMT is enhanced by cytoplasmic La

We employed a cellular HCC model based on p19ARF-/- hepatocytes transformed with oncogenic H-Ras (MIM-R) which synchronously undergo epithelial to mesenchymal transition (EMT) in response to TGF-β treatment (MIM-RT; [25]). Upon EMT, LamB1 was found to be translationally upregulated by an IRES element in the LamB1 5'-UTR [27, 28]. La was identified as an ITAF that interacts with the LamB1 IRES [28]. Increased levels of La were bound to the minimal IRES motif in EMT-transformed cells suggesting a role of La in the regulation of LamB1 IRES translation during EMT. In this study, we verified the increase of LamB1 expression in mesenchymal cells by Western blot analysis. Quantification of LamB1 protein levels revealed a 2,5 fold upregulation during EMT (Figure 1A). In contrast, qPCR displayed similar LamB1 mRNA levels in epithelial MIM-R and mesenchymal MIM-RT cells (Figure 1B). Protein levels further showed that shRNA mediated knock-down of La significantly reduced LamB1 expression in mesenchymal MIM-RT cells but not in epithelial MIM-R cells indicating that La enhances LamB1 IRES activity after EMT (Figure 1A; [28]).

La is a nuclear protein that has been described to shuttle to the cytoplasm during apoptosis or cellular stress, where it activates IRES translation of target mRNAs [31-35]. We investigated the cytoplasmic localization of La during EMT by Western blot analysis of subcellular fractions (Figure 1C). Cytoplasmic fractions showed accumulation of La in EMT-transformed cells whereas total La levels were not changed (Figure 1A and C). Interestingly, we found increased cytoplasmic La not only after EMT but also in epithelial cells after TGF-β treatment for 24 hours (Figure 1C). Since EMT is associated with ER-stress [36] and La translocates to the cytoplasm upon induction of cellular stress [34], we asked whether the regulatory mechanism responsible for La translocation is connected to TGF-β induced cellular stress. Therefore, epithelial MIM-R cells were stimulated with TGF-β and IRES mediated LamB1 expression was analyzed after expression of a bicistronic β-gal/CAT reporter during EMT kinetics (Figure 1D). β-gal activity of the first reporter shows cap-dependent translation whereas CAT activity indicates the translation of the second reporter that is regulated by the LamB1 IRES. Reporter activities were normalized to mRNA levels of the bicistronic transcript allowing an independent estimation of cap- and IRES-dependent translation. Interestingly, β-gal levels decreased during the first 48 hours of TGF-β treatment, indicating downregulation of cap-dependent translation. Reduced cap-dependent translation correlated with decreased expression of the translation initiation factor (eIF)4E and increased eIF2α phosphorylation, suggesting cellular stress (Supplementary Figure S1). In contrast, cap-dependent β-gal expression was even slightly elevated at later time points of TGF-β treatment as well as in EMT transformed cells (RT; Figure 1D), correlating with increased phosphorylation of 4E-binding protein (4E-BP; Supplementary Figure S1). These data

indicate that epithelial cells respond to TGF-β treatment with a transient downregulation of cap-dependent translation that does not persist after EMT transformation. Cellular stress and decreased availability of eIFs or cytoplasmic accumulation of La might therefore contribute to the increase of LamB1 IRES translation in response to TGF-β (Figure 1C). However, EMT-transformed cells are devoid of cellular stress and other mechanisms must be responsible for the enhanced LamB1 IRES translation.

Recently, PDGF was reported to modulate phosphorylation and subcellular localization of La in glioma [37]. Furthermore, PDGF signaling was found to be activated upon EMT and involved in cellular stress. TGF-β induced EMT of malignant hepatocytes shows upregulation of PDGF signaling components including PDGF-Rα (Supplementary Figure S1A and S1C; [26]). Kinetics of epithelial cells showed immediate activation of PDGF-Rα expression after TGF-β treatment and in EMT-transformed cells (Figure E). Expression of PDGF-Rα correlated with LamB1 protein levels (Figure 1E) and IRES activity (Figure 1D), pointing to an involvement of PDGF signaling in the regulation of LamB1 IRES translation. Investigation of pathways downstream of PDGF that are activated during EMT revealed that MAPK/ERK rather than PI3K/AKT signaling (Figure 1F) correlated with the regulation of LamB1 expression (Figure 1 E).

In summary these data show that LamB1 IRES translation is enhanced by La which accumulates in the cytoplasm of EMT-transformed cells. Stimulation of epithelial cells with TGF-β leads to a transient downregulation of cap-dependent translation and cytoplasmic localization of La. PDGF-Rα expression is induced by TGF-β during EMT and correlates with LamB1 expression and IRES activity, suggesting a role in the regulation of LamB1 translation.

The cytoplasmic accumulation of La in EMT-transformed cells is regulated by PDGF

La was reported to translocate from the nucleus to the cytoplasm where it functions as an ITAF by enhancing IRES translation of target mRNAs [32, 38, 39]. Multiple phosphorylation sites or cleavage of the C-terminal nuclear localization signal have been described to regulate the subcellular localization of La [35, 40, 41]. Yet, less is known about the regulation of La distribution, apart from a recent report showing that PDGF signaling triggers La phosphorylation and cytoplasmic accumulation in glioma cells [37]. To examine whether PDGF signaling is responsible for cytoplasmic accumulation of La and enhancement of LamB1 IRES translation, we used hepatocytes expressing a dominant negative version of PDGF-Rα (dnP) [30]. During PDGF stimulation, cells were simultaneously treated with TGF-β inhibitor to interfere with TGF-β driven endogenous PDGF signaling and to exclude the involvement of other TGF-β activated pathways. Western blot analysis of subcellular fractions revealed that cytoplasmic accumulation of La and enhanced LamB1 expression is induced by PDGF in mesenchymal MIM-RT but not in epithelial MIM-R cells (Figure 2A).

Expression of dnP impaired the upregulation of cytoplasmic La and LamB1. Immunocytochemical stainings confirmed the impact of PDGF signaling on the cytoplasmic localization of La (Figure 2B).

To investigate whether the cytoplasmic localization of La affects IRES mediated translation of LamB1, we assessed IRES activity with the bicistronic reporter assay. MIM-RTdnP showed decreased LamB1 IRES activity compared to MIM-RT, indicating a role of PDGF in LamB1 IRES regulation (Figure 2C). Impairment of TGF-β and downstream PDGF signaling by treatment with TGF-β inhibitor resulted in decreased LamB1 IRES activity in mesenchymal MIM-RT cells but not after dnP expression (RTdnP; Figure 2C). In contrast, LamB1 IRES activity was not reduced in MIM-RT cells upon the simultaneous treatment with TGF-β inhibitor and PDGF stimulation. These data indicate that PDGF is downstream of TGF-β signaling and that LamB1 IRES translation in EMT-transformed cells depends on PDGF signaling. Noteworthy, epithelial MIM-R cells express only low levels of PDGF-Rα. Thus, treatment with PDGF had no effect on LamB1 IRES translation (R, RdnP; (Figure 2C), whereas stimulation with TGF-β induced PDGF-Rα expression and LamB1 IRES activity in MIM-R cells. As expected, expression of dnP impairs the TGF-β mediated induction of LamB1 IRES translation (RdnP). Together, these data indicate that induction of EMT by TGF-β activates PDGF signaling which triggers cytoplasmic accumulation of La to enhance LamB1 IRES translation.

PDGF signals via PI3K and MAPK pathways

To characterize the pathways that are involved in the PDGF mediated regulation of La, Western blot analysis of epithelial MIM-R, mesenchymal MIM-RT cells and those expressing dnP (RdnP, RTdnP) was performed (Figure 3). Treatment with TGF-β resulted in the activation of PI3K/AKT as well as MAPK/ERK signaling and LamB1 expression in epithelial and EMT-transformed cells. Expression of dnP significantly reduced activation of these pathways and impaired the enhancement of LamB1 expression, suggesting that PDGF contributes to TGF-β-induced PI3K and MAPK signaling and regulates LamB1 expression. In order to estimate the effect of PDGF stimulation, the TGF-β triggered endogenous PDGF expression was pharmacologically inhibited. In contrast to TGF-β, PDGF was exclusively able to induce AKT and ERK signaling in mesenchymal MIM-RT but not in epithelial MIM-R hepatocytes which express low levels of PDGF-Rα. Interference with PDGF signaling by dnP expression impaired PDGF induced AKT as well as MAPK signaling and enhancement of LamB1 expression. In contrast, the expression of La was not changed (Figure 3). Interestingly, expression of dnP strongly impaired AKT and ERK signaling in response to PDGF. Thus, the correlation of both ERK and AKT phosphorylation with LamB1 expression point to their involvement in PDGF mediated regulation of LamB1 IRES translation during EMT.

LamB1 IRES translation is predominantly regulated by MAPK signaling

To analyze PDGF downstream signaling for the regulation of LamB1 IRES translation, we used cell lines expressing mutated Ras versions. C40-V12-Ras predominantly activates PI3K/AKT while S35-V12-Ras induces the MAPK/ERK signaling [25]. Cells that activate the MAPK/ERK pathway undergo TGF-β induced EMT resulting in MIM-ST cells, whereas those activating PI3K/AKT signaling acquire a scattering phenotype in response to TGF-β and fail to undergo complete EMT (MIM-CT; [25]). Cells were treated with TGF-β inhibitor and PDGF to trigger PDGF but not TGF-β induced signaling. As expected, PDGF treatment stimulated PI3K/AKT and MAPK/ERK signaling (Figure 4A). Activation of AKT was stronger in MIM-CT and MIM-RT while activation of ERK was higher in MIM-ST and MIM-RT cells. All cell lines expressed comparable levels of total La, thus being not regulated in response to PDGF treatment. LamB1 expression was increased in PDGF-stimulated MIM-ST but not in MIM-CT cells, suggesting the involvement of MAPK/ERK signaling in the regulation of LamB1 expression. Accordingly, PDGF treatment significantly elevated LamB1 IRES translation in MIM-ST and MIM-RT cell lines but showed less effect in MIM-CT cells (Figure 4B). In agreement with these data, cytoplasmic localization of La was induced by PDGF treatment in MIM-ST and MIM-RT but not in MIM-CT cells (Figure 4C and Supplementary Figure S2). These data suggest that the PDGF-induced IRES mediated translation of LamB1 is predominantly regulated by MAPK/ERK signaling, which triggers the cytoplasmic accumulation of La.

PI3K affects MAPK-dependent regulation of cytoplasmic La localization

To further examine the PDGF signaling on LamB1 IRES translation, we selectively inhibited PI3K/AKT or MAPK/ERK signaling in EMT-transformed MIM-RT cells using small molecule inhibitors targeting PI3K or MEK1/2. Inhibition of the PI3K/AKT signaling did neither affect LamB1 expression nor cytoplasmic levels of La (Figure 5A). In contrast, MEK1/2 inhibition resulted in a significant reduction of LamB1 and cytoplasmic La levels, which was even more lowered by combined inhibitor treatment. In accordance, inhibition of MEK1/2 reduced LamB1 IRES activity which was even more inhibited through the combined treatment while interference with PI3K/AKT alone had no significant effect (Figure 5B). These results suggest that cytoplasmic accumulation of La and the enhanced IRES driven expression of LamB1 is mainly regulated by MAPK/ERK signaling with an additional impact of the PI3K/AKT pathway.

Inhibition of the MAPK/ERK pathway leads to reduced eIF4E activity [42] which could be involved in the downregulation of cap-dependent LamB1 expression. To estimate whether decreased eIF4E availability could influence the expression of LamB1, a reporter assay was performed in the presence of 2A protease. This enzyme cleaves the eIF4E binding site on eIF4G and therefore disrupts the association of eIF4E with the translation preinitiation

complex [43]. We expressed 2A protease (2Awt) or the inactive mutant (2Amut) together with a reporter plasmid containing the LamB1 5'-UTR in front of a Firefly luciferase reporter. Firefly luciferase activity therefore reflects both the cap-dependent and IRES mediated translation of LamB1. To detect the impairment of cap-dependent translation by 2A protease, 2Awt or 2Amut was co-transfected with the empty reporter vector expressing Firefly luciferase. Interestingly, cap-dependent translation was significantly reduced by 2A protease activity while translation of the LamB1 5'-UTR containing reporter was not impaired (Figure 5C). Similar experiments were performed recently with a bicistronic vector showing that 2A protease activity leads to increased IRES translation of LamB1 [27]. Together, these results suggest that reduced eIF4E availability leads to enhanced IRES mediated translation of LamB1 which compensates a possible reduction of cap-dependent translation and stabilizes protein expression of LamB1. Notably, the decrease of eIF4E activity has been reported to reduce cap-dependent and to induce IRES mediated translation [44]. Diminished LamB1 expression upon inhibition of MAPK/ERK signaling can therefore not be explained by the reduction of cap-dependent translation. Thus, inhibition of MAPK/ERK does not induce a possible switch to IRES translation of LamB1. Rather, MAPK/ERK pathway regulates IRES mediated translation of LamB1 by the modulation of La translocation.

PDGF regulated subcellular localization of La correlates with LamB1 levels *in vivo*

We next addressed the question whether the localization of La correlates with LamB1 expression and the tumor formation of EMT-transformed cells in vivo. Therefore, epithelial MIM-R or mesenchymal MIM-RT cells as well as those expressing dnP or a stable knockdown of La (shLa84, shLa87) or the non-target control (shNT) were xenografted for subcutaneous tumor formation. Interestingly, the knock-down of La as well as the intervention with PDGF signaling significantly reduced subcutaneous tumor growth of EMT-transformed cells (Figure 6A and Supplementary Figure S3). Expression of LamB1 was analyzed in cell lysates or subcellular fractions obtained from tumor tissues. The knock-down of La in EMT-transformed cells decreased LamB1 expression, showing that enhanced LamB1 expression depends on La even under in vivo conditions (Figure 6B). Analysis of subcellular fractions from mesenchymal MIM-RT-derived tumors revealed a cytoplasmic accumulation of La and increased LamB1 expression in vivo (Figure 6C). Furthermore, intervention with PDGF signaling decreased the cytoplasmic accumulation of La as well as LamB1 expression. No changes in La localization could be observed in cell lysates from tumors generated by epithelial cells (data not shown). Immunohistochemical analysis further confirmed the link between cytoplasmic La and increased LamB1 levels in tumors derived from EMT-transformed cells. Tumors generated from MIM-RT cells expressing dnP showed a lack of cytoplasmic La which correlated with lowered LamB1 expression upon EMT as compared to control or shNT (Figure 6D). Similarly, reduced levels of LamB1 were observed

in tumors with a knock-down of La. In summary, these data provide strong evidence that endogenous, tumor-derived TGF-β levels are sufficient to induce PDGF mediated accumulation of La and to enhance LamB1 expression upon hepatocellular EMT.

9.5. Discussion

EMT is a crucial step in the metastatic cascade that provides carcinoma cells with the ability to remodel and cross the ECM of the basement membrane as well as to enter circulation [7]. Hepatocellular EMT is induced by the cooperation of oncogenic Ras and TGF-β signaling and essentially depends on the activation of PDGF signaling [26]. Here we present evidence that PDGF triggers the cytoplasmic accumulation of La during EMT in a PDGF-Rα dependent way. Cytoplasmic La acts as an ITAF and enhances IRES mediated translation of LamB1 in cells that have undergone EMT [28]. To our knowledge these are the first data describing the cytoplasmic shuttling of La during EMT and its regulation by PDGF-Rα that controls IRES activity.

Autocrine secretion of TGF-β results in a persistent PDGF expression in EMT transformed hepatocytes. Interference with TGF-β and PDGF signaling with small molecule inhibitors or expression of a dnP revealed that cytoplasmic accumulation of La and IRES driven translation of LamB1 depends on active TGF-β signaling and downstream PDGF-Rα expression. Furthermore, we found that PDGF contributes to the activation of MAPK/ERK and PI3K/AKT pathways in mesenchymal cells. Experiments with hepatocytes expressing mutated Ras versions clearly indicate that La translocation and LamB1 IRES translation during EMT is regulated by MAPK/ERK signaling. Yet, combined pharmacological inhibition of MAPK/ERK and PI3K/AKT pathways had a more significant effect than sole inhibition of MEK1/2, suggesting that active PI3K/AKT signaling contributes to La translocation. Accordingly, kinetics of TGF-β induced EMT show a more prominent upregulation of LamB1 IRES activity at later time points correlating with elevated PI3K/AKT signaling (Figure 1D and F). Importantly, an assay employing 2A protease expression showed that the effects observed upon MEK1/2 or AKT inhibition are not merely a result of decreased eIF4E availability, but rather depend on cytoplasmic accumulation of La.

PDGF signaling was not only activated in EMT transformed cells, but also shortly after TGF-β stimulation of epithelial cells (Figure 1E). Cap-dependent translation is reduced during 48 hours post TGF-β treatment. This reduction correlates with increased eIF2α phosphorylation and activation of the p38/MAPK pathway, pointing to cellular stress (Figure 1F, Supplementary Figure 1D). Indeed, a link between EMT and ER-stress has been reported in thyroid cells [36]. Activation of PDGF-Rα expression as well as cytoplasmic localization of La in response to cellular stress has been described recently [33, 34]. PDGF-Rα was found to signal via Src during cellular stress. However, experiments with pharmacological inhibitors against Src or Janus kinases had no effect on LamB1 IRES activity (data not shown). Therefore, we exclude the involvement of the Src or JAK/Stat pathway in regulating cytoplasmic accumulation of La during TGF-β induced stress and the increase of LamB1 IRES translation. Our data rather indicate that MAPK/ERK signaling

regulates the immediate translocation of La upon TGF-β stimulation. It remains to be clarified whether PDGF is generally involved in regulating La translocation via MAPK/ERK during cellular stress or whether it is a TGF-β mediated effect. Cellular stress associating with a switch to PDGF-Rα signaling and IRES mediated translation is activated in this context, suggesting that PDGF signaling could be involved in the regulation of further ITAFs in addition to La. This opens the question whether other ITAFs are regulated upon EMT and possibly contribute to the enhanced IRES translation of LamB1.

The significance of these findings is underlined by recent findings demonstrating that cytoplasmic La contributes to the translation of oncogenes. Aberrant regulation of La has been particularly described in chronic myeloid leukaemia, where BCR/ABL mediated activation of La supports tumorigenesis [45, 46]. Phosphorylation dependent shuttling of La and translational activation of target genes that include tumor promoting factors was found in glioma cells [37]. However, La modulates translation in diverse ways including binding to the 3'-UTR or to the cap of oligopyrimidine containing mRNAs depending on the phosphorylation status [37, 47]. Cytoplasmic La in EMT transformed cells might therefore affect translation of target genes on multiple levels. So far, La mediated modulation of IRES translation during apoptosis and cell cycle progression has only been described for XIAP, BIP and Cyclin D1 [32, 38, 39]. Truncation as well as phosphorylation were found to trigger the cytoplasmic translocation of La during apoptosis or cellular stress, suggesting that multiple signaling events are involved in regulating subcellular La distribution [35, 40, 41]. Recently, Brenet et. al. published a PDGF-B induced, AKT dependent phosphorylation of La. In contrast to these findings, PDGF induced translocation of La rather depends on the MAPK/ERK pathway during hepatocellular EMT.

LamB1 signaling includes binding to LamR and β1 integrin receptors, which were both found to be overexpressed in HCC [21, 22]. Rearrangement of integrin receptor allocation and abundance is an essential step during EMT that leads to enhanced cell motility and survival [9, 14, 48]. Consistently, LamB1 was found to be involved in the regulation of cell migration suggesting that increased IRES translation of LamB1 during EMT may contribute to tumor cell migration and metastasis [18, 49]. Furthermore, EMT is associated with the activation of cell programs that regulate dedifferentiation during embryogenesis and has been suggested to be involved in the generation of tumor stem cells [8, 50]. Interestingly, LamB1 is the first laminin subunit to be expressed during early embryogenesis and is essentially involved in cell differentiation [51]. While embryonic cells expressing LamB1 alone have a mesenchymal phenotype, additional activation of Laminin α expression during E4 stage induces epithelial cell polarization via α6β1 integrin signaling. Accordingly, a recent publication reported the cultivation of embryonic stem cells on laminin 10, which contains the LamB1 subunit, without the need of feeder cells [52]. These data indicate that the context of

laminin expression might be important for the generation of stem cell niches. Finally, Laminin 1 as well as LamB1 peptides were found to regulate tube formation and sprouting during tumor vascularization [53, 54]. Angiogenesis is induced by hypoxic conditions and is driven by the increased IRES translation of VEGF-A, PDGF and bFGF mRNAs in response to HIF1α activation [55, 56]. Thus, it is promising to investigate the involvement of IRES driven LamB1 translation during tumor angiogenesis and metastasis.

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9.7. References

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9.8. Figures

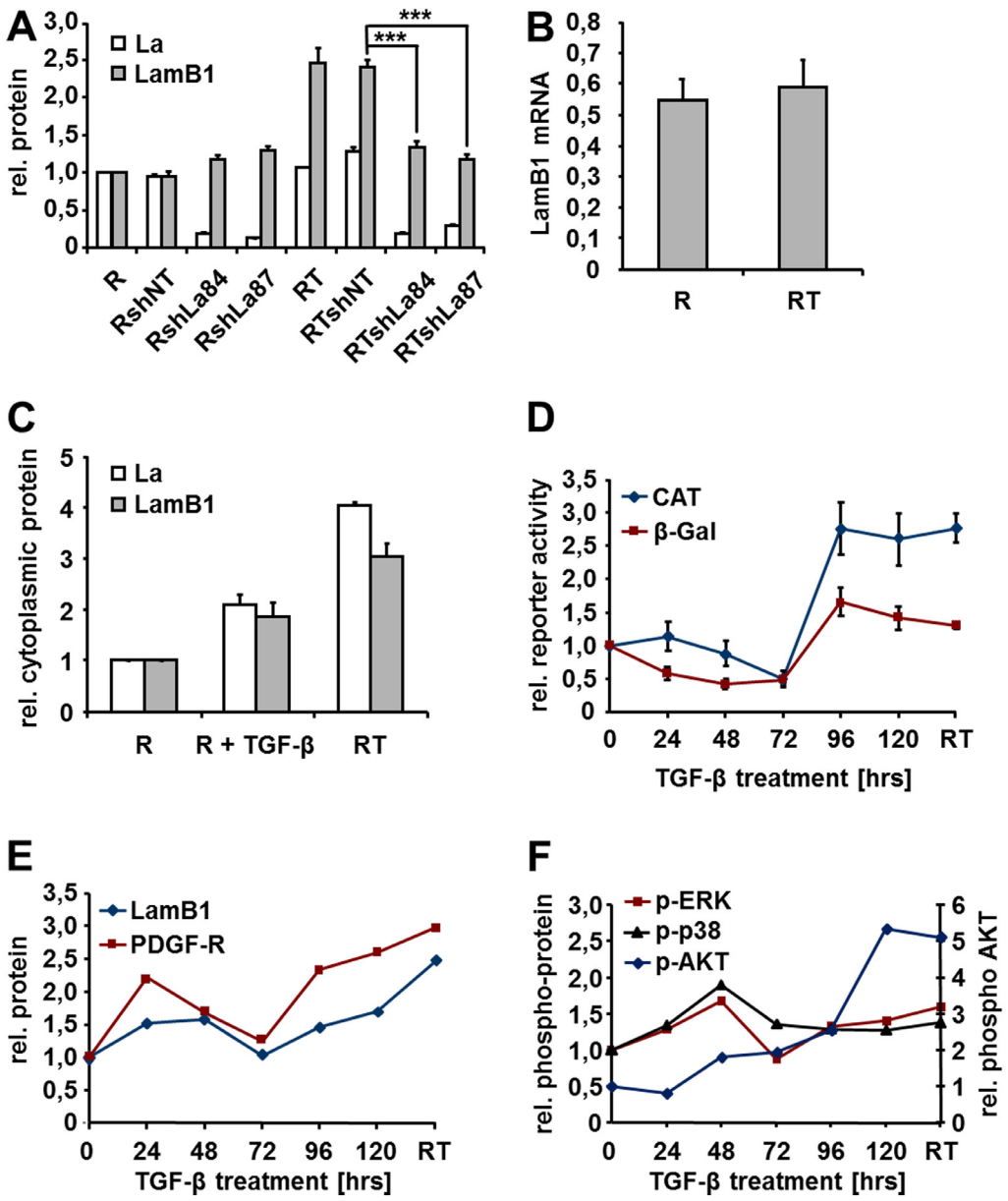


Figure 1. LamB1 IRES translation is enhanced by La during EMT. (A) Expression of shRNA against La (sh84, sh87) resulted in a stable knock-down of La in epithelial MIM-R (R) and fibroblastoid MIM-RT (RT) cells. Non-target shRNA (shNT) was used as control. Protein levels of La and LamB1 were detected by Western blotting and were quantified. The protein levels of MIM-R cells were set to a value of 1 for normalization. (B) qPCR of LamB1 mRNA levels in MIM-R and MIM-RT cells. (C) Cytoplasmic fractions of MIM-R and MIM-RT cells as well as MIM-R cells treated for 24 hours with 2,5 ng/ml TGF-β were analyzed by Western blotting and protein levels were quantified. The protein levels of MIM-R cells were set to a value of 1 for normalization (D) EMT of epithelial MIM-R cells was induced by administration of 2,5 ng/ml TGF-β in intervals of 24 hours. Cells were transfected with the bicistronic vector

containing the LamB1 5'-UTR between the β-gal and CAT reporter. β-gal and CAT activities were detected 48 hours after transfection and normalized to mRNA levels as quantified by qPCR. Relative reporter activities of MIM-R cells were set to a value of 1 for normalization (E) Total protein or (F) phospho-protein levels were detected by Western blotting and were quantified. The protein levels of MIM-R cells were set to a value of 1 for normalization ***P<0.005.

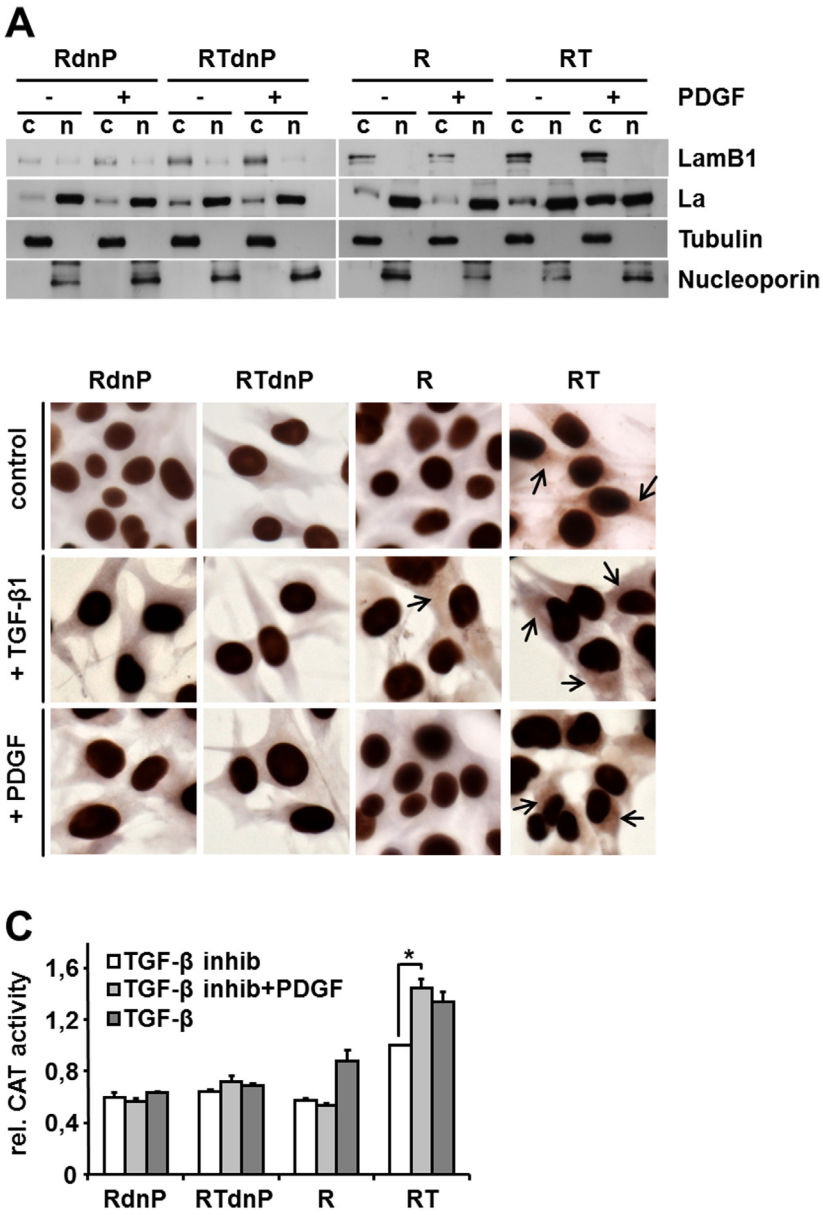


Figure 2. The EMT-induced cytoplasmic accumulation of La is regulated by PDGF. Epithelial MIM-R and mesenchymal MIM-RT hepatocytes and those expressing dominant negative PDGF-Rα (dnP) were employed to study the subcellular localization of La. All cells were treated with 20 μM TGF-β inhibitor or stimulated with 10 ng/ml PDGF for 32 hours in the presence of 20 μM TGF-β inhibitor. (A) Cytoplasmic (c) and nuclear fractions (n) were analyzed by Western blotting. The purity of subcellular fractions was assessed by detection of tubulin (c) and nucleoporin (n). (B) Immunocytochemical staining of La. Arrows indicate cytoplasmic localization of La. (C) The effect of impaired PDGF signaling on LamB1 IRES activity was analyzed with a bicistronic reporter assay. Cells were transfected with a bicistronic vector containing the LamB1 5'-UTR. β-gal and CAT activities were detected 48 hours after transfection. Relative reporter activities of MIM-RT cells treated with TGF-β inhibitor were set to a value of 1 for normalization *P<0.05

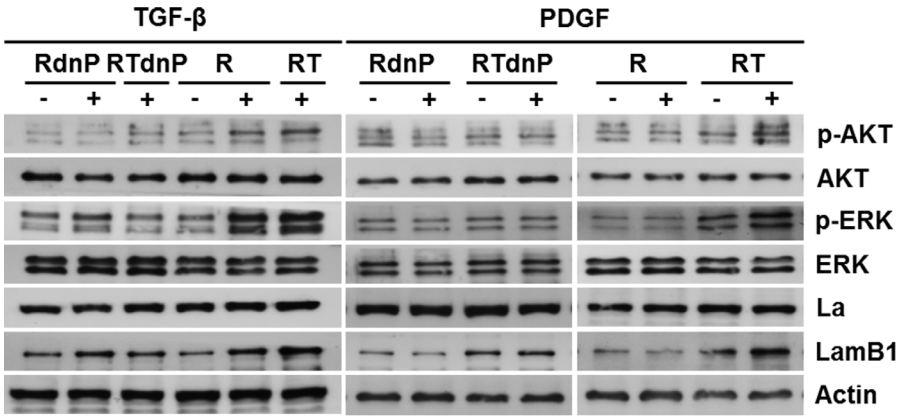


Figure 3. PDGF activates PI3K and MAPK signaling in EMT-transformed cells. MIM-R and MIM-RT cells or those expressing dnP were employed to analyze PDGF induced signaling by Western blotting. MIM-R and MIM-RdnP cells were treated with 2,5 ng/ml TGF-β for 24 hours (RdnP, R) or persistently cultured with 1 ng/ml TGF-β (RTdnP, RT) to analyze TGF-β signaling. Cells were cultured with 20 μM TGF-β inhibitor and simultaneously treated with 10 ng/ml PDGF for 32 hours to study PDGF signaling.

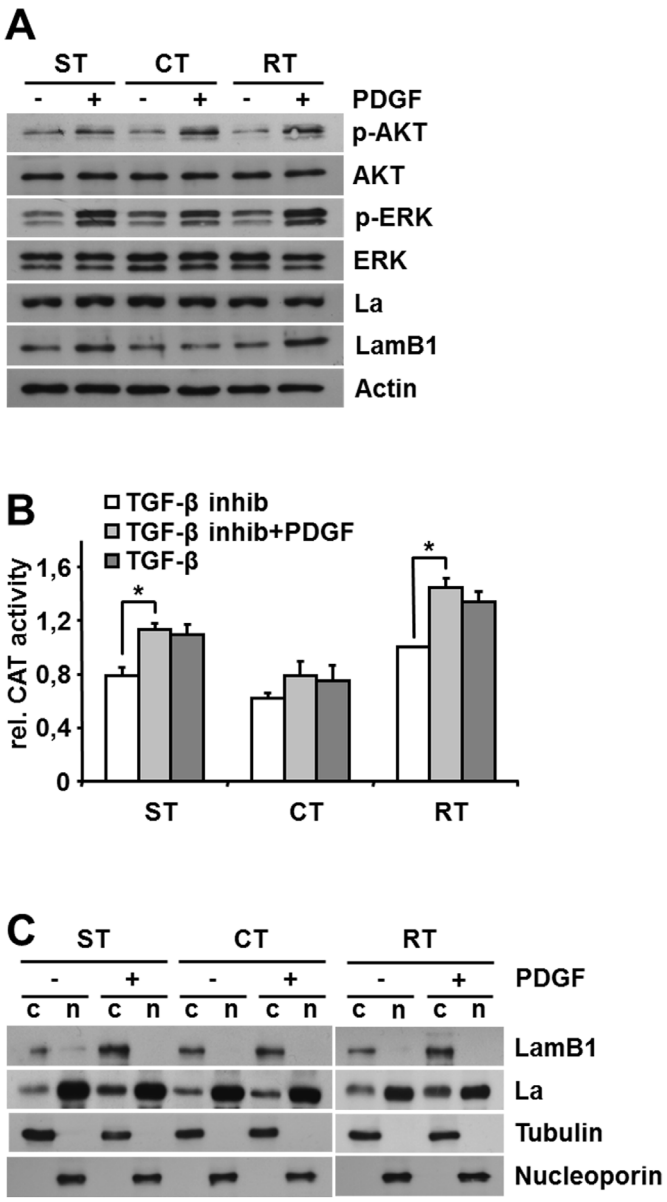


Figure 4. LamB1 IRES translation is enhanced by PDGF driven MAPK signaling. Hepatocytes expressing S35-V12-Ras undergo EMT in response to TGF-β (MIM-ST) whereas cells expressing C40-V12-Ras acquire a scattering phenotype (MIM-CT). All cells were treated with 20 μM TGF-β inhibitor or stimulated with 10 ng/ml PDGF for 32 hours in the presence of 20 μM TGF-β inhibitor. (A) Western blot analysis of cells in response to PDGF. (B) Cells were transfected with a bicistronic vector containing the LamB1 5'-UTR to analyze LamB1 IRES activity. β-gal and CAT reporter activities were measured 48 hours post transfection. Relative reporter activities of MIM-RT cells treated with TGF-β inhibitor were set to a value of 1 for normalization (C) The subcellular localization of La was analyzed by Western blotting of cytoplasmic (c) and nuclear (n) fractions. Tubulin and nucleoporin served as cytoplasmic or nuclear marker, respectively. *P<0.05.

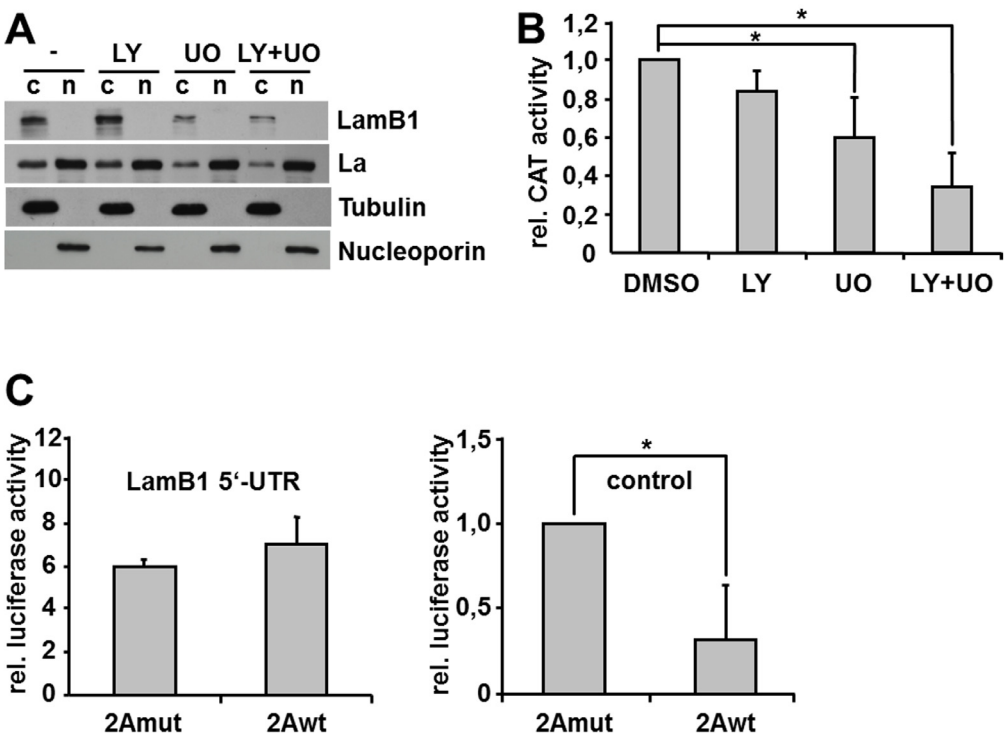


Figure 5. Cytoplasmic accumulation of La depends on the cooperation of MAPK and PI3K signaling. MIM-RT cells were treated with 10 µM of PI3K (LY) and/or MAPK (UO) inhibitor. DMSO was used as control. (A) Western blotting of cytoplasmic (c) and nuclear (n) fractions to analyze subcellular localization of La. (B) Cells were transfected with the bicistronic vector containing the LamB1 5'-UTR to study LamB1 IRES activity. CAT reporter activities were measured 48 hours after transfection and normalized to mRNA levels as quantified by qPCR. Relative reporter activities of MIM-RT cells treated with DMSO were set to a value of 1 for normalization (C) MIM-RT cells were co-transfected with a vector expressing 2A protease (2Awt) or an inactive mutant (2Amut) together with either a plasmid containing the LamB1 5'-UTR in front of a Firefly luciferase reporter or the empty Firefly luciferase reporter as control. Relative reporter activities of MIM-RT cells that where co-transfected with the 2Amut and Firefly luciferase control vector were set to a value of 1 for normalization *P<0.05.

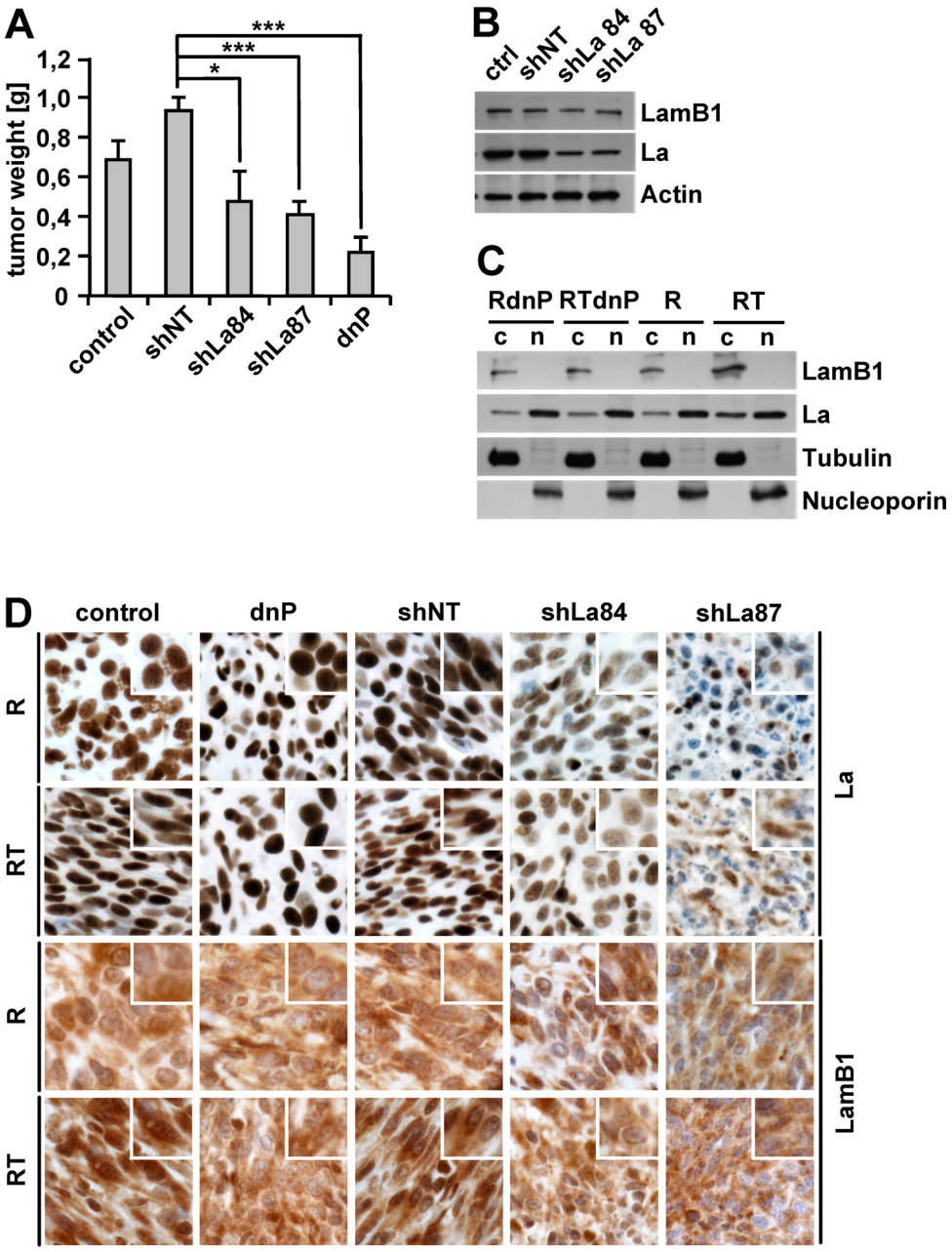


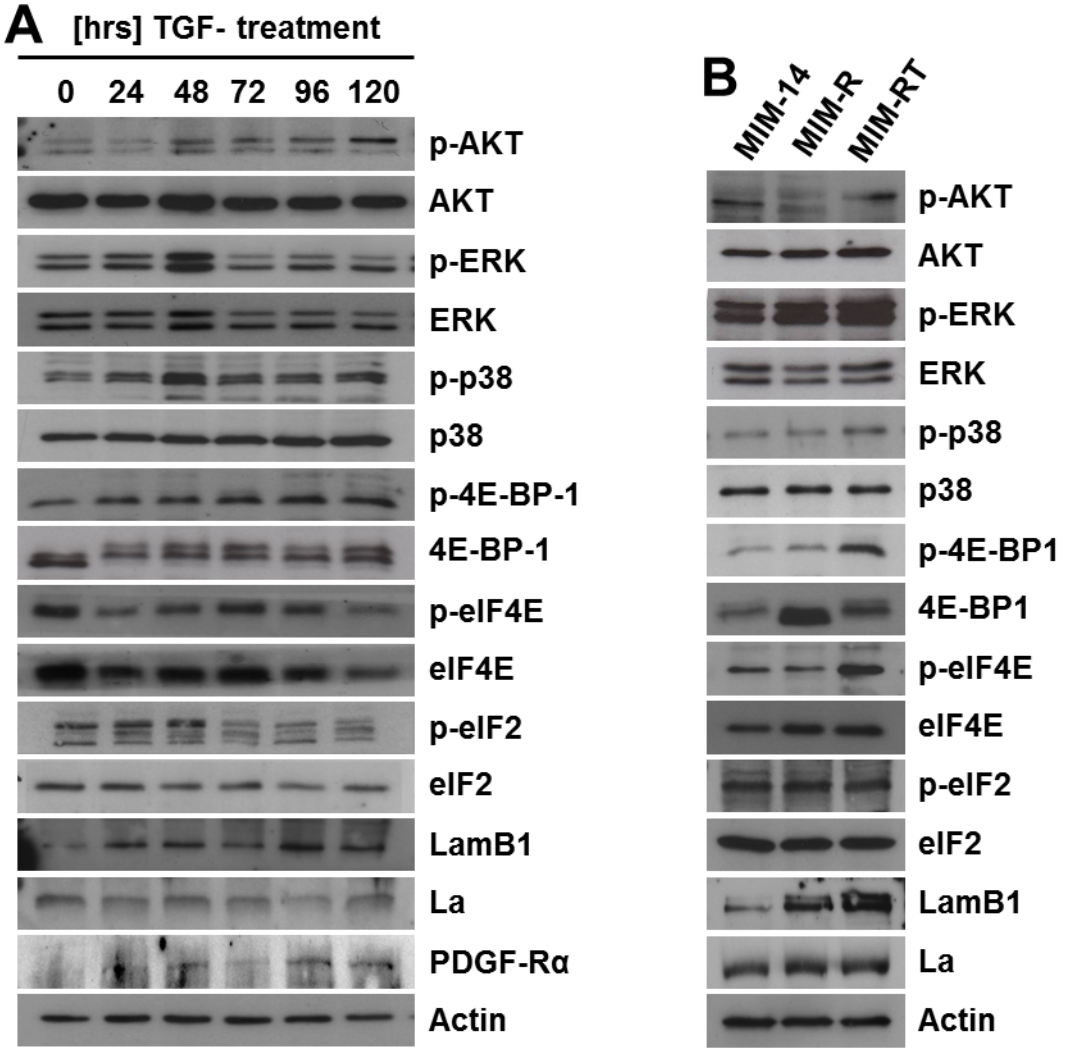
Figure 6. Stable knock-down of La reduces LamB1 expression and impairs tumor formation of EMT-transformed hepatocytes. Epithelial (MIM-R, R) or mesenchymal (MIM-RT, RT) hepatocytes expressing dnP or a stable knock-down of La (sh84, sh87) or the non-target control (shNT) were subcutaneously injected into SCID mice. (A) Mean weight of subcutaneous MIM-RT tumors after 16 days of growth in SCID mice. (B) Western blot analysis of MIM-RT-derived tumor tissue. (C) Western blotting of cytoplasmic (c) and nuclear (n) fractions isolated from tumor tissue. (D) Histological stainings of subcutaneous tumors. Insets show stainings at higher magnification. *P<0.05 or ***P<0.005.

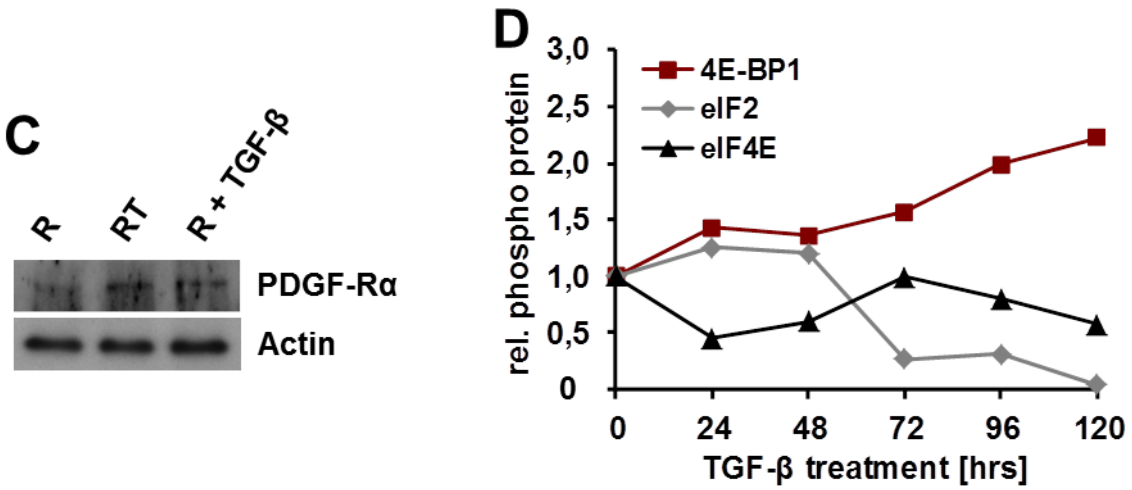
Supplementary Data

Supplementary Table S1. Primer for quantitative RT-PCR.

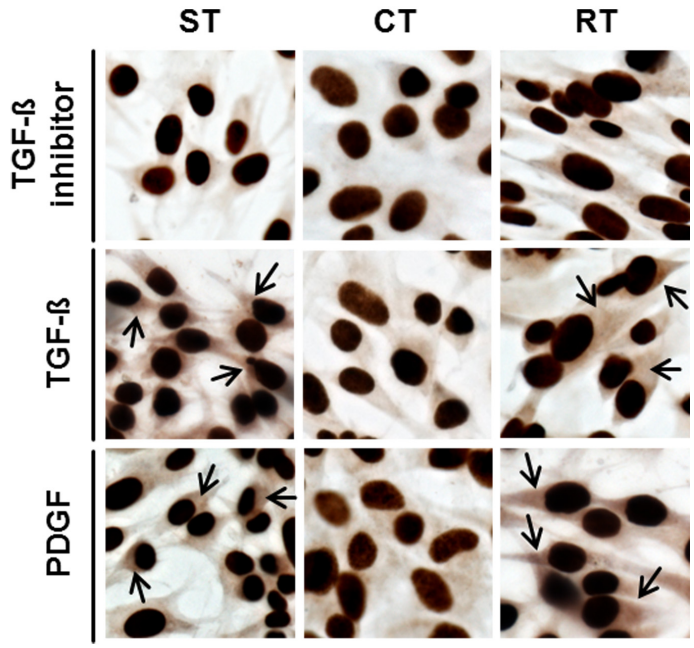
description	forward	reverse
β-gal reporter	actatcccgaccgccttact	ctgtagcgctgatgttgaa
CAT reporter	gcgtgttacggtgaaaacct	gggcgaagaacttgtccata
RhoA	ggaagaaactggtgattgttggtg	tcgtggttggcttctaaatactgg
murine LamB1	caggaaccggagttcagcta	gcaggtggctaacaatacag

Supplementary Figure S1. Western blot analysis as quantified and shown in Figure 1E and 1F. (A) EMT was induced in MIM-R cells by treatment with 2,5 ng/ml TGF-β for 120 hours. Cells were passaged every 24 hours and supplied with TGF-β. (B) Western blot analysis of immortalized (MIM-1-4), Ras-transformed (MIM-R) and EMT-transformed (MIM-RT) hepatocytes. (C) Western blot analysis of MIM-R (R) and MIM-RT (RT) cells or MIM-R cells stimulated with 2,5 ng/ml TGF-β for 24 hours (R+TGF-β). (D) Quantification of phospho-eIFs levels that are shown in Supplementary Figure 1A.

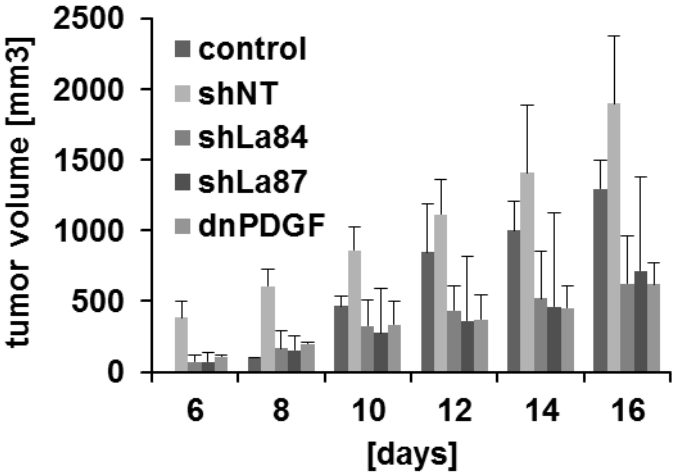




Supplementary Figure S2. Immunocytochemical stainings for La. EMT- transformed cells that preferentially activate MAPK/ERK (MIM-ST) or PI3K/AKT (MIM-CT) signaling in response to cognate signals were cultured with 1 ng/ml TGF-β, treated with TGF-β inhibitor or co-treated with TGF-β inhibitor and PDGF for 32 hours. Cells were fixed on collagen coated glass slides and stained for La. Arrows indicate cytoplasmic localization of La.



Supplementary Figure S3. Mesenchymal MIM-RT (control) hepatocytes or those MIM-RT cells expressing a stable knock-down of La (shLa84, shLa87) or the non-target control (shNT) or dnP were subcutaneously injected into SCID mice. Tumor length and width were measured every two days and the tumor volume was calculated by the formula (length x width2) x ½.



10. Acknowledgements

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Publications

Petz, M., Them, N., Huber, H., Beug, H., Mikulits, W., (2012) **La enhances IRES mediated translation of laminin B1 during malignant epithelial to mesenchymal transition.** *Nucleic Acids Res*., Vol. 40, No. 1, p. 290-302

Grusch, M., Petz, M., Metzner, T., Öztürck, D., Schneller, D., Mikulits, W. (2010) **The crosstalk of RAS with the TGF-ß family during carcinoma progression and its implications for targeted cancer theraphy.** *Curr. Cancer Drug Targets*, Vol. 10, No. 8, p 849-857

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Petz, M., Kozina, D., Huber, H., Siwiec, T., Seipelt, J., Sommergruber, W. Mikulits, W. (2007) **The leader region of Laminin B1 mRNA confers cap-independent translation.** *Nucleic Acids Res* , Vol. 35, No. 8, p. 2473–2482

Poster and Talks

2011 Nov. **RNA Club 2011**
Petz, M., Them, N., Huber, H., Mikulits, W., PDGF drives IRES mediated translation of LamB1 by regulating cytoplasmic localization of La during tumorigenesis, **talk**

2011 Jun. **7th YSA Ph.D. Symposium**
Petz, M., Them, N., Huber, H., Mikulits, W., IRES mediated regulation of Laminin B1 translation during carcinoma progression, **poster, presentation award**

2010 Sept. **2nd Annual Meeting Austrian Association of Molecular Life Sciences and Biotechnology**

Petz, M., Huber, H., Mikulits, W., Cap-independent translation of Laminin B1 during liver cancer progression, **poster**

2010 Sept. **Cold Spring Habor Meeting: Translational Control**

Petz, M., Huber, H., Mikulits, W., IRES mediated regulation of Laminin B1 translation during carcinoma progression, **poster**

2010 Jun. **6th YSA Ph.D. Symposium**

Petz, M., Hau, M., Huber, H., Mikulits, W., Cap-independent translation of Laminin B1 during liver cancer progression, **poster**

2010 Apr. **45th Annual Meeting of the European Association for the Study of the Liver**

Petz, M., Hau, M., Huber, H., Mikulits, W., Cap-independent translation of Laminin B1 during liver cancer progression, **poster, young Investigator bursary**

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2009 Jun. **5th YSA Ph.D. Symposium**

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2007 Dec. **RNA Biology Symposium**

Translation regulation by miRNAs, **talk**

2007.Oct. **3rd Joint Meeting on Experimental and Clinical Cancer Research**

Petz, M., Kozina, D., Huber, H., Siwiec, T., Seipelt, J., Sommergruber, W. Mikulits, W. The leader region of Laminin B1 mRNA confers cap-independent translation, **poster**

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Petz, M., Kozina, D., Huber, H., Siwiec, T., Seipelt, J., Sommergruber, W. Mikulits, W. The leader region of Laminin B1 mRNA confers cap-independent translation, **poster**

2007 Jun. **YSA Ph.D. Symposium**

Petz, M., Kozina, D., Huber, H., Siwiec, T., Seipelt, J., Sommergruber, W. Mikulits, W. The leader region of Laminin B1 mRNA confers cap-independent translation**, poster**

2007 Mar. **Forschungssoirée Initiative Krebsforschung**

Petz, M., Pils, D., Gugerell, A., Pangerl, T., Wittinger, M., Mikulits, W., Krainer, M. Functional characterization of N33 (TUSC3), a novel gene with high relevance for ovarian cancer prognosis, **talk**

Lecturing and Supervision

2009 **student lecture:**

Molecular mechanisms in hepatocellular carcinoma

co-supervision of diploma thesis:

2010-2011 Nicole Them; Translation regulation of Laminin B1 during hepatocellular EMT

2009-2010 Mara Hau; Translational regulation of LamB1 and ILEI during cancer progression

2007-2008 Alfred Gugerell; Characterization of the potential tumor suppressor gene N33 (TUSC3) in ovarian cancer