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Translational regulation of Laminin B1 and ILEI during cancer progression

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„Der Mensch wird vom Gedanken geschaffen.
Ein Mensch wird das, woran er denkt.
Denke, du bist stark; Du wirst stark werden.
Denke, du bist schwach; Du wirst schwach werden.
Denke, du bist töricht; Du wirst ein Dummkopf werden.
Denke du bist Gott; Du wirst Gott.“

Swami Sivananda

Table of Contents

1. Summary / Zusammenfassung

1.1. Summary

Translation control is an important mechanism to regulate polypeptide production during physiological processes. Extrinsic and intrinsic factors control the availability of the translation machinery including eukaryotic initiation factors (eIFs), thus influencing the different steps in messenger RNA (mRNA) translation. Besides cap-dependent initiation mechanisms of translation, several mRNAs harbor an internal ribosome entry site (IRES) within the 5´-untranslated region (UTR) for alternative translation, which is suggested to have severe implications in tumorigenesis.

Laminin B1 (LamB1) represents an extracellular matrix (ECM) protein involved in ECM-cell interactions which affects cell migration, proliferation and differentiation. Recent studies correlated enhanced LamB1 receptor expression with cancer cell invasiveness. Furthermore, LamB1 was detected to be translational upregulated during hepatocellular epithelial to mesenchymal transition (EMT). In addition, it has been suggested that the LamB1 5´-UTR contains an IRES element that is responsive to changes during malignant progression of hepatocytes.

In this study, we show that the LamB1 5´-UTR is able to direct translation of a bicistronic reporter construct in murine neoplastic hepatocytes. Furthermore, both mitogen-activated protein kinase and phosphatidylinositol-3-kinase signaling might be crucial for cap- and cap-independent LamB1 translation during EMT of hepatocytes. Notably, expression of bicistronic reporter constructs containing deletions of the LamB1 5´-UTR revealed that the region from 155 to 335 cannot retain a full IRES activity. Therefore, we suppose that the nucleotide sequence 1 to 155 of the LamB1 5´-UTR might be indispensable for LamB1 IRES activity. Recent data suggest that 3´-UTRs of mRNAs are able to modulate cap-dependent translation via interaction of the poly(A)-binding protein with eIF4G. Therefore, we investigated the regulatory role of LamB1 3´-UTR on translation in a monocistronic reporter assay. Notably, we found that the effect of the LamB1 3´-UTR might depend on the sequence located upstream of the reporter gene. In particular, an inhibitory role of the LamB1 3´-UTR on the LamB1 5´-UTR-dependent translation was detected in hepatocytes that have undergone EMT. On the contrary, the 3´-UTR of the interleukin like EMT inducer (ILEI) was able to promote translation in mammary carcinoma cells. Altogether, these data show novel insights into the *cis*-acting regulatory motif of the LamB1 IRES and the impact of the trailer in the translation control of LamB1.

1.2. Zusammenfassung

Die Kontrolle der Translation ist eine wichtige regulatorische Ebene der Genexpression während vieler physiologischer Prozesse. Extrinsische und intrinsische Faktoren, wie zum Beispiel eukaryotische Initiationsfaktoren (eIF), kontrollieren die Aktivität des Translationsapparates und damit die Translation der messenger RNA (mRNA). Einige mRNAs besitzen eine „ Internal Ribosome Entry Site" (IRES) in ihrer 5´untranslatierten Region (5´-UTR), die eine alternative Initiation der Translation zu cap-abhängigen Mechanismen ermöglicht und eine wichtige Rolle in der Tumorigenese einnimmt.

Laminin B1 (LamB1) ist ein Protein der extrazellulären Matrix (ECM), welches die Migration, Proliferation und Differenzierung der Zellen beeinflusst. Kürzlich konnte ein Zusammenhang zwischen der verstärkten Expression des LamB1 Rezeptors und der Tumorinvasion nachgewiesen werden. Weiters wurde festgestellt, dass LamB1 während der epithelialen zu mesenchymalen Transition (EMT) von Hepatozyten translationell aktiviert wird. Nach letzten Untersuchungen ist es sehr wahrscheinlich, dass ein IRES Element innerhalb der 5´-UTR von LamB1 für dessen Translation während der Tumorprogression verantwortlich ist.

Die vorliegende Untersuchung zeigt, dass der 5´-UTR von LamB1 die Translation eines bicistronischen Reporterkonstrukts in neoplastischen Hepatozyten steuert. Zudem sind zwei Signalwege, der „Mitogen-Activated-Potein-Kinase" –Weg und der „Phosphoinositid-3-Kinase" -Weg wesentlich an der cap-abhängigen und cap-unabhängigen Translation von LamB1 während der Hepatozyten EMT beteiligt. Interessanterweise zeigt der Sequenzbereich 155 bis 335 des LamB1 5´-UTR im bicistronischen Reporterassay funktionell nicht die volle IRES Aktivität. Damit scheint die Sequenz 1 bis 155 für die volle LamB1 IRES Aktivität unentbehrlich zu sein. Neue Ergebnisse deuten darauf hin, dass die 3´-UTRs die cap-abhängige Translation durch eine Interaktion des Poly(A)-bindenden Proteins mit dem eIF4G modulieren können. Daher haben wir die regulatorische Funktion der LamB1 3´-UTR in der Translation mittels monocistronischen Reporterassays untersucht. Wir konnten zeigen, dass dessen Funktion möglicherweise von der, dem Reportergen vorgelagerten Sequenz abhängig ist. Der LamB1 3´-UTR wirkte hemmend auf die LamB1 5´-UTR gesteuerte Translation in Hepatozyten nach EMT. Im Gegensatz dazu konnte der 3´-UTR des „Interleukin Like EMT Inducer" (ILEI) die Translation in Brustkrebszellen verstärken. Diese Daten geben einen neuen Einblick in die „*cis*-agierenden" regulatorischen Elemente des LamB1 IRES und der Auswirkung des 3´-UTR auf die Kontrolle der Translation von LamB1.

2. Introduction

2.1. Tumorigenesis

Tumorigenesis is considered as a complex sequence of events usually evolving over many years [1-3]. The generally accepted model describes a multistage process divided into three phases: tumor initiation, tumor promotion and tumor progression [3]. Genetic alterations in the genome, caused by e.g. chemical carcinogens, designate the initial phase of tumorigenesis. Particularly, mutations in genes involved in the regulation of cell growth and differentiation, which are known as proto-oncogenes and tumor suppressor genes, display a hallmark in the onset of cancer [1, 3]. Activated oncogenes support tumorigenesis by promoting cell growth and cell division. In contrast, mutational inactivation of tumor suppressor genes enable a cell to evade apoptosis and overcome senescence [3-4]. Together, these irreversible alterations of the genome drive the progressive transformation of normal cells into malignant tumor cells [1-3]. Increased cell growth and clonal expansion of neoplastic cells allow the formation of macroscopic tumors. Inflammation processes and disintegrated cytokine signaling in the surrounding tissue promote tumor malignancy [3]. Finally, additional spontaneous genomic mutations accompanied by alterations in the tumor microenvironment facilitate tumor invasion and progression [5-6]. During this third stage, the highly malignant cancer cells acquire capabilities allowing them to invade the surrounding tissue and intravasate into the bloodstream in order to form distal metastasis. Epithelial to mesenchymal transition (EMT) is a central event in this context, contributing to the tumor cell heterogeneity and metastatic progression [6].

During the last decades, cancer research has generated a rich body of knowledge concerning tumorigenesis and its underlying mechanisms. Multiple mechanisms that govern the transformation of normal human cells into malignant cancer cells have been revealed [3, 7]. In general, six or even seven essential alterations in cell physiology of normal cells collectively promote malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis as well as the inflammatory tissue context (Figure 1) [3, 7].

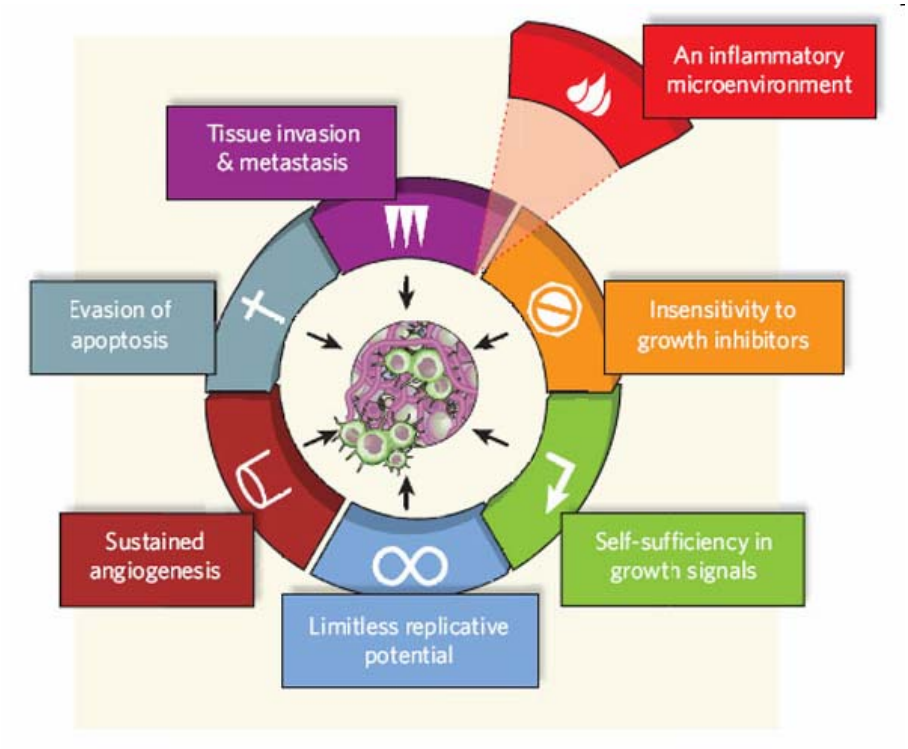

Figure 1. Mechanisms of tumorigenesis [3, 7].

Each of the listed alteration reflects the successful evasion of complex control and anticancer defence mechanisms present in normal cells. Notable, the particular sequence in which capabilities are acquired can vary among tumors types and individuals [3].

Normal cells require mitogenic growth signals (GS), such as diffusible growth factors or extracellular matrix (ECM) components in order to initiate proliferation via specific receptors [8]. In tumor development, some oncogenes mimic normal growth signaling, thus liberating cells from the dependence on stimulation by their microenvironment. Consequently, mechanisms that mediate tissue homeostasis are frequently disrupted [3, 7]. There are various molecular strategies in order to achieve GS autonomy. Some cancer cells acquire the ability to stimulate their own proliferation by creating an autocrine positive feedback loop. For example, platelet-derived growth factor (PDGF) and the tumor necrosis factor (TNF)α are frequently regulated by autocrine stimulation in tumors [3, 7]. Other cancer cells overexpress growth factor (GF) receptors, thus amplifying growth-stimulatory signals. The expression of truncated receptor versions lacking the cytoplasmic domain displays another mechanism to achieve a constitutive active ligand-independent signaling [3, 7]. Furthermore aberrant regulation of downstream signaling pathways is frequently observed. In normal cells, signals from

ligand-activated GF receptors and integrins are transduced via cytoplasmic downstream effectors into the nucleus, where gene expression is regulated. The SOS-Ras-Raf-MAPK cascade plays a central role in this context [3]. This signal transduction cascade is linked to several other pathways, thus influencing multiple cellular processes. Notably, Ras proteins are structurally altered in about 25% of human tumors, leading to a constitutive activation of mitogenic signaling [3, 9]. In addition to these cell autonomous mechanisms, malignant cell proliferation requires also tumor-stroma mediated paracrine growth stimulation. Tumor cells frequently acquire the abilitiy to secrete GFs in the surrounding tumor microenvironment [3, 10].

Soluble inhibitors are also involved in the maintenance of tissues homeostasis. These anti-growth signals transfer cells from active proliferation into a quiescent state (G_0) or induce the irreversible transition into a postmitotic state, which is usually accompanied with cell modulation of differentiation [3]. In general, anti-proliferative signals are transduced by the retinoblastoma protein (pRb) which is hypophosphorylated and subsequently blocks proliferation by altering the function of E2F, a transcription factor that controls the expression of genes essential for the progression from G1 into S phase [11]. pRb signaling is disrupted in many tumors, enabling the cells to proliferate despite of anti-growth signals. A prominent soluble signaling molecule in this context is transforming growth factor ß (TGF-ß). Among its various functions, TGF-ß prevents Rb inactivating phosphorylation by induction of cell cycle inhibitors such as $p15^{INK4B}$. In this line, it blocks cell cycle progression at the G1 checkpoint [3, 12]. Interestingly, some tumors acquire the ability to overcome the blocked cell cycle progression. To evade the TGF-ß mediated homeostasis, tumor cells frequently lose their TGF-ß responsiveness by mutational inactivation or downregulation of TGF-ß receptor expression [3, 13]. Additionally, functional pRb and other important regulators can be lost by gene mutation [3].

Programmed cell death represents another mechanism in order to maintain tissue homeostasis [3]. The apoptotic machinery contains sensors and effectors. Specific survival factors such as Insulin-like growth factor (IGF)-1/-2 and death factors such as FAS ligand bind to their corresponding receptors and determine cell fate. Importantly, cell survival can be ensured by the maintenance of tissue architecture which is controlled by cell-matrix and cell-cell adherence-based survival signals [3, 14]. Additionally, intracellular sensors such as p53 or members of the Bcl-2 family monitor changes within the cell and activate the death pathway in response to genomic abnormalities, signaling

imbalance, survival factor insufficiency or hypoxia [3, 15-16]. Once the death pathway is activated, intracellular proteases termed caspases execute the death program through selective and well-ordered destruction of subcellular structures, organelles and the genome [3]. Tumor cells developed strategies to impair proapoptotic pathways and acquire resistance towards apoptosis. One prominent example is the loss of the tumor suppressor p53, which happens in approximately 50 % of human cancers [3]. Furthermore, the activation of JAK/STAT, PI3K/AKT and RAS/ERK pathways is frequently enhanced in many tumors including hepatocellular carcinoma (HCC) [17]. In fact, alterations of the apoptotic machinery can dramatically affect the dynamics of tumor progression.

An indispensable event during progressive transformation of normal cells towards malignancy is the acquisition of a limitless replicative potential [3, 7]. Normal cells show a finite replicative potential that limits their cell doublings. Once a cell has progressed through a certain number of doublings, it stops growing, a process which is called senescence [3, 18]. Senescence can be circumvented by disabling the pRb and p53 tumor suppressor proteins allowing the cell to continue replication until it enters the state of crisis [3]. Crisis is characterized by massive cell death as well as the occasional occurrence of an altered (1 in 10^7) cell that acquires the ability of limitless replication and immortalization [3]. At this point, the cell has completely disrupted the primary intrinsic cell-autonomous program that limits cell proliferation. The immortalized phenotype of the cell is essential for the development of a malignant growth state [3]. Immortalized cells require an enhanced maintenance of telomeres, which is supported by the upregulated expression of telomerase enzyme in malignant cells [3, 19].

Survival of tumor cells depends as well on adequate supply with oxygen and nutrients [3]. In order to progress in size, emerging neoplasias must develop angiogenic abilities. Multiple observations indicate that neovascularization is a prequisite to the rapid clonal expansion associated with the formation of macroscopic tumors [3, 20]. Angiogenesis is regulated and kept in balance by positive and negative signals. A dimension of regulation comprises the proteases which control the bioavailability of angiogenic activators and inhibitors [3]. Tumor cells are able to induce an angiogenic switch by altering the balance of angiogenic inducers and inhibitors [3].

It is well recognized that all cancers acquire the same capabilities: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, apoptosis, limitless replicative potential and sustained angiogenesis [3]. These capabilities are indispensable for a

tumor cell to invade the surrounding tissue, to spread throughout the body and to colonize at distant place as metastasis. Tissue invasion and metastasis are essential steps during tumor progression [6]. E-cadherin, an important adherence protein links adjacent cells and therefore serves as a suppressor of invasion and metastasis. The generation of cell contacts by E-cadherin induces antigrowth signals which are transduced via cytoplasmic bound ß-catenin affecting many intracellular signaling pathways [3]. The functional loss of E-cadherin represents a key step in the acquisition of the capability to invade and to metastasize. During the invasion process, malignant cells are supported by matrix-degrading proteases produced by stromal and inflammatory cells. However, successful cancer cells additionally alter their binding specifities for cadherins, cellular adhesion molecules (CAMs), and integrins in order to acquire invasive and metastatic abilities. Notably, the molecular mechanisms and responsible regulators that govern cell invasion are tissue and context dependent [3, 6].

Mutations and changes within the genome of cancer cells are the cause of an increase in mutability [21]. Mutation of specific genes is not sufficient enough to cause cancer. Repair enzymes and DNA protecting factors maintain the genomic integrity and ensure that DNA sequence information remain pristine. Therefore, malfunction of these genomic “caretakers” has been proposed as an explanation for the increased mutability in malignant cells [22]. Accordingly, genomic instabilities appear to enable evolving populations of premalignant cells to reach these six or even seven capabilities. Moreover cancer development should be considered as a rather complex process that is also controlled and affected by the tumor microenvironment [3, 5].

2.2. Hepatocellular carcinoma (HCC)

Hepatocellular carcinoma (HCC) is the third most lethal cancer and represents the fifth most common cancer worldwide [23-25]. The prevalence of HCC is very high in developing countries, like from Asia and Africa but current data foresee a steady increase of HCC incidences in the Western countries [26]. The wide geographical distribution of HCC can be explained by the heterogeneity of risk factors [25, 27]. Generally, any agent that leads to chronic liver injury is considered as a risk factor for HCC. Hepatitis C infections and excessive alcohol abuse are the leading causes for HCC in Western countries, whereas aflatoxin B1 ingestion and hepatitis B infections are mainly responsible for the high HCC incidence in Asia and Africa [24, 27].

The development of HCC takes about several decades. In the majority of cases no obvious symptoms are present at early stages, hindering an early diagnosis. Generally, chronic hepatitis, fibrosis and/or cirrhosis, caused by the mentioned risk factors, are accompanied by phenotypic alterations of hepatocytes, an event considered to be pivotal for the onset of HCC (Figure 2) [23-25]. Inflammatory processes and the incipient destruction of liver structure challenge liver regeneration systems [24].

The molecular events which promote the development and progression of HCC are still not completely understood. Consequently, current therapies are yet not as successful as for other cancer types leading to poor survival of HCC patients after diagnosis [24, 27]. Untreated intermediate or advanced stage HCCs are characterized by a median survival of six to sixteen months [27]. Furthermore, the incidence of metastases is very high and predominantly occur in bones, lung, peritoneum, pancreas and brain [28-30].

Actually, the therapy options mostly depend on macroscopic parameters such as tumor size and number [23]. Surgical resection of the tumor presents an option for patients diagnosed at early tumor stages, showing solid tumor nodules with well-preserved liver function. After successful surgery, the five years survival rate lies between 60 – 70 %, but unfortunately in up to 70 % of the cases, HCC recurrence is observed [23]. Liver transplantation, a potent therapeutic strategy, achieves the same outcome concerning the five years survival rate, and shows a lower recurrence rate compared to surgical therapy [23]. Another treatment option is the percutaneous ablation, where tumor tissue is ablated by ethanol injection or radiofrequency. The success of this therapy mostly depends on the tumor size, but under optimal conditions it leads to a recovery in 80% of cases [23]. Additionally, the trans-arterial percutaneous chemoembolization (TACE),

aims to reduce symptoms as a palliative therapy during late tumor stages [23, 31]. Therefore, a chemotherapeutic drug is injected into hepatic arteries, leading to the obstruction of the tumor feeding vessels [27, 30].

Sorafenib, is a new promising therapeutic drug for the treatment of advanced HCC stages [32]. The agent blocks important molecular mechanisms by targeting Raf signaling, PDGF-receptor and vascular endothelial growth factor receptor (VEGF-R). PDGFR-α is involved in tumor angiogenesis and maintenance of the tumor microenvironment, and has been found to be implicated in the development and metastasis of HCC [32-34].

Due to the predicted increase of HCC for the next several decades, the identification of a potential prognostic factor is of particular importance. Extensive tumor hypervascularisation as well as the invasiveness of HCC have recently been linked to high VEGF levels [35-36]. Therefore VEGF is discussed as a prognostic marker [25].

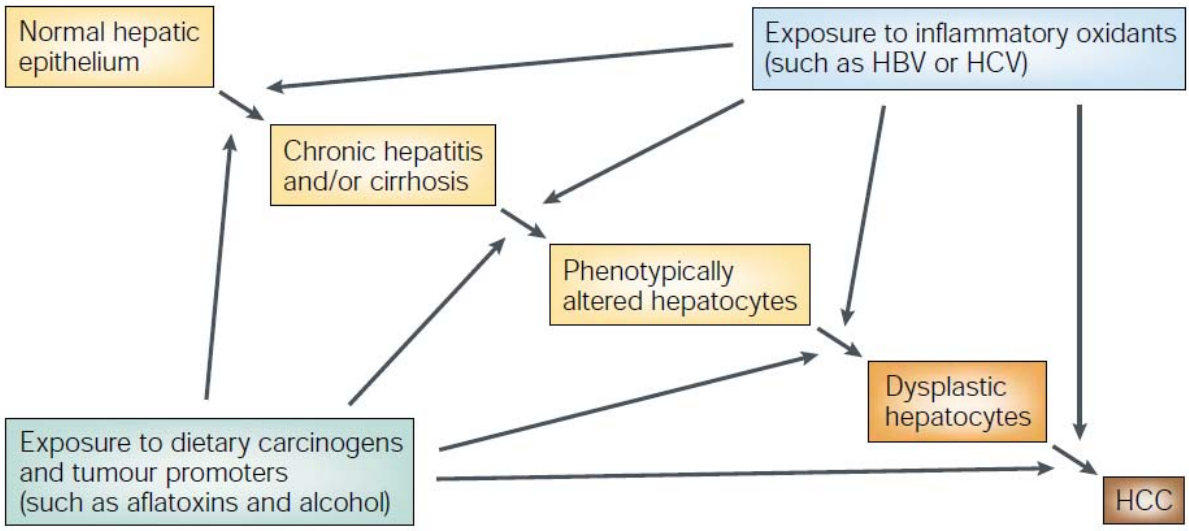

Figure 2. Pathogenesis of hepatocellular carcinoma (HCC) [24].

Generally, hepatocytes are highly differentiated epithelial cells which are of paramount relevance for glucose, amino acid and lipid metabolism. These cells are mitotically inactive under physiological conditions. However, in response to liver damage, hepatocytes show an extraordinary proliferative capacity for regeneration [37-38]. Interestingly, aberrant proliferation and dedifferentiation of hepatocytes are observed during the pathogenesis of HCC. While our knowledge of genetic and epigenetic alterations leading to HCC is rapidly growing, the molecular pathogenesis of HCC is still

poorly understood. Nevertheless, various studies on human HCCs revealed some molecular events that may play crucial roles in human hepatocarcinogenesis [39-41]. Most frequently occurring molecular alterations in HCC include (i) the loss of tumor suppressors such as p53, pRB, $p14^{ARF}$, $p16^{INK4A}$ and cyclins/cdks, (ii) the loss of the cell-cell adhesion protein E-cadherin, (iii) the constitutive activation of signaling pathways – e.g. Erk/MAPK and PI3K pathway, (iv) the nuclear accumulation of Wnt/ß-catenin, and (v) the aberrant regulation and secretion of cytokines such as TGF-ß [38-41].
In several tumor models including human HCC models, the cooperation of oncogenic Ras and TGF-ß induces progression to undifferentiated invasive tumors which are characterized by migratory and fibroblastoid cell phenotypes [38, 42-43]. This phenotypical conversion of cells, termed epithelial to mesenchymal transition (EMT), is increasingly recognized as a central process during cancer progression and metastasis [43-44].

2.3. Epithelial to mesenchymal transition (EMT) in embryonic development

EMT is a highly conserved and fundamental process that plays key roles in many steps of normal morphogenesis [45-46]. Differentiated epithelial cells switch into motile mesenchymal cells allowing the cells to migrate and generate new tissues during embryogenesis (Figure 3) [47]. EMT is described as a morphological conversion of epithelial monolayered cells into single dispersed fibroblastoids. In particular, epithelial polarized cells, which are merely able to move laterally in the epithelial layer by retaining contact to the basal lamina, undergo a phenotypical conversion characterized by the gain of mesenchymal, fibroblastoid-like properties such as increased motility combined with the loss of intercellular adhesion [46, 48-49].

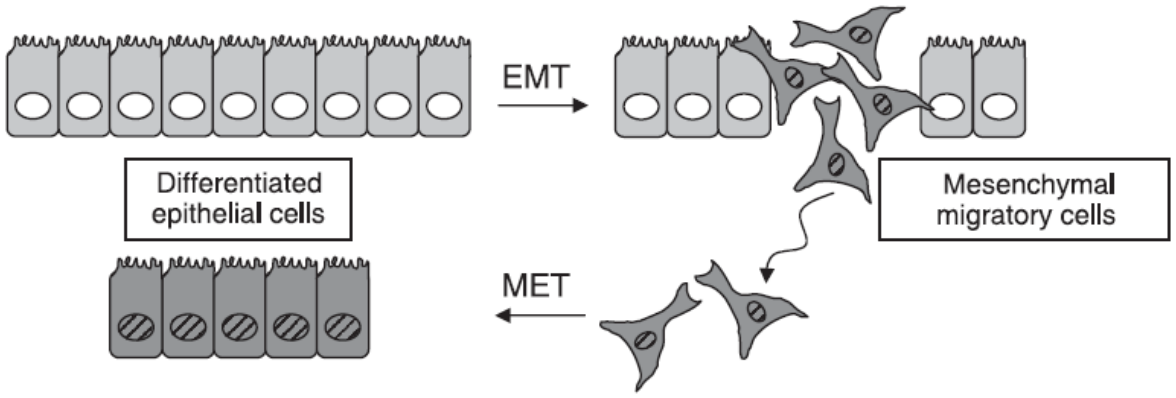

Figure 3. The epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) [47].

This phenomenon has been first described as an indispensable process during distinct steps of embryogenesis, including gastrulation and the migration of cells from the primitive neural crest throughout the embryo [6, 48].

During the last years, investigations have shown that EMT is programmed and regulated by a variety of selected transcription factors (TF) and cytokines which have the potential to activate EMT at specific sites within the embryo [6, 50]. The persistent expression of these TFs in EMT transformend cells indicates that a downregulation of the TFs may lead to the loss of EMT-inducing effects reverting the EMT. This morphogenetic reversion is referred to as mesenchymal to epithelial transition (MET) [46, 48, 51]. Observations of EMT events that are restricted to specific locations within the embryo suggested that EMT is a consequence of specific contextual signals received from the local microenvironment [6]. Interestingly, similar epithelial changes can be observed during different physiological processes such as wound healing and pathological processes such as chronic inflammation which is a preliminary stage to fibrotic diseases [52]. During the last years, investigations have revealed an important role of EMT in tumorigenesis [48]. The fact that many TFs that orchestrate EMT during embryogenesis are also found to be expressed in various human cancers, additionally supports its relevance to cancer pathogenesis [6]. Today the EMT process is considered as a crucial step in tumor progression allowing cancer cells to disseminate from the primary tumor and to cause local invasion and metastasis at distant sites [6, 46, 48].

2.4. Epithelial to mesenchymal transition (EMT) in tumor progression

EMT describes a developmental event that is increasingly recognized to play an essential role during cancer progression and metastasis [6, 38, 48]. The generally accepted model of carcinogenesis and metastasis describes a step by step development of the malignant increase (Figure 4) [48]. Normal epithelial cells, lined by a basement membrane display an apical - basal polarization and are functionally differentiated. Epithelial cells are organized via cell-cell contacts such as tight and adherence junctions in order to form intact epithelial layers. Initial malignant transformations induced by epigenetic and molecular alterations cause a carcinoma *in situ*, which is characterized by an intact basement membrane [3, 48]. Subsequently, a cascade of alterations leads to the formation of cells which escaped physiological cell control [3]. For example, by evading apoptosis and growth control, cells establish and maintain highly malignant

properties. Further, the malignant cells gain invasive properties, possibly enabled by undergoing the EMT process. Finally, less differentiated, invasive cells break through the basement membrane, intravasate into the lymphatic or blood system and subsequently spread throughout the organism [3, 48].

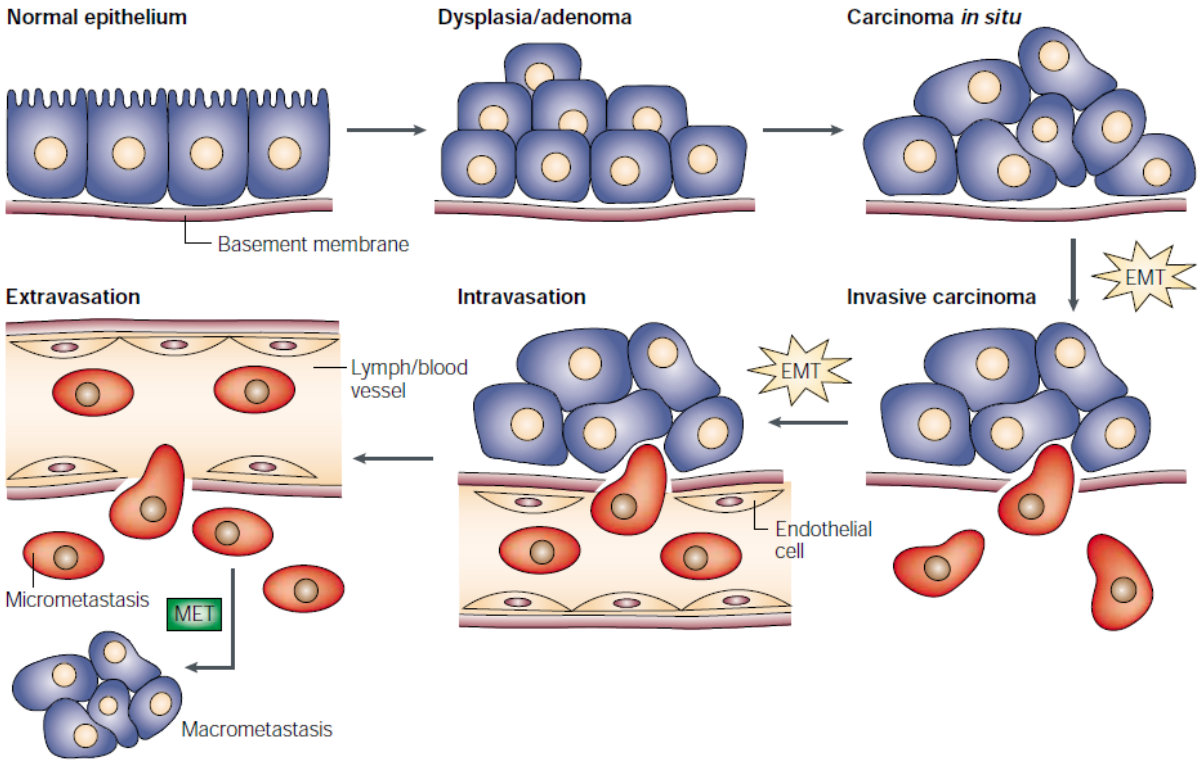

Figure 4. Model of epithelial tumor development and metastasis [48].

In distant organs, small cell accumulations can form micrometastasis or the invaded cells establish a new carcinoma through the MET process [46, 53]. Therefore, EMT and its reverse mechanism, the MET, play a crucial role for tumor progression, by allowing malignant cells to escape from the solid tumor [47-48].

As already mentioned above, the expression of EMT-inducing TFs in cancer cells in response to heterotypic signals by surrounding cells seems to play a crucial role during the process of carcinoma progression. These signals such as Wnts, Hedgehogs, members of the TGF-ß family as well as ligands of tyrosine kinase receptors are released by mesenchymal cells that form the tumor-associated stroma [6, 51]. It is assumed that these mesenchymal cells which are recruited either from the tissue stroma or the bone-marrow, become increasingly activated and reactive as tumor progression proceeds. The inflamed tumor-stroma may be the source of the heterotypic signals that evoke EMT. However, at distant sites of metastasis and in the absence of these stimulating signals, the cancer cells will decline expression of EMT-inducing TFs and

therefore undergo MET leading to an epithelial phenotype [6]. The subsequent fate of such disseminated tumor cells is still unclear. The cancer cell is confronted with a new microenvironment to which it is poorly adapted. It seems likely that out of thousands of micrometastasis only some few successfully grow into macroscopic metastasis [6, 48].

2.5. Signaling networks guiding EMT

Within the EMT process, epithelial cells regulate epithelial-specific markers down, leading to the loss of typical epithelial features such as the ability to form adherens and tight junctions (Figure 5) [51]. Tight junctions are localized at the lateral side close to the apical surface enabling membrane fusions of epithelial cells. Maintained by occludins and claudins, the cytoplasmic components Zonula Occludens (ZO)-1,-2,-3 and p120 attach to actin filaments, thereby contributing to the integrity of tight junctions [54]. Adherens junctions, located adjacent to the tight junctions in the basolateral surface compartments, are established by homotypic E-cadherin interactions. The cytoplasmic domains of E-cadherin bind tightly to ß-catenin that in turn anchors to the actin cytoskeleton. Structurally similar to the adherens junctions are the desmosomes which connect the cadherins to the intermediate filament cytoskeleton [51]. At the onset of EMT, tight junctions are dissocciated and E-cadherin gets lost which leads to a reorganization of the cytoskeleton [51, 55]. Subsequently, the mesenchymal phenotype becomes apparent by the expression of mesenchymal cytoskeletal proteins such as vimentin and the increased deposition of ECM proteins including fibronectin and collagens. These secreted ECM components stimulate integrin signaling and induce the formation of focal adhesion complexes (FAK), which facilitate cell migration [56]. Further, degradation of the basement membrane and remodeling of the actin cytoskeleton from cortical actin towards actin stress fibers takes place, which are a hallmark of migratory mesenchymal cells [29]. Besides non-transcriptional changes, all these processes additionally underly a plexus of changes in transcriptional regulation, leading to a repression of epithelial genes expression and an activation of mesenchymal gene expression [51].

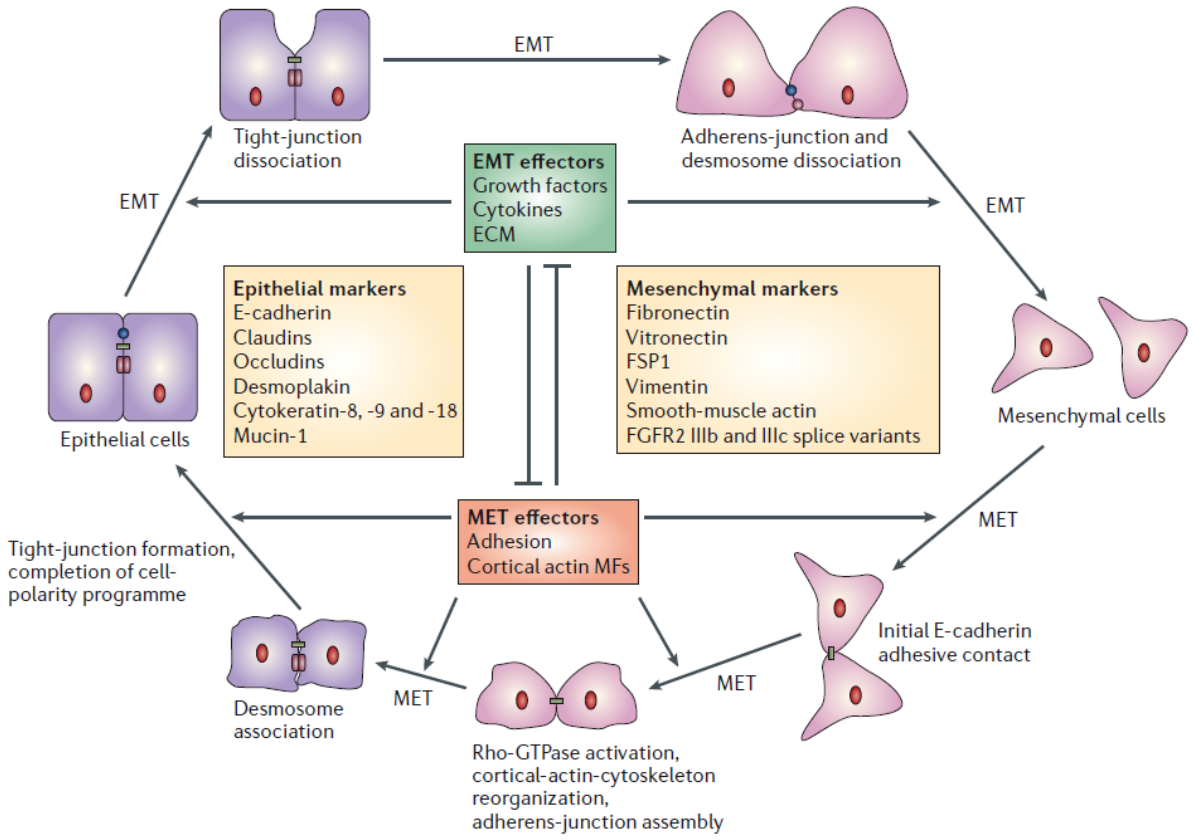

Figure 5. The cycle of epithelial-cell plasticity [51].

E-cadherin, the epithelial membrane protein that provides cell-cell contacts, can be considered a tumor suppressor inhibiting invasion and metastasis [48]. Corroborating this theory, the re-expression of E-cadherin in carcinomas has been shown to be sufficient to reduce the aggressiveness of tumor cells in *in vitro* experiments [57]. The loss of functional E-cadherin is described as a consequence of mutations within the gene or as a result of epigenetic changes including promoter hypermethylation and transcriptional repression by prominent repressors such as Snail, Slug, ZEB1, ZEB2 and E12/E47 [48, 58]. Additionally, the serin-threonine kinase AKT which is frequently activated in human epithelial cancer was recently shown to regulate mRNA and protein levels of E-cadherin [59-60]. Another study revealed a collarboration of AKT-mediated activation of Rab5 protein with E-cadherin sequestration [61].

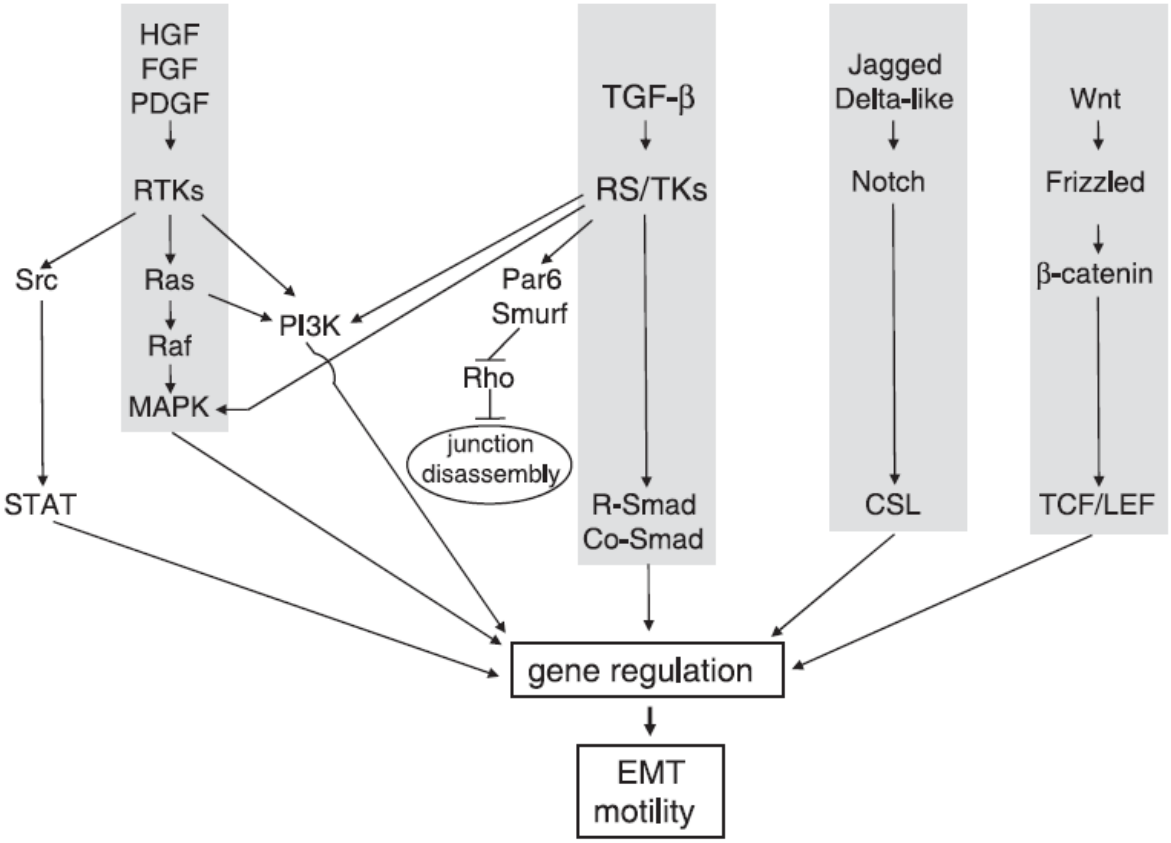

Figure 6. The major signaling pathways inducing epithelial-mesenchymal transition (EMT) [47].

However, loss of E-cadherin should not be considered as the sole pivotal event in EMT. In tumor progression as well as in development, several signal transduction pathways such as Src, Ras, Ets, integrin, Wnt/ß-catenin and Notch are involved in the onset and regulation of the EMT process [49]. Experimental data revealed a complex network of signaling pathways that cooperatively regulate epithelial plasticity (Figure 6) [47]. A major signaling pathway that is constitutively activated in tumors is the Ras/MAP-Kinase pathway. Growth factors such as hepatocyte growth factor (HGF), fibroblast growth factor (FGF) and PDGF bind their corresponding receptor tyrosine kinases (RTK) and activate Ras-Raf-MAPK, Posphatidylinositol-3´-kinase (PI3K) or Src-STAT signaling pathways [49]. Paracrine activation of TGF-ß signaling in cells with constitutive active Ras leads to the induction of EMT [62]. PI3K is a critical intracellular mediator of RTK signaling providing a cross-talk between growth factor signaling, integrin receptors and small GTPases of the Rho family in order to control cytoskeletal organization and to induce EMT [58]. Rho-GTPases and the expression of proteases such as matrix-metalloproteinases (MMPs) and urokinase-type plasminogen activator (uPA) have a

strong impact on cell motility by remodelling the actin cytoskeleton and by degrading the ECM [58].

Beside the established role of RTK signaling in EMT, observations in various cancers demonstrate a complex interplay of multiple signaling pathways including TGF-ß, PDGF, WNT and Notch, which cause activation of downstream Erk MAPK, ß-catenin and nuclear factor-κB signaling (NF-κB) [63].

Numerous *in vivo* as well as *in vitro* models were established in order to study the EMT process. *In vitro* models are used to investigate individual signaling pathways by simple genetic manipulation, but the relevance of the data is still questionable [48-49, 62]. In this aspect, *in vivo* models reflect the human tumors more closely due to the presence of tumor microenvironment, which proved to have a strong regulatory influence on tumor progression and metastasis [64-67]. Despite the limitations of *in vivo* models, these experimental settings allow a more detailed study of the tumor-host interplay than *in vitro* models [68].

2.6. TGF-ß signaling in cancer progression

Under physiological conditions, TGF-ß is involved in the regulation of cell proliferation, differentiation and apoptosis. Soluble TGF-ß binds to the TGF-ß receptor (TßR) II which results in heterodimerization and phosphorylation of the regulatory GS domain within TßRI [69]. Subsequently TßRI activates Smad2 and Smad3 through direct C-terminal phosphorylation and enables Smad2/Smad3 to bind Smad4, and translocate into the nucleus. In the nucleus numerous co-activators such as p300, CBP or SMIRF or co-repressors such as p107 join the Smad complex in order to activate or repress target gene expression (Figure 7) [70-72]. Smad6 and Smad7 show inhibitory functions by preventing the activation of the receptor-regulated Smads [73]. Among all the various growth factors and differentiation factors, TGF-ß received much attention as a major inducer of EMT during embryogenesis, tumor progression and fibrosis. In fact, TGF-ß is a double-edged sword in tumorigenesis; on one hand acting as a tumor suppressor and on the other hand as a tumor promoter [74]. During the early phase of epithelial tumorigenesis, suppressive TGF-ß signaling prevents epithelial cell transformation by inducing apoptosis. In contrast, in late stages of tumor progression, tumor cells can become resistant to the growth-inhibitory effects of TGF-ß due to inactivation of the TGF-ß signaling [75]. Furthermore, TGF-ß can promote cancer progression via increased

motility, invasiveness and metastasis [74, 76-77]. The development of resistance to TGF-ß mediated inhibition of proliferation is frequently observed in various human cancers [75].

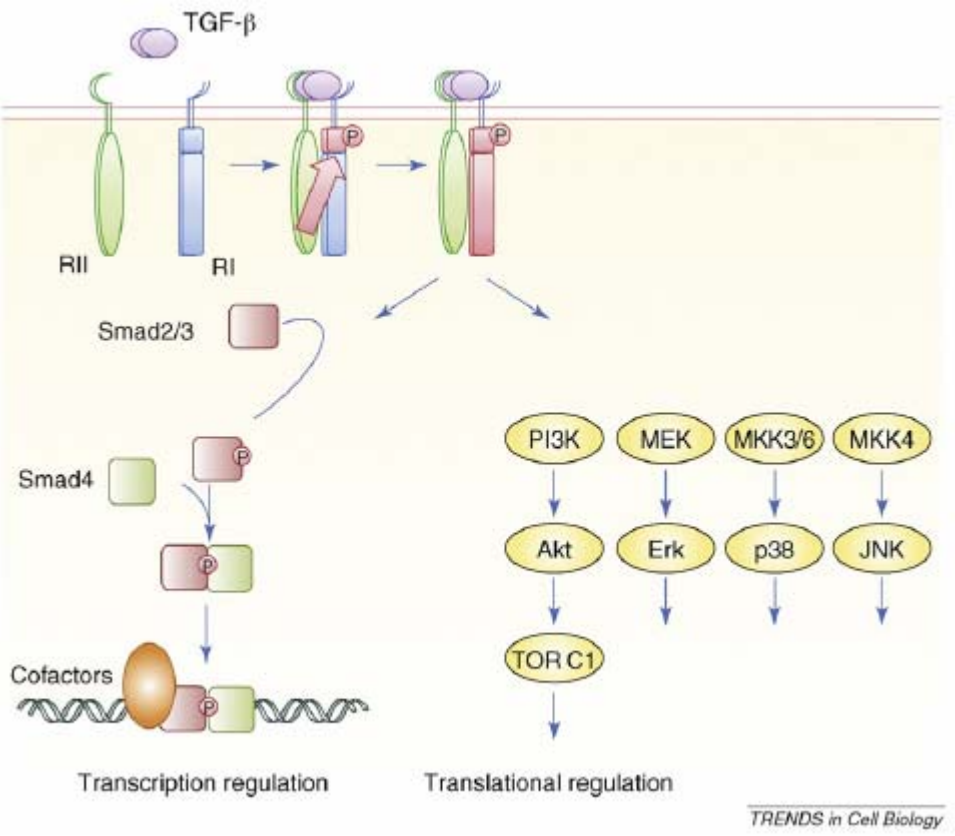

Figure 7. Schematic representation of TGF-ß signaling pathways [71].

During EMT, TGF-ß induced Smad signaling regulates the transcription of three prominent transcription factor families, the Snail, ZEB and bHLH (helix-loop-helix) families, resulting in repression of epithelial marker gene expression and activation of mesenchymal gene expression [29, 78].

Several *in vivo* and *in vitro* experiments were performed in order to reveal the possible roles of TGF-ß signaling mediators during EMT [78]. For example, Smad2$^{-/-}$ hepatocytes appear phenotypically mesenchymal *in vivo* and their migratory capacities are higher compared to wild-type cells, whereas Smad3$^{-/-}$ hepatocytes retain their epithelial characteristics [79]. Therefore, it was suggested that TGF-ß driven EMT of hepatocytes depends on Smad3 and not Smad2. Smad2 may play an antagonistic role in dedifferentiation and EMT of hepatocytes [79]. However, the expression of activated Smad2 promotes invasion of spindle tumor cells, compared to the dominant negative

form, which has no invasive properties. Consequently, researchers conclude that Smad2 may promote EMT *in vivo* [80]. *In vitro* models revealed an indispensable role of Smad4 for the transcriptional mechanism that downregulates the expression of E-cadherin in response to TGF-ß [81].

Interestingly, the expression of a novel cytokine, the secreted interleukin-like EMT inducer (ILEI), is stimulated by TGF-ß at the translational level [82]. Stable overexpression of ILEI caused EMT, invasive growth of carcinomas and metastasis of breast cancer models. Actually, the cellular processes and signaling pathways influenced by ILEI are still unknown [82].

In various cancers the suppressive effect of TGF-ß is lost due to either TGFßR mutations such as in colorectal cancer, gastric tumors, pancreatic cancers, breast cancer and in T-cell lymphomas or due to mutated Smads such as in familial juvenile polyposis [8, 83-85]. However, loss of TGF-ß growth inhibitory effects in cancer cells occurs more often downstream of the core TGF-ß signaling pathway. Increased production of TGF-ß is a very common feature in various tumors and correlates with the tumor aggressiveness [86]. Importantly, the tumor-derived TGF-ß affects various cell types located in the surrounding tissue of the tumor as well as the tumor cells themselves (Figure 8) [87].

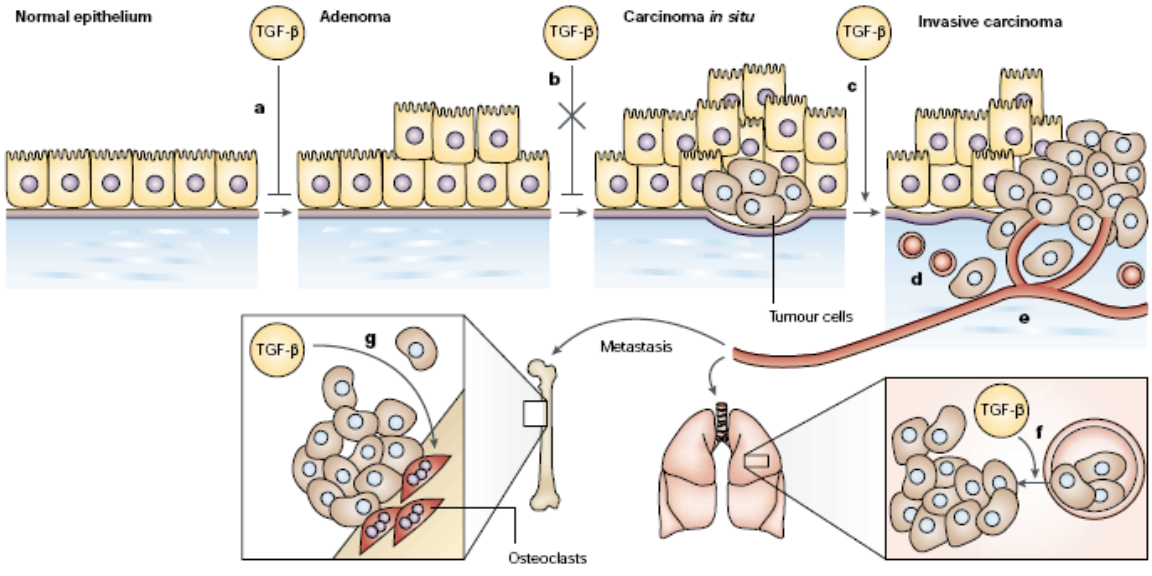

Figure 8. Functions of TGF-ß during cancer progression [87].

TGF-ß establishes an immune-suppressive environment that influences the function of immune cells and thus allowing tumor cells to escape immune cells, such as cytotoxic T

lymphocytes. In addition, recruitment of new blood vessels towards the tumor tissue enables sufficient supply with nutrients and oxygen and facilitates metastasis. This process is regulated by tumor secreted TGF-ß which induces the expression of VEGF and connective tissue growth factor (CTGF) in both, epithelial cells and fibroblasts [88-89]. TGF-ß regulates the growth of normal epithelium and early stage tumors, whereas it promotes the progression of a carcinoma *in situ* towards an invasive carcinoma in tumors that established TGF-ß resistance but still retained TGF-ß signaling components [87]. Frequently, tumor cells achieve an invasive phenotype by undergoing TGF-ß induced EMT and thus are more likely to locally infiltrate the surrounding tissue and to spread throughout the organism.Several signaling pathways have been shown to be involved in TGF-ß induced EMT [29, 78]. TGF-ß is capable to activate Smad- and non-Smad signaling pathways [29, 90]. All non-Smad pathways cooperate with TGF-ß/Smad signaling in order to orchestrate responses that constitute TGF-ß induced EMT. For example, independent of Smad activation, TGF-ß receptors interact with alternative signaling effectors, such as mitogen activated protein kinase (MAPK), phosphatidylinositol-3'-kinase (PI3K) and small GTPases of the Rho family, which are important regulators involved in cell motility, apoptosis and the EMT process (Figure 6) [91-93]. Various observations such as enhanced TGF-ß-induced EMT in the presence of mutant Ras, support the hypothesis of an interplay of the Ras-Erk MAP kinase pathway and TGF-ß signaling in the induction of EMT [42, 48-49, 94]. In this context, specific transcription responses are triggered, leading to the downregulation of E-cadherin and the upregulation of N-cadherin and MMP expression [95]. Additionally, hyperactive oncogenic Ras leads to enhanced TGF-ß signaling, concomitant by increased autocrine TGF-ß secretion and nuclear accumulation of phosphorylated Smads [96]. The activation of Erk/MAP kinase signaling seems to be required for TGF-ß induced EMT [95, 97]. In addition, TGF-ß signaling induces other players of the EMT process, such as c-Jun amino-terminal kinase (JNK) and p38 MAP kinase [78]. The small GTPase RhoA and its effector kinase ROCK, which promote stress fiber formation, acquisition of mesenchymal morphology and enhanced migration are also activated in response to TGF-ß [98]. Moreover, activation of the PI3K/Akt pathway by TGF-ß plays a major role in EMT. Akt is a central regulator of pathways involved in cell growth, survival and migration. During EMT, activation of the mTOR/S6 kinase pathway by PI3 kinase/Akt results in enhanced protein synthesis [99].

Another, highly conserved signaling pathway that cooperates with TGF-ß signaling in the elaboration of the EMT response is the Wnt/ß-catenin pathway [29, 78]. Cytoplasmic ß-catenin, a component of adherens junctions, connects E-cadherin to the cytoskeleton. In the absence of Wnt, ß-catenin is phosphorylated by GSK-3ß, a component of the adenomatous polyposis coli (APC) destruction complex, leading to its proteasomal degradation by E3 ubiquitin ligase [100]. In the case of active canonical signaling, induced by binding of Wnt to its corresponding Frizzled receptor, GSK-3ß is inhibited by a yet not fully understood mechanism, leading to cytoplasmic accumulation of ß-catenin. Subsequently, ß-catenin translocates into the nucleus and interacts with Tcf/Lef transcription factors, where they regulate target gene transcription. The cross-talk between TGF-ß/Smad signaling and Wnt signaling during EMT has been documented, but still remains to be elucidated [101]. However, also Notch signaling is suggested to contribute to EMT during tumor progression and cardiac development [78]. Binding of the membrane-bound receptor to its ligand presented on the surface of a neighbouring cell, triggers the proteolytic cleavage of the Notch receptor, liberating a Notch intracellular domain (NIC). The released NIC enters the nucleus where it regulates, together with transcription activators, EMT-related gene transcription [29, 78].

2.7. EMT in murine hepatocellular model

To establish a *in vitro* EMT model, immortalized $p19^{ARF}$ null hepatocytes were isolated from murine liver [23, 94]. Due to this genetic alteration the repression of Mdm2 is abolished leading to the loss of the growth suppressing functions of p53. These immortalized, but non-tumorigenic hepatocytes are termed MIM-1-4. They express liver-specific marker proteins and are able to reconstitute liver parenchyma after spleen injection into Fas-L damaged livers of severe combined immuno deficient (SCID) mice [102]. Noteworthy, MIM-1-4 cells have the potential to develop a progenitor phenotype that can form hepatocytes or cholangiocytes [103]. MIM hepatocytes were transfected with a bicistron expressing constitutively active Ha-Ras and green fluorescent protein (GFP) [94]. Accordingly, these defined tumorigenic hepatocytes are termed MIM-Ras. Upon TGF-ß treatment MIM-Ras undergo EMT endowing them with metastatic properties. These EMT transformed hepatocytes are referred to as MIM-RT (Figure 9) [94]. Importantly, the immortalized MIM-RT hepatocytes which are able to repopulate the liver, promote tumor growth upon expression of oncogenic Ras and undergo EMT

through the synergism of Ras and TGF-ß [94, 103]. These three hepatic cell lines have been employed to establish a murine EMT model. Changes in epithelial plasticity were associated with cytoplasmic dislocation or loss of E-cadherin, nuclear accumulation of ß-catenin as well as the activation of Smad2/3 signaling [104-106].

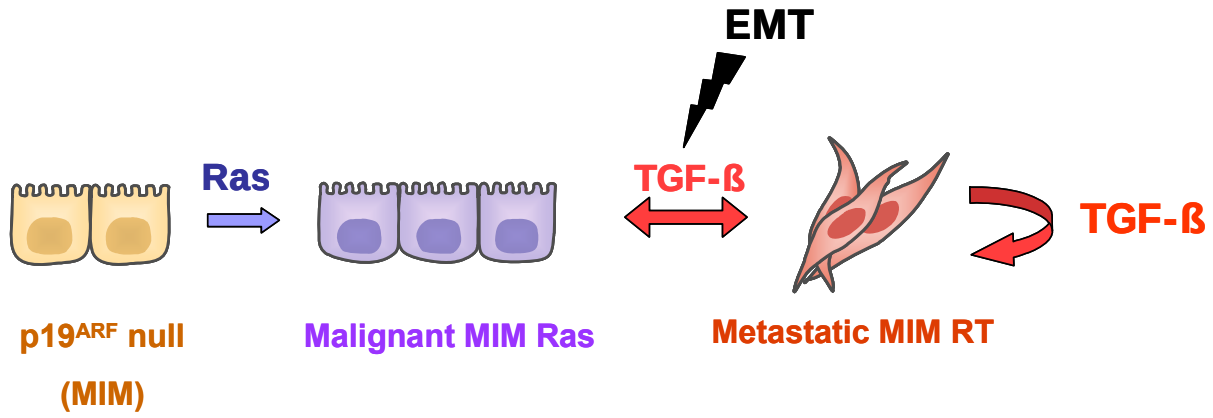

Figure 9. Model of hepatic inflammation and liver tumorigenesis.

These findings are of particular relevance since they are known as hallmarks of human HCC [94]. Interestingly, about 50% of human HCC display TGF-ß secretion and nuclear accumulation of ß-catenin [106-108]. The novel EMT regulator, ILEI (interleukin-like EMT inducer), which is able to induce EMT in murine mammary tumor model was tested in this hepatic tumor model. Overexpression of ILEI had no detectable effect in MIM-1-4 cells, but in cooperation with oncogenic Ras, the cells induce and maintain EMT in a TGF-ß-independent fashion [82]. In contrast, ILEI overexpression in MIM-C40 cells, which are characterized by hyperactivate PI3K, failed to induce EMT, but enabled these cells to form tumors [82]. Importantly, another study revealed the essential role of PDGF in TGF-ß-mediated EMT of neoplastic hepatocytes [106]. Inhibition of PDGF signaling leads to a decrease of cell migration *in vitro* and tumor suppression *in vivo* [109]. Furthermore the collaboration of oncogenic Ras and ILEI during the fibroblastoid conversion of Ras-transformed hepatocytes leads to the upregulation of PDGF signaling [109]. Additionally, studies on tumor-stroma cross-talk revealed a crucial role of myofibroblasts concerning the TGF-ß-dependent induction as well as the PDGF-mediated maintenance of EMT [38, 110].

2.8. EMT in murine mammary tumor model

Spontaneously immortalized murine mammary gland cells isolated from midpregnant mice retain complete epithelial polarization [82]. These non-tumorigenic epithelial cells are termed EpH4. Upon TGF-ß treatment, EpH4 cells enter cell cycle arrest and undergo apotosis. After transformation with oncogenic hyperactive Ras, the resulting EpRas cells retain full epithelial polarity but become tumorigenic [82]. In the presence of TGF-ß, EpRas cells undergo complete EMT *in vivo*, endowing them with metastatic properities. These cells, named Ep-XT are characterized by an fibroblastoid phenotyp, accompanied by the loss of epithelial markers, such as E-cadherin and ZO-1 and the upregulation of mesenchymal markers, such as vimentin [82]. *In vivo* studies demonstrated that EpRas cells undergo EMT in response to TGF-ß, which is stabilized by an autocrine TGF-ß loop [43, 80]. TGF-ß-induced EMT was accompanied by upregulation of PDGF ligands and receptors, thus leading to PI3K activation and pro-survival signals [111]. Additionally, it was shown that Ras-dependent MAPK pathway hyperactivation in EpH4 cells (EpS35) is essential for EMT and metastasis. In contrast, a Ras-induced, hyperactive PI3K pathway (EpC40) was required to accelerate tumor growth and to prevent apoptosis, but could not cause EMT [82].

Recently, expression profiling of polysome-bound mRNA was performed in order to molecularly characterize the EMT phenotyp and to reveal novel putative EMT regulators [112]. The cytokine ILEI, a facultatively secreted protein was identified within this study to be translationaly upregulated in EMT transformed Ep-XT cells [82]. In the EpH4/EpRas model, overexpression of ILEI causes EMT, accelerated tumor growth and metastasis upon tail vein injection. RNAi-mediated knockdown of ILEI in EpRas prevents EMT and metastasis [82]. Therefore, the experimental observations suggest that ILEI is both necessary and sufficient for EMT and late events in metastasis in epithelial cells [82].

2.9. Initiation of translation

Mechanism of Cap-dependent initiation

Gene expression is regulated at multiple steps including transcription, splicing, mRNA transport, mRNA stability, translation, protein modification and protein stability [113-114]. In eukaryotes, the regulation at the level of translation plays an important role in various

biological processes and events such as stress response, development and differentiation, aging and disease [115]. In comparison to transcriptional regulation, the translational control of mRNAs allows rapid changes in the abundance of specific proteins. Therefore it enables an immediate response to new cellular conditions such as cellular stress due to nutrient deprivation. The process of translation is a sequence of highly conserved steps namely initiation, elongation, termination, and ribosome recycling [113]. Initiation of translation by ribosome recruitment is the rate-limiting step of protein synthesis and thus an effective target for regulatory mechanisms to control the process [115].

The ribosome recruitment to the mRNA in the cytoplasm is mediated by a large number of eukaryotic initiation factors (eIFs) (Figure 10) [113, 116]. Initially, the heteromultimeric complex eIF4F, consisting of cap-binding protein eIF4E, the RNA helicase eIF4A and eIF4G, recognizes the cap structure located at the 5´ terminus (m^7GpppN) of the mRNA. eIF4G serves as scaffolding protein that bridges the whole eIF4F complex via eIF3 with the small ribosomal subunit. eIFA, promoted by eIF4B and eIF4H, unwinds secondary structures within the 5´-untranslated region (5´-UTR) in order to facilitate the binding of the 43S preinitiation complex [113]. This 43S preinitiation complex contains the 40S ribosomal subunit associated with eIF3, eIF1A and the ternary complex of eIF2, GTP and the methionyl-initiator tRNA. Once bound to the mRNA, the preinitiation complex starts to scan along the 5´-UTR up to the first start codon (AUG). Start codon recogition by codon-anticodon base-pairing triggers the hydrolysis of eIF2-bound GTP, the dissociation of the initiation factors and the subsequent binding of the 60S ribosomal subunit to form the functional 80S ribosomal complex. The 80S translation-competent ribosome initiates protein synthesis [113, 115, 117].

However, certain features of the 5´-UTR are known to interfere with the ribosomal scanning process. Long GC-rich and highly structured RNAs reduce the ribosomal scanning efficiency by inhibiting the migration of the 43S preinitiation complex along the 5´-UTR [118-119]. Several cap-dependent mechanisms to overcome such limitations have been described in various organisms [120-121]. Strong secondary structures which can not be disintegrated by the eIF4A helicase may be bypassed by skipping these segments, a process termed ribosome shunting in diverse viruses. The mechanism of reinitiation enables a second open reading frame (ORF) located within the same mRNA to be translated after translation of the first ORF. Thereby the 40S ribosomal subunit keeps attached to the mRNA, ready for scanning a new start codon [118-119, 122].

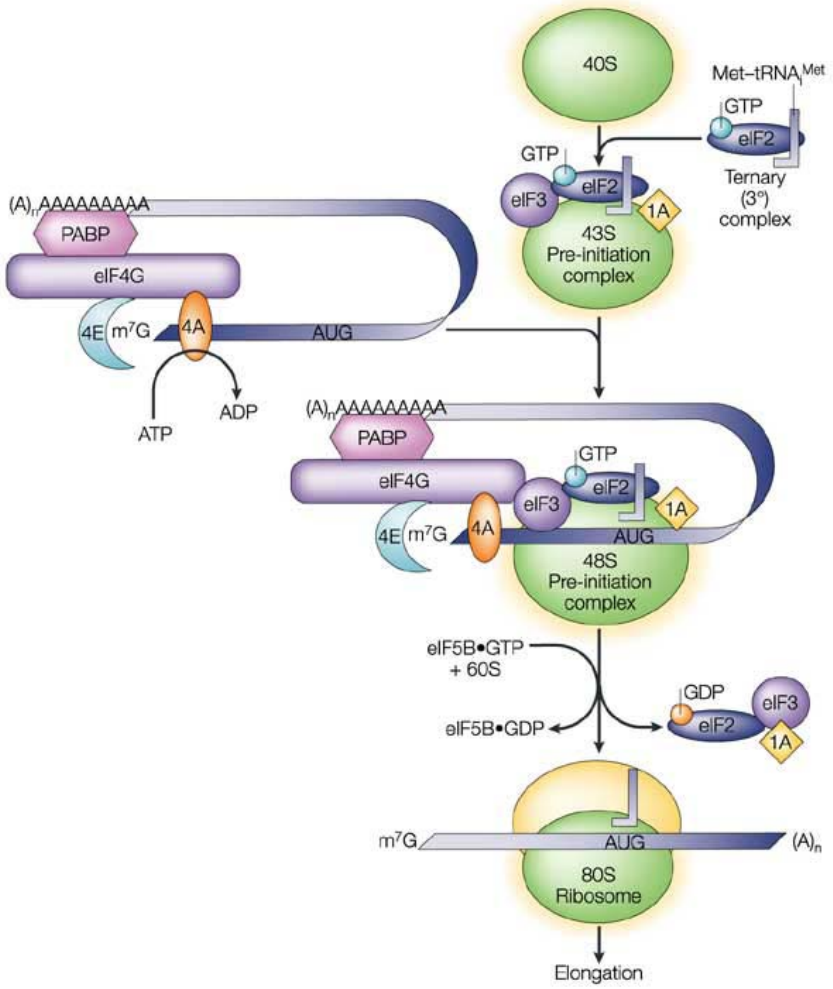

Figure 10. Initiation of translation in eukaryotes [116].

Translational regulation

The regulation of translation is essential for stress response, cell growth, proliferation and differentiation [115]. Thus the translational control machinery depends on extrinsic and intrinsic signal molecules such as growth factors, cytokines and ECM proteins which modulate various signaling pathways within the cell. These signaling pathways establish a complex network that tightly regulates the activity of specific translation initiation factors and control protein synthesis [115, 120-121]. An extensively used mechanism in eukaryotes to control the rate of translation involves the cap-recognition process. The key player of this process is the mRNA cap-binding protein eIF4E which is the rate

limiting factor of the eIF4F complex. The availability of active eIF4E is regulated by two distinct mechanisms [122]. Firstly, the assembly of eIF4E with eIF4G within the eIF4F complex is inhibited by members of a family of heat stable repressor proteins, termed eIF4E-binding proteins (4E-BPs). In detail, hypophosphorylated 4E-BPs compete with eIF4G for a shared binding site on eIF4E and consequently inhibit cap-dependent translation. In contrast, phosphorylation of 4E-BPs weakens the interaction with eIF4E, and thereby enables cap-dependent translation. Secondly, upon mitogenic and/or stress stimuli, eIF4E is phosphorylated by Mnk1 and 2 downstream of Erk and p38 MAPK signaling. Importantly, phosphorylation of eIF4E directly correlates with translation rates [121-122]. Another well-documented mechanism to downregulate protein synthesis involves eIF2 [115, 122]. eIF2 associates with GTP and Met-tRNA in order to transfer the initatior tRNA to the 40S subunit. At a later step during the initiation process, after GTP hydrolysis, the eIF2-GDP complex is released. Before eIF2 can promote a new round of translation initiation, the remaining eIF2-GDP requires an exchange for GTP, a reaction catalyzed by eIF2B. Phosphorylation of the eIF2α subunit stabilizes the eIF2-GDP-eIF2B complex and inhibits the turnover of eIF2B. Induction of PKR by interferon (IFN)-γ and TNF-α causes potent phosphorylation of eIF2α, a known mechanism which is important for the regulation of cell growth and apoptosis [115, 123].

Generally, Ras and PI3K are two major pathways which mediate growth factor, hormone or cytokine dependent translational alterations. Ras signaling activates the MAP kinases Erk1/2, which in turn activate the eIF4E kinases Mnk1 and 2, leading to an increase of translational initiation [115, 123]. A critical kinase that phosphorylates 4E-BPs is mammalian target of rapamycin (mTOR) [124-125]. mTOR is a downstream kinase of PI3K/AKT signaling which is responsible for the phosphorylation of several substrates relevant for translation, including S6 kinases (S6Ks). Phosphorylated S6Ks activate S6 ribosomal proteins which stimulate general translation. Prominent inhibitors of mTOR are PTEN and rapamycin [115, 123, 125].

Mechanism of Cap-independent initiation

The majority of mRNA translation in eukaryotic cells is initiated via cap-dependent ribosomal scanning mechanism. Nevertheless, under certain conditions such as cellular stress, proteins that are involved and required for cap-dependent translation initiation are compromised due to protein modifications or degradation [122]. In order to enable a

continous protein synthesis under stress conditions, mRNA translation relies on an alternative initiation mechanism [115]. This cap-independent initiation process is directed by a complex RNA structural element referred to as internal ribosome entry site (IRES) [115, 120-121, 126-128]. This structural motif, located within the 5´-UTR, enables the ribosome to bind directly upstream of the start codon and to initiate translation by bypassing cap-dependent ribosome scanning. The first IRES was discovered in 1988 during studies of poliovirus and encephalomyocarditis virus (EMCV) [129-131]. Since then, the list of viral and cellular mRNAs harboring an IRES in the 5´-UTR is still growing [127, 132-133]. Generally, IRESs allow maintenance of translation of certain mRNAs under conditions of reduced cap-dependent translation. Besides, the involvement of mRNAs containing IRES elements in apoptosis, angiogenesis, development, differentiation and cell cycle progression emphasizes important functions in cell physiology [115, 122-123, 129].

Features of IRES

IRESs are phylogenetically conserved structures that are often found in long GC rich and highly structured 5´-UTRs (Figure 11) [118-119, 122]. However, actually no universally conserved IRES sequence or structure has yet been identified. Therefore, scientists agree that ´diverse´ is the word that best describes IRES structures. Existing functional data in combination with structural information reveal a rich structural diversity of viral IRES RNAs supporting the idea of functional diversity. The *Dicistroviridae* intergenic region (IGR) IRES presents the most highly structured yet identified IRES [134]. This specific and compact three-dimensional structure that does not require any initiation factor and operates as an all RNA-based ribosome recruitment apparatus [135]. A prefold IRES conformation binds and actively manipulates both ribosomal subunits, possibly by mimicking a hybrid state tRNA, and directs translation initiation. In contrast to the IGR IRES, the hepatitis C virus (HCV) IRES additionally binds to eIF3, and requires initiator tRNA, eIF2, and GTP hydrolysis to initiate translation [134]. Detailed studies showed that the HCV IRES interacts with different components of the translation initiation machinery. Another class of viral IRESs does not fold globally compact structures but retains some conformational flexibility. Typically, these less-structured IRESs require various eIFs and IRES-trans acting factors (ITAFs) to recruit the ribosome [136-138].

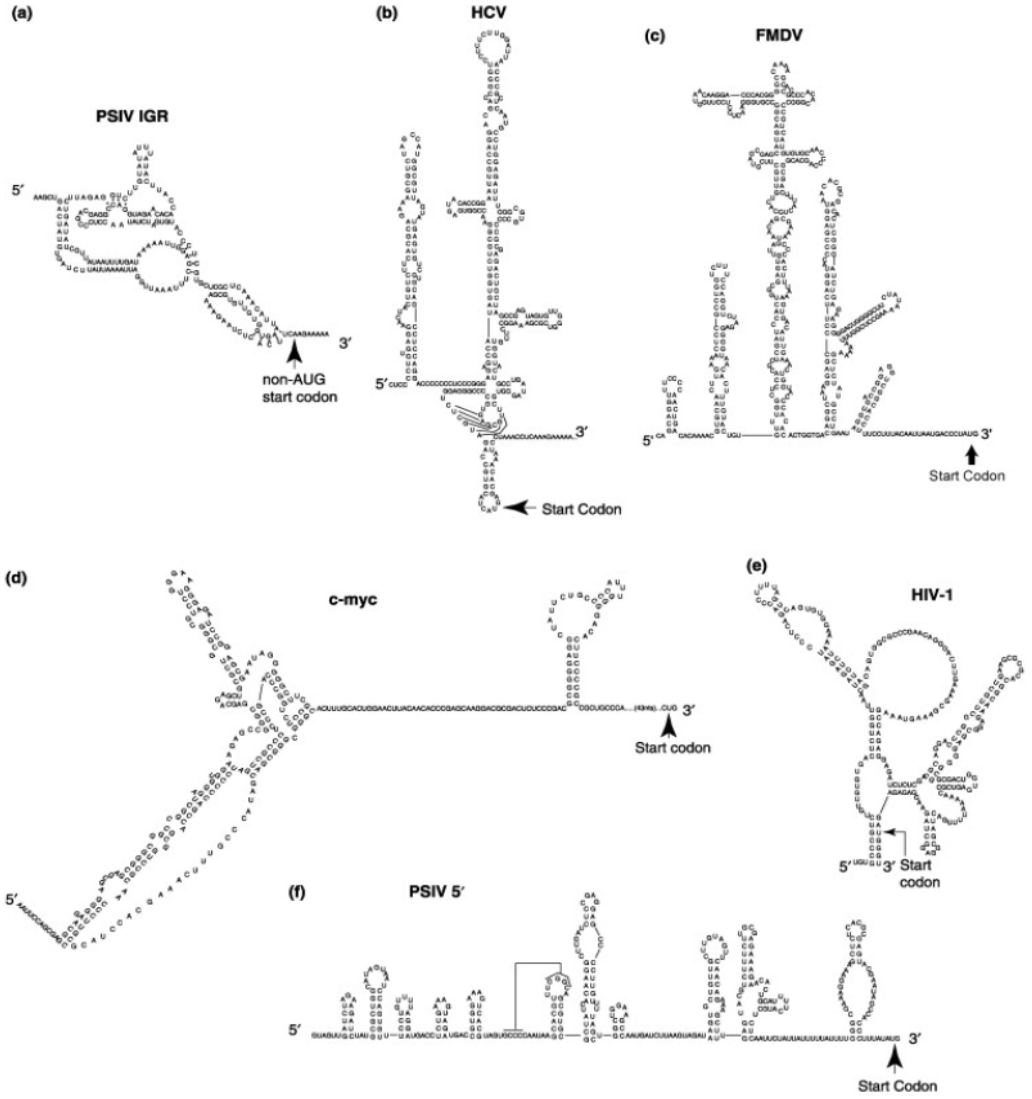

Figure 11. Examples of viral and cellular IRES secondary structures. (a) *Plautia stali* intestine virus (PSIV) IGR IRES, (b) HCV IRES, (c) FMDV IRES, (d) c-*myc* IRES, (e) Human immunodeficiency virus-1 (HIV-1) gag-IRES, (f) PSIV 5′ IRES, the black line indicates a proposed pseudoknot interaction [118].

ITAFs are not components of the canonical translation initiation machinery but essential proteins for the function of many IRESs. The structural integrity of IRES elements may be supported by ITAFs. Together, it is suggested that ITAFs are a part of a multi-component ribonucleoprotein (RNP) complex that enables and directs ribosome recruitment to the mRNA. However, the mechanisms by which ITAFs facilitate ribosomal recruitment is still poorly understood [136-137].

Cellular IRESs are characterized by a great variability in length, sequence and secondary structure [118]. The length ranges from 60 to 1000 nucleotids (nts), although

also 22 nts long, full active elements have been described. Structurally, cellular IRESs vary strongly from viral ones, but employ similar ITAFs. For example, some ITAFs that were initially identified in a viral context are also used by cellular IRESs including polyprimidine tract binding protein (PTB), heterogeneous nuclear ribonucleoprotein A1 (hnRNP-A1), La and upstream of N-ras (UNR) [136-138]. However, it should be mentioned, in some cases that ITAFs such as PTB are inhibitory to IRES function [139]. Numerous cellular mRNAs harboring an IRES have been already identified and associated with various physiological important processes such as differentiation, proliferation and angiogenesis [137, 140]. The observation that ITAFs can induce structural changes in cellular IRESs suggests that changes in expression and subcellular localization of specific ITAFs enable IRES-containing transcripts to respond precisely to changing cellular conditions [138]. Under specific cellular conditions such as stress, protein distribution and protein levels are altered, thus affecting structural state and activation of a subset of cellular IRESs [122, 137, 140].

Biological significance of internal initiation

IRES-mediated translation initiation represents an alternative mechanism to cap-dependent translation initiation in a wide range of cellular processes [118, 120, 127, 140-141]. Observations suggest that IRES-containing mRNA transcripts are predominantly translated via internal initiation under conditions of reduced cap-dependent translation due to e.g. stress, apoptosis or viral infections. In some cases such as for fibroblast growth factor 2 (FGF2), the balance of cap-dependent and IRES-mediated protein expression is determined by the availability of ITAFs and regulatory eIFs and the activity of other cis regulatory elements [139]. The presence of ITAFs and other regulatory factors has been confirmed to be essential for the activity of some cellular IRESs in *in vitro* studies [138]. Thus, these IRES-containing transcripts show general low cap-dependent and –independent translation under normal conditions, but are inducible in response to changing cellular conditions. Interestingly, internal initiation is mainly triggered during cellular processes such as differentiation, proliferation, angiogenesis and apoptosis or under conditions when cap-dependent translation is diminished such as heat shock, hypoxia or nutrient deprivation [122]. It is assumed that IRES-mediated translation control might regulate the cellular response in transient stress conditions in order to avoid programmed cell death [137].

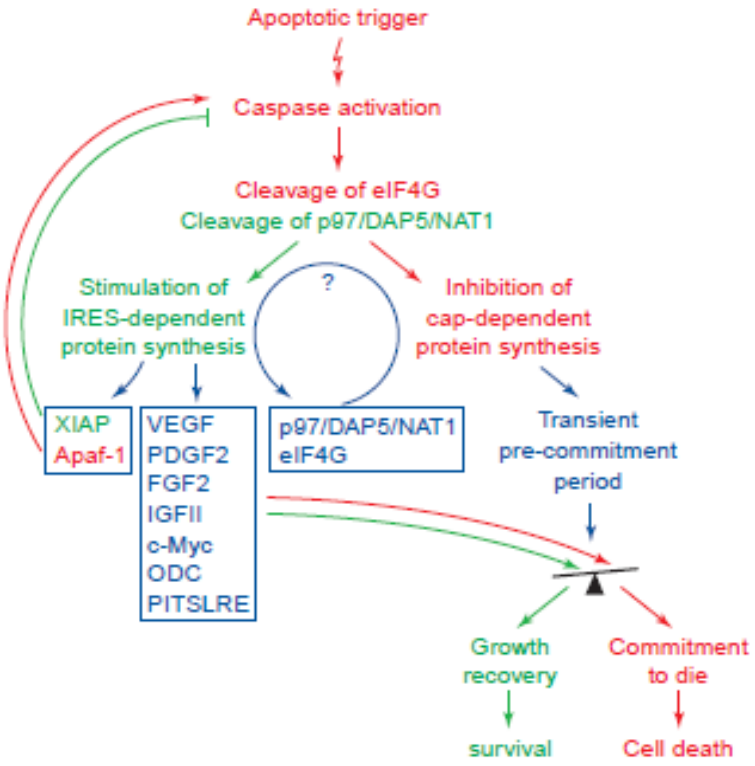

Figure 12. The proposed model for IRES-dependent translational regulation of cell death [142].

However, cap-independent translation has been implicated in the regulation of apoptosis itself (Figure 12) [126, 141-142]. During apoptosis, several factors required for the cap-dependent translation are reduced due to caspase cleavage. Under these circumstances, IRES-mediated translation displays an essential alternative method to synthesize proteins involved in the apoptotic process. Some important regulatory proteins related to apoptosis include c-myc, X-linked inhibitor of apoptosis (XIAP), (DAP5) and apoptotic protease activating and factor 1 (ApaF1) [126, 141, 143-144]. Furthermore, IRES-mediated initiation is involved in the synthesis of Bag-1 during the recovery phase of heat shocked cells, or the synthesis of alpha subunit of hypoxia-inducible factor-1 (HIF-1α) and FGF2, both growth factors promote blood vessel formation in hypoxic cells via IRES-mediated upregulation of VEGF [122-123, 126, 139, 145-146]. These above listed proteins are only few examples, which support the idea that cellular IRESs may be evolved as an alternative regulatory mechanism to respond precisely to certain states of the cell such as stress.

Internal initiation during carcinogenesis

The fact that IRES elements are observed in transcripts corresponding to regulatory proteins confirms an important function of internal translation initiation in many cellular processes [136]. The relevance of IRES-mediated translation in cancer is particularly provided by c-myc, one prominent pro-oncogene that is frequently transcriptional deregulated in many cancers. Interestingly, oncogenic gain-of-function provided by a C-T mutation located in the IRES of c-myc, correlates with increased c-myc translation in cells derived from patients with multiple myeloma [143, 147]. Futhermore, various proteins that contain an IRES element in the 5´-UTR are associated with carcinogenesis including survival factors such as Bag-1 and growth factors such as FGF2, PDGF and VEGF [122-123, 126, 139, 145-146]. Many genes that drive tumorigenesis are not mutated, but are translationally deregulated. This observation is supported by the fact that central signaling pathways of translation regulation, namely Ras/ERK and PI3K/Akt/mTOR pathway are frequently activated in tumors [148]. Both pathways regulate protein synthesis by activating the ribosomal S6 protein and / or translation initiation factors for the ribosome recruitment to the mRNA. In tumors, Ras is frequently activated by mutation, whereas enhanced PI3K signaling is provided by the inactivation of the tumor suppressor PTEN. eIF4E, a rate limiting factor of cap-dependent translation initiation is a major target of both signaling pathways. eIF4E is activated by phosphorylation as well as by the inactivation of its inhibitory counterpart, the 4E-BP. Under normal conditions, mRNAs may have to compete for the availability of eIF4E [24, 59, 123-124, 149-150]. Generally, it is supposed that short unstructured 5´-UTRs are more likely to become translated than those with long, GC rich and highly structured 5´-UTRs such as IRES containing mRNA transcripts. Experimental overexpression of eIF4E increases cap-dependent translation in various types of tumors including breast, head and neck, bladder, liver and colon cancers [151]. Importantly, elevated eIF4E levels selectively enhances the translation of IRES-containing and metastasis-related mRNAs such as VEGF, ornithine decarboxylase (ODC) or FGF-2. Accordingly, it was demonstrated that overexpression of eIF4E induces malignant transformation in epithelial cells. Another study could connect phosphorylated eIF4E with tumor cell survival. In a mouse model of B cell lymphoma it was shown that activated eIF4E targets the antiapoptotic protein Mcl-1, and thus suppresses apoptosis [151]. Enhanced eIF4E activity might also influence the process of epithelial to mesenchymal transition (EMT), a

frequent event in late stage tumorigenesis [151-152]. Although, it was shown that overexpression of eIF4E is required for the induction of EMT [123], little is known about translational regulation and the impact of IRES mediated translation during EMT. Interestingly, siRNA-mediated downregulation of eIF4E expression could revert the Ras-oncogene transformed phenotype in a cell culture model [153]. The transforming activity of eIF4E can be in part explained by its ability to maintain the IRES-mediated expression of several oncogenes under stress conditions. In many cancers the amount of eIF4E is elevated, supporting the idea of an indispensable role during tumor progression [123].

Translational control by the 3´-UTR

The 3´-UTR with its proximity to the termination codon and the poly-A tail offers a diversity of regulatory mechanisms [113, 154]. For example, polyadenylation signals within the 3´-UTR regulate mRNA stability, whereas other signals regulate the subcellular localization of specific transcripts. Additionally, processed miRNAs, loaded into RNA-induced silencing complexes (RISC), are able to target specific miRNA-complementary sites within the 3´-UTR and inhibit protein expression [115, 155-157]. However, the 3´-UTR has been demonstrated to communicate with the 5´-cap-complex via the interaction of PABP with the N-terminal part of eIF4G in order to modulate cap-dependent translation. Little is known about the involvement of the 3´-UTR on IRES-mediated translation [113, 144].

2.10. Laminin B1 in tumor progression

Laminin B1 (LamB1) is one of the three ß subunits that form together with α and γ chains several heterotrimeric laminin isoforms which perform diverse functions in different tissues [158]. The α-, ß- and γ-polypeptide chains build a triple-helical coiled structure that is organized by disulfide bridges, resulting in a cruciform shaped glycoprotein composed of three short arms, each formed by a different chain, and one main arm consisting of all three chains. LamB1 belongs to the extracellular matrix (ECM) proteins and is involved in many ECM - cell interactions, thus affecting multiple cellular processes such as cell adhesion, migration, proliferation and differentiation [159]. During tumor invasion and metastasis malignant cells cross the basement membrane in order to leave the primary tumor, to invade surrounding tissue and to intravasate into the vascular

system. The interaction of malignant cancer cells with LamB1, which is a main component of the basement membrane, is a key step in cell migration. Notably, a study with neoplastic cells revealed that the expression of the 67 kDA Laminin binding protein, a receptor that interacts with the LamB1 subunit, is enhanced and directly correlates with invasiveness [159]. Therefore LamB1 emphasizes its particular interest in cancer progression. Laminin signaling is mediated by integrins and its corresponding laminin receptor, leading to the activation of central signaling pathways such as MAPK and PI3K [160]. Both pathways are known to be involved in EMT related events such as microfilament rearrangements or regulation of cell growth and differentiation. In a recent study, expression profiling was performed in order to determine translationally controlled mRNAs during hepatocellular EMT [161]. Among the 84 translationally upregulated mRNAs, LamB1 was detected. Bicistronic reporter assays provided first evidence that the 5´-UTR of LamB1 contains a bona fide IRES that mediates the translational regulation during stress conditions and neoplastic progression of hepatocytes [110].

2.11. Working Hypothesis

Concerning the ECM protein LamB1, data of previous monocistronic reporter assays revealed a strongly enhanced reporter activity of constructs carrying the LamB1 5´-UTR, thus proposing an initiation mechanism alternative to ribosomal scanning [110]. Additionally, analysis of the LamB1 5´-UTR based on Zukers algorithm predicts a highly stable secondary structure with a minimal free energy of -154 kcal / mol that may impair cap-dependent translation. In agreement with existing data, experiments with bicistronic reporter constructs showed an activation of LamB1 protein expression via internal ribosome initiation upon EMT in murine MIM-Ras hepatocytes and in human SW480 colon carcinoma cells (Figure 13) [110]. Therefore, we propose that an IRES motif, located in the 5´-UTR, is essentially required for the cap-independent translation of the LamB1 mRNA. Additionally, we suggest a possible role of the LamB1 3´-UTR in regulating IRES-dependent translation. Furthermore, we suppose that MAPK and PI3K signaling is involved in IRES-mediated LamB1 translation in the murine hepatic EMT model.

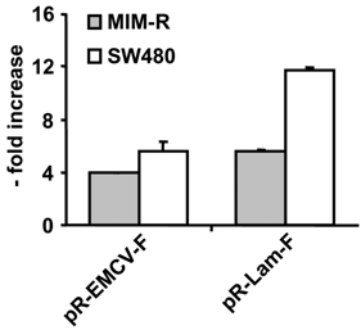

Figure 13. LamB1 5´-UTR upregulates reporter gene expression in murine and human cancer [110].

Recently, expression profiling was performed in order to identify EMT-specific genes. The interleukin like EMT inducer (ILEI), a cytokine-like secreted protein was revealed to be exclusively upregulated at the translational level [82]. Furthermore it was shown that ILEI is both necessary and sufficient for EMT, tumorigenesis and metastasis of epithelial cells [82, 162]. Besides the assumption that the leader region of ILEI mRNA confers cap-independent translation, we propose a regulatory role of the 3´-UTR on translational efficiency of cells undergoing EMT.

2.12. Aim of study

The general aim of the project is the identification of the cis-acting IRES motif of LamB1 mRNA. Therefore we aimed to determine the minimal sequence of the IRES motif which mediates cap-independent translation. In order to localize the IRES sequence , we established bicistronic reporter constructs carrying deletion fragments of the LamB1 5´-UTR. To accomplish our tasks we used the well studied malignant murine MIM-Ras hepatocytes. Furthermore, a monocistronic assay using the Firefly Luciferase (Luc) reporter gene was employed to investigate a regulatory role of the 3´-UTR of LamB1 in malignant MIM-Ras and metastatic MIM-RT hepatocytes in the context with IRES-driven translation. Within this experimental setup, we additionally examined a possible role of the ILEI 3´-UTR on translational efficiency in a murine breast cancer model. In order to examine LamB1 translational regulation in a human EMT model, we performed a monocistronic reporter assay in human colon cancer cell lines. To complete our investigations we elucidated the molecular mechanisms involved in IRES-mediated translation by pointing out the crucial role of the MAPK and PI3K signaling during EMT.

Aims of the study

- to determine the minimal sequence of the IRES motif which mediates cap-independent translation
- to investigate the translational upregulation of Laminin B1 in human colon cancer cells
- to examine the regulatory role of the 3´-UTR of LamB1 and ILEI
- to elucidate the signaling involved in IRES-mediated translation

3. Materials and Methods

3.1. Cell culture

The $p19^{ARF-/-}$ murine hepatocyte cell lines MIM-1-4 and MIM-Ras were seeded on rat tail collagen-coated cell culture dishes and grown in RPMI 1640 plus 10% fetal calf serum (FCS) and 1% antibiotics [103]. Medium for MIM-1-4 cells was additionally supplemented with 40 ng/ml recombinant human transforming growth factor (TGF)α (Sigma, St.Louis, USA), 30 ng/ml recombinant human insulin-like growth factor II (IGF-II, Sigma, St. Louis, USA) and 1,4 nM insulin (Sigma, St.Louis, USA) [103]. Malignant epithelial MIM-Ras cells were established by the stable retroviral transmission of the parental MIM-1-4 cells with oncogenic v-Ha-Ras [94]. Fibroblastoid MIM-RT cells, derived from MIM-Ras cells after long term treatment with TGF-ß1, were additionally supplied with 1 ng/ml human TGF-ß1 (R&D Systems, Minneapolis, USA) as described previously [94].
The murine mammary cell lines EpH4, EpRas and Ep-XT were cultured in Dulbecco´s Modified Eagle Medium (DMEM) plus 15% FCS and 1% antibiotics. Epithelial EpH4 cells tansformed with oncogenic v-Ha-Ras are termed EpRas cells, which retain full epithelial polarity [43]. Upon TGF-ß1 (1 ng/ml human TGF-ß1) treatment, these cells undergo EMT in collagen gels and show a fibroblastoid morphology, which is stabilized by autocrine TGF-ß signaling [82]. The human colon carcinoma cell lines HT-29, SW480 and SW620 were grown in DMEM supplemented with 10% FCS and 1% antibiotics.
All cells were kept at 37°C and 5% CO_2 and routinely screened for the absence of bacteria and mycoplasma.

3.2. Cloning of plasmids

Bicistronic reporter constructs carrying LamB1 5´-UTR deletions

Deletions of the LamB1 5'-untranslated region (UTR) were generated by PCR using the pLamF vector as template. Three different forward primer containing a NheI restriction site were designed which targeted the nucleotide positions 155, 200 and 235 within the LamB1 5´-UTR. The reverse primer, which contains a XhoI restriction site was positioned at the 3´-end of the LamB1 5´-UTR at nucleotide 335. Primers were designed according to GenBank sequence NM_002291. The generated amplicons were cloned into the

multiple cloning site (MCS) of a bicistronic reporter construct (p-ßgal-CAT) using the NheI and XhoI restriction sites. The resulting p-ßgal-155-CAT, pßgal-200-CAT and pßgal-253-CAT deletion contructs, the empty reporter construct (p-ßgal-CAT), a construct containing a 162bp long X-linked inhibitor of apoptosis (XIAP) 5'-UTR fragment (p-ßgal-XIAP-CAT) and the full length LamB1 5´-UTR (p-ßgal-Lam-CAT) were each transformed into *E.coli* GT116. The bicistronic reporter construct carrying the minimal IRES sequence of XIAP served as positive control and was a gift from M. Holcik.

Primer for LamB1 5'-UTR deletions:

5´-UTR_155_fw	5´-CCTAGCTAGCAGGCGCCTCCCT-3´
5´-UTR_200_fw	5´-CCTAGCTAGCTTCTTTGGGCTCGGG-3´
5´-UTR_253_fw	5´-CCTAGCTAGCGGAAGACGGGAAG-3´
5´-UTR_335_rv	5´-GCGCTCGAGGCCGGCTCCCT-3´

Monocistronic reporter constructs carrying the 5´-UTR and the 3'-UTR of LamB1 mRNA

Monocistronic reporter constructs carrying either the LamB1 5´-UTR upstream (pLam-F) or the LamB1 3´-UTR downstream (pF-Lam) of a firefly luciferase reporter gene were established. Additionally, a construct that harbors both the LamB1 5´-UTR and the 3´-UTR was generated (pLam-F-Lam). SW480 cDNA was used as template to amplify the LamB1 3´-UTR by PCR. PCR primers contained XbaI restriction site and were designed according to the GenBank sequence NM_002291. The amplified 3'-UTR was cloned into the XbaI restriction site of the pLam-F vector resulting in pLam-F-Lam containing the LamB1 5'UTR upstream as well as the 3'UTR downstream of the firefly reportergene. Subsequently, the monocistronic construct pF-Lam was generated by excising the LamB1 5´-UTR from the pLam-F-Lam vector using NheI and XhoI restriction sites. The pLam-F monocistronic reporter construct was already previously generated [110]. A monocistronic vector exclusively harboring the firefly luciferase reporter gene served as negative control and was together with pLam-F, pF-Lam and pLam-F-Lam transformed into *E.coli* GT116.

Primers used:

3´-UTR_LamB1_fw	5´-TCTAGATAGCACATGCTTGTAA-3´
3´-UTR_LamB1_rv	5´-TCTAGAACAAAAGCACTGTACT-3´

Monocistronic reporter constructs carrying the 3´-UTR of ILEI mRNA

The ILEI 3´-UTR was amplified from SW480 cDNA. PCR primers contained XbaI restriction sites and were designed according to the GenBank sequence NM_014888.2. The amplified ILEI 3´-UTR was cloned into the XbaI site of the monocistronic pF vector downstream of the firefly luciferase reporter gene, resulting in pF-ILEI. The generated constructs as well as the empty control vector pF were transformed into *E.coli* GT116.

Primers used:

3´-UTR_ILEI _fw	5´-TCTAGAGAAAGCGCACTTTCA -3´
3´-UTR_ILEI_rv	5´-TCTAGACAAAGCAAAACCAAGAG-3´

Ligation

Before carrying out the ligation reaction, purified linearized vectors were dephosphorylated (CIP-reaction) to prevent religation and to facilitate insertion of the purified amplicon.

CIP-reaction

10 µl purified DNA, 10 µl 10x Dephosphorylation buffer, 77,5 µl ddH_2O and 2,5 µl calf intestinal phosphatase (CIP [20 U/µl]) were mixed together in a microcentrifuge tube and incubated for 30 minutes at 37°C. In order to boost dephosphorylation, another 2,5 µl CIP were added and the mixture was incubated for additional 45 minutes at 55°C. Subsequently, the phosphatase was heat-inactivated by incubating for 15 minutes at 85°C. Dephosphorylated plasmids were purified using a DNA purification kit (QIAGEN, Hilden, Germany) according the manufacturers instructions.

Ligation

50-100 ng vector DNA were mixed with insert at different molar ratios (1:1, 1:2, 1:4) and incubated over night at 16°C or over weekend at 4°C.

Vector DNA: 50 – 100 ng
Insert DNA: 2 – 5 fold amount of the vector DNA

x µl vector-DNA
1 µl 10x Ligation Buffer
x µl Insert
x µl H2O
1 µl T4 Ligase
10 µl final volume

All cloned plasmids were sequenced.

3.3. Microbiology

Preparation of *E.coli* for transformation

50 ml *E.coli* - lysogeny broth (LB) suspension (OD_{600}=0,375) was centrifuged for 7 minutes at 3000 rpm at 4°C and obtained pellet was resuspended into 10 ml 0,1 M $CaCl_2$ solution. *E.coli* suspension was centrifuged for 5 minutes at 2500 rpm at 4°C and pellet was again resuspended into 10 ml 0,1 M $CaCl_2$ solution and then kept on ice for 30 minutes. Afterwards, *E.coli* suspension was centrifuged for 5 minutes at 2500 rpm at 4°C. The generated pellet was resuspended into 2 ml 0,1 M $CaCl_2$ solution, snap frozen in liquid nitrogen and stored at -80°C.

Transformation of *E.coli*

A 3 or 5 µl (10 ng) aliquot of vector DNA was resuspended into 100 µl of $CaCl_2$ - competent *E.coli* GT116 and kept for 10 minutes on ice. Cells were heat shocked at 42°C for 2 minutes and then placed back on ice. 1 ml LB -medium was added to regenerate *E.coli*. Cells were incubated for 1 hour at 37°C, shortly centrifugated at 110 g

and the supernatant was poured off. The cell pellet was resuspended in the remaining (~100 µl) supernatant, plated on LB/ampicillin plates and incubated at 37°C over night. All plasmids expressed ampicillin resistance as selection marker.

Plasmid DNA preparation

Single *E.coli* colonies were picked and grown in 3-5 ml LB/ampicillin-medium at 37°C for 3 hours by moderate shaking. 100 µl of these pre-cultures were inoculated in 200-500 ml LB/ampicillin and cultured at 37°C over night by moderate shaking. The plasmid DNA was isolated using a QIAfilter Plasmid Midi/Maxi Kit (QIAGEN, Hilden, Germany) as recommended by the manufacturer. The integrity of isolated DNA plasmids was confirmed by restriction analysis and separation of DNA fragments in an 1% agarose/ethidium bromide (EtBr) -gel.

Restriction digests of plasmids

Preparative plasmid digests

Reactions using DNA restriction enzyms were performed to excise DNA sequences from plasmids or to linearize plasmids for subsequent ligation reactions. Therefore, 30 µl vector DNA, 10 µl restriction buffer, 54 µl H_2O and 6 µl restriction enzyme were mixed and incubated for 2 – 4 hours at 37°C. For some double digests, reactions were optimized by altering digest conditions, or a step-by-step digestion was performed by inclusion of an additional purification step using QIAgen purification kits as recommanded by manufacturer (Fermentas, Burlington, Canada). Afterwards, the digested plasmids were separated via agarose gel electrophoresis. DNA fragments of the correct sizes were cut out with a scalpel and the DNA was purified using a Gelex purification kit (QIAGEN, Hilden, Germany).

Control plasmid digests

Control restriction analysis was performed to check the size and orientation of cloned DNA fragments. In general, 2 µl vector DNA, 1 µl restriction buffer, 6 µl H_2O were incubated with 0,5 µl restriction enzyme for 1 – 2 hours at 37°C. DNA fragments were separated by agarose gel electrophoresis and visualized by EtBr staining.

3.4. Agarose gel electrophoresis

Electrophoresis

Generally, 1% agarose gels were prepared. In case of smaller DNA fragments with a size < 300 bp, 2% agarose gels were employed. Agarose was dissolved in 1 x TAE (40 mM Tris-acetate, 1 mM EDTA) by heating up in the microwave. After cooling down to approximately 60°C, EtBr (1 µg/ml) was added. The gel was poured immediately afterwards and was allowed to polymerize for 45 minutes before use.

Sample preparation

DNA was mixed with sample buffer (0,313 M Tris-HCl, pH 6,8; 10% SDS; 0,05% bromophenol blue; 50% glycerol) and loaded onto the gel. Gels were run in 1 x TAE running buffer at 100 Volt. In the case of a preparative gel, DNA bands were excised with a razor blade under UV light. Subsequently, DNA was isolated using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany).

3.5. Polymerase chain reaction (PCR)

PCR to generate cloning fragments

PCR was employed to generate amplicons carrying at both ends restriction sites for cloning. cDNA or linearized plasmids served as templates for amplification.

First PCR program example:

2x (95°C 2´/ 56°C 1´10´´/ 72°C 1´55´´)
2x (95°C 50´´/ 54°C 52´´/ 72°C 1´40´´)
30x(95°C 42´´/ 52°C 45´´/ 72°C 1´10´´)
72°C 10´/ 8°C

PCR product received from first round of PCR was then used as template in a further PCR in order to generate high amounts of amplicon. The applied Taq beads (GE Healthcare, Buckinghamshire, UK) contained all substances needed for PCR reaction.

Second PCR program example:

2x (95°C 2´/ 56°C 1´10´´/ 72°C 1´55´´)
2x (95°C 50´´/ 54°C 52´´/ 72°C 1´40´´)
36x (95°C 42´´/ 52°C 45´´/ 72°C 1´10´´)
72°C 10´/ 8°C

First PCR

Template / PCR product	1 µl
Pfu-Buffer	5 µl
dNTPs (mM)	1 µl
Primer Mix (30 nmol)	30 µl
Pfu-Polymerase	0,5 µl
H_2O	18,5 µl
Final volume	50 µl

Second PCR

PCR product	5 µl
ddH_2O	5 µl
Primer Mix (30 nmol)	15 µl
Taq-Bead	1x
Final volume	25 µl

Control PCR

Besides control plasmid digestion, the plasmid DNA of transformed *E.coli* was checked directly by control PCR. Only the *E.coli* suspension containing the plasmid with insert in correct orientation provided a PCR product with a correct fragment length.

***E.coli*-suspension**	1 µl
ddH_2O	8 µl
Primer Mix (30 nmol)	15 µl
Taq-Bead	1x
Final volume	25 µl

PCR program example:

95°C 5´
2x (95°C 2´/ 60°C 1´10´´/ 72°C 1´55´´)
2x (95°C 50´´/ 58°C 52´´/ 72°C 1´40´´)
36x (95°C 42´´/ 52°C 45´´/ 72°C 2´)
72°C 10´/ 8°C

Primer pair used for verification of p-ßgal-155/200/253-CAT:

p-ßgal-155/200/253-CAT_fw	5´-GCGTCTTCTCCACTCCTCTG-3´
p-ßgal-155/200/253-CAT_rv	5´-CGTAACACGCCACATCTTGC-3´

Primer pair used for verification of pF-Lam:

pF_Lam_fw	5´-TTGCTAACGCAGTCAGTGCTTC-3´
pF_Lam_rv	5´-CCTTGTTCACCTCAGCCATT-3´

3.6. Transient transfection with Lipofectamine Plus™

Transient transfections of different cell lines were performed under the described cell culture conditions.

Cells were seeded on 6-well plates at a density of 3 x 10^5 cells one day before transfection in order to reach a cell confluence of 70%. Lipofectamine Plus™ was used for transient transfections as recommended by the manufacturer (Invitrogen, Carlsbad, USA). Relative Firefly Luciferase activity of monocistronic plasmids was determined by co-transfection of 0,75 µg plasmid and 0,25 µg ß-galactosidase reporter. Together, 1 µg vector DNA was mixed with 100 µl unsupplemented medium and 6 µl Plus reagent. In parallel, 4 µl Lipfectamine reagent were mixed with 100 µl unsupplemented medium. Both mixtures were carefully resuspended and incubated for 15 minutes at room temperature. Subsequently, both suspensions were mixed by pipetting gently up and down and incubated another 15 minutes at room temperature. Meanwhile, cells were washed with 1 x PBS (phosphate buffered saline: 8 g NaCl; 0,2 g KCl; 1,15 g Na_2HPO_4 and 0,2 g KH_2PO_4 in 1 liter H_2O) and 800 µl unsupplemented medium were added to each well. 200 µl of the incubated DNA-Lipofectamine Plus mixture were dropwise added to cells. After incubation of 3 hours at 37°C, cells were washed with 1 x PBS and supplied with 2 ml of fresh medium containing FCS and 1% antibiotics. Transfected cells were incubated for 48 hours at 37°C and 5% CO_2 before cell lysates were prepared.

ß-Galactosidase assay

The whole procedure was performed on ice. After washing cells with 1x PBS, 200 µl lysis buffer (0,25 M Tris/HCl pH 7,5; 0,5% Triton X-100) were added. Cell lysis was performed by pipetting up and down. The lysate was subsequently centrifuged at 12000 g for 10 minutes at 4°C and the supernatant was transferred into a new tube. Extracts were immediately used to perform assays or snap frozen in liquid nitrogen and stored at -20°C.

To analyze the ß-galactosidase activity, 20 µl cell extract or 20 µl H_2O as blank were incubated with 268 µl 0,1 M sodium phosphate solution (0,06 M Na_2HPO_4; 0,04 M NaH_2PO4; pH 7,5), 88 µl 1x 2-Nitrophenyl-β-D-galactopyranosid (ONPG) (Sigma, N-1127; 4 mg/ml of ONPG was dissolved in 0,1 M sodium phosphate solution; pH 7,5) and 4 µl 100 x Mg^{2+} solution (0,1 M $MgCl_2$; 4,5 M ß-mercaptoethanol) at 37°C for 30 minutes

until a faint yellow color developed. Optical density was photometrically measured at a wavelength of 420 nm (linear range is 0,2 – 0,8 O.D.). All assays were done in triplicate.

Luciferase reporter assay

Assay buffer was always freshly prepared by mixing 6,28 ml H_2O with 2,5 ml 0,1 M glycylglycine (Fluka 50199; pH 7,8), 150 µl 1 M $MgSO_4$ and 500 µl 0,1 M ATP (disodium salt, Boehringer). The injection buffer consisting of 6 ml H_20, 2 ml 0,1 M glycylglycine pH 7,8 and 2 ml 1 mM luciferin (D-Luciferin Sigma Sodium salt L-6882) was stored at -20°C.
Cell extracts were generated as described for the ß-galactosidase assay. 50 µl assay buffer was mixed with 20 µl cell extract or water as blank in a 96 well plate and immediately measured with a luminometer (Labsystems, Farnborough, UK). The assay was performed in triplicate.
Luminometer settings were adjusted as follows: The reagent volume of injection buffer to be automatically added before each measurement was set to 50 µl. Luciferase activity was measured in an integral curve of 10 seconds. The lag time between each measurement was set to 1 second. Measured luciferase activities were normalized to the corresponding ß-galactosidase values. Results represent the average of three independent experiments.

Chloramphenicol acetyltransferase (CAT) ELISA

The quantitative determination of the chloramphenicol acetlytransferase (CAT) activity in cell extracts was performed by photometry of Enzyme-linked Immunosorbent Assay (ELISA) plates.

Sample preparation for CAT ELISA

Cell extracts were generated according to the CAT ELISA protocol (Roche, Mannheim, Germany). The ß-galactosidase assay and CAT ELISA were performed with the same cell extract. Cells were washed 3 x with pre-cooled PBS and lysed with 500 µl CAT lysisbuffer per 6-well plate. Cells were subsequently scraped, transferred into microcentrifuge tubes and immediately snap frozen in liquid nitrogen and stored at -20°C. Frozen cell lysates were thawed up and centrifuged for 15 minutes at 4°C at 10000 g.

Supernatants were then transferred into new microcentrifuge tubes and employed for CAT and ß-gal assays.

CAT ELISA

Each prepared sample was measured in duplicate. The working procedure was performed according to the CAT ELISA manufacture protocol (Roche, Mannheim, Germany).

3.7. Western blot analysis

Sample preparation for Western blotting

All steps were performed on ice. RIPA+ lysis buffer containing 0,95 ml RIPA buffer (150 mM NaCl; 50 mM Tris pH 7,4; 0,5% Nadeoxycholate; 1 mM ß-Glycerophosphat pH 7,2; 1% Nonidet P-40), 50 µl 20x Complete and 17 µl inhibitor cocktail (1 mM NaF; 1 mM Na_3VO_4; 1 mM PMSF; 10 µg/ml Leupeptin; 10 µg/ml Aprotinin) was freshly prepared. Confluent cells were washed twice with ice-cold PBS and then lysed with 100 or 200 µl RIPA+ buffer. Afterwards, cells were scraped off using a police rubber, resuspended several times and transferred into microcentrifuge tube. Subsequently, the cell lysate was centrifuged for 10 minutes at 10 600 g at 4°C. The generated supernatant was transferred into a new microcentrifuge tube and snap frozen in liquid nitrogen and stored at -20°C.

Bradford assay

Bradford assay was performed to determine the protein concentration of cell lysates. A standard curve was generated with bovine serum albumin (BSA) dilutions (0,1; 0,2; 0,4; 1,8; 1,0; 1,2 mg/ml). All samples were diluted (1:10 or 1:20) with cell lysisbuffer in order to receive absorption values inside the linear range. Lysisbuffer served as blank. To perform the assay, 2 µl sample dilution, BSA dilution or water were mixed with 98 µl Bradford solution in a 96 well plate. Finally, absorption was measured at 595 nm. Protein concentrations of the cell lysates were then calculated with the standard curve.

Western blotting

Protocol

Western blotting was performed to detect proteins in RIPA+ cell lysates. Protein concentrations were determined by Bradford as described. 40 µg protein lysate was mixed with 2x sodium dodecyl sulfate (SDS) sample buffer, denaturated for 5 minutes at 95°C and then loaded onto a 7,5%, 10% or 12% polyacrylamide (PAA) gel. To compare protein weights, a prestained marker (Fermentas, Burlington, Canada) was additionally loaded on each gel. Separation gels and stacking gels were prepared according to the tables below.

for separation gels

	7,5%	**10%**	**12%**
30% PAA	2,5 ml	4,45 ml	5,2 ml
2 M Tris pH 8,8	1,87 ml	2,5 ml	2,25 ml
ddH_2O	5,63 ml	6,2 ml	4,42 ml
Total	10 ml	13 ml	12 ml

45 µl APS (10%); 7,5 µl TEMED were added; gels were overlaid with Isopropanol

for stacking gels

30% PAA/1% PDA	0,5 ml
1M Tris pH 6,8	0,5 ml
ddH_2O	3 ml
Total	4 ml

20 µl APS (10%); 4 µl TEMED were added

Under denaturing conditions, electrophoresis run for 2 hours at 100 constant voltage (V_{const}). Afterwards, separated proteins were blotted onto a nitrocellulose membrane for 1 hour at 400 mA. Nitrocellulose membrane was then washed with H_2O and stained with Ponceau to visualize proteins. To allow different protein stainings, nitrocellulose membrane was cut into parts if needed. Further, all membranes were blocked for 1 hour (at room temperature) or over night (at 4°C) with 5% BSA – Tris buffered saline-Tween (TBST) (150 mM NaCl; 10 mM Tris pH 8; 10% Tween 20) solution and afterwards directly stained with a primary antibody - TBST solution for 2 hours at room temperature. After 3x washing with TBST, the membranes were incubated with the secondary antibody – TBST solution for 1 hour at room temperature. Membranes were further washed for several times to remove the antibodies surplus. Finally, enhanced chemiluminescent (ECL) solution (Thermo scientific, Rockford, USA) was added,

incubated for 2 minutes and membranes were developed. In the presence of ECL solution, the horseradish peroxidase linked to secondary antibody performed an enzymatic reaction, which generates luminescence detectable on a X-ray film. The observed signal intensity corresponded to the amount of proteins presented in the loaded sample. The Actin protein served as loading control in order to ensure that comparable protein amounts were loaded. Due to the instability of protein phosphorylations, nitrocellulose membranes were first analyzed for the phosphorylated proteins and afterwards checked for the unphosphorylated proteins.

Solutions

2x SDS sample buffer:
1 ml Tris / HCL
5 ml 10% SDS
0,5 ml ß-mercaptoethanol
2 ml glycine
10 mM DTT
dd H_2O to 10 ml

10x Tris / Glycine:
30 g Tris
144 g glycine
dd H_2O to 1 l

TBST:
10 ml 10% Tween in
1 l 1x TBS

Electrophoresis buffer:
100 ml 10x Tris / Glycine
5 ml 20% SDS
final volume 1 l

Blotting buffer:
100 ml 10x Tris / Glycine
150 ml Methanol
1 ml 20% SDS
final volume 1 l

Western blot stripping

Membranes were incubated with pre-warmed stripping-buffer for 30 minutes at 50°C. Then buffer was poured off and membranes were washed with TBST for several times. Subsequently, membranes were blocked for 1 hour (at room temperature) or over night (at 4°C) with 5% BSA - TBST solution and incubated with antibodies as described above.

Stripping-buffer:
100 mM ß-Mercaptomethanol
2 % SDS
62,5 mM TRIS

List of used antibodies:

1st antibody	Label	kDa	source	Dilution
Laminin B1	Neomarker	210	Rat	1:1000
ERK 1/2	Cell signal.	42; 44	Rabbit	1:1000
pERK1/2	Cell signal.	42; 44	Rabbit	1:1000
eIF4E	Cell signal.	25	Rabbit	1:1000
peIF4E	Cell signal.	25	Rabbit	1:1000
AKT	Transd. Lab	59	Mouse	1:1000
pAKT	Cell signal.	59	Rabbit	1:1000
4E-BP1	Cell signal.	15 – 20	Rabbit	1:1000
p4E-BP1	Cell signal.	15 – 20	Rabbit	1:1000
Actin	Sigma	42	Rabbit	1:2500

2nd antibody	conjugate	Label	source	Dilution
Mouse IgG	Peroxidase	Vector Lab	Horse	1:10000
Rabbit IgG	Peroxidase	Vector Lab	Goat	1:10000
Rat IgG	HRP	Santa cruz	Goat	1:5000

4. Results

Minimal sequence required for IRES-mediated translation of LamB1

To gain insight into the changes of translation control during hepatocellular EMT, expression profiling of neoplastic epithelial MIM-Ras and metastatic fibroblastoid MIM-RT cells has been performed previously [161]. Evaluation of the data revealed a list of mRNAs suggested to be translationally up- or down-regulated. In particular, several components of the ECM including LamB1 were translationally upregulated upon hepatocellular EMT [110, 161]. Analysis of the 335 nt long and GC rich (68%) LamB1 5´-UTR predicted a highly stable secondary structure with a minimal free energy of -154 kcal/mol within the 5´-UTR [110]. Stable secondary structures within the 5´-UTR of mRNAs are generally assumed to hinder the ribosomal scanning process, thus impairing cap-dependent translation [119, 129, 163]. Investigations of the LamB1 5´-UTR translation efficiency by using reporter assays provided unexpected data. The 5´-UTR of human LamB1 could enhance the activity of a monocistronic reporter gene and additionally direct the translation of a bicistronic mRNA in murine hepatic carcinoma cells [110]. In summary, these data suggest that the leader region of LamB1 confers cap-independent translation by internal ribosome entry site (IRES) [110].

In order to localize the sequence responsible for the IRES activity, bicistronic reporter assays were performed. Multiple alignment of the LamB1 5´-UTR revealed that the sequence element which ranges from nucleotide position 150 to 335, is highly conserved between different species (Figure 14). Therefore, we generated bicistronic reporter constructs carrying different deletions of the 5-UTR. In particular, we examined three deletion fragments corresponding to region between nucleotide position 253 to 335, 200 to 335 and 155 to 335 of the human LamB1 5´-UTR. The resulting deletions having a length of 82, 135 and 180 base pairs (bp), were cloned in the bicistronic vector between the ß-galactosidase and CAT reporter genes (Figure 15A). All deletions of the human LamB1-5´-UTR were generated by PCR and contained NheI and XhoI restriction sites for forced cloning. While the translation of the upstream ß-galactosidase reporter is cap-dependent, the downstream CAT reporter is only translated in the presence of an IRES-containing and upstream positioned 5´-UTR (p-ßgal-Lam-CAT, Figure 15B, 16A). Bicistronic reporter constructs carrying one of the three deletion fragments of the LamB1 5´-UTR were generated (p-ßgal-155/ 200/ 253-CAT, Figure 15B and 16A). Furthermore,

a bicistronic plasmid containing a 162 bp long segment of the XIAP 5´-UTR provided a positive control for IRES-mediated translation (p-ßgal-XIAP-CAT) [164]. An empty bicistronic vector was used as negative control (p-ßgal-CAT) (Figure 16A). Each of the six vectors were transfected into MIM-Ras hepatocytes (Figure 16B). Subsequently, the ratios of CAT to ß-galactosidase activities were evaluated. Figure 17A and 17B shows a 30-fold upregulation of the p-ßgal-Lam-CAT plasmid compared to the negative empty control p-ßgal-CAT. Furthermore, the p-ßgal-Lam-CAT activity was 2-fold higher than the activity generated by the IRES-containing positive control p-ßgal-XIAP-CAT. Compared to p-ßgal-Lam-CAT activity, the analysis of the three constructs carrying LamB1 5´-UTR deletion fragments revealed decreased CAT activities, correlating with the length shortening of the analyzed deletions. The bicistronic construct containing the smallest deletion, p-ßgal-155-CAT, generated approximately 60% of the cap-independent CAT activity provided by the full length LamB1 5´-UTR. The p-ßgal-200 / 253-CAT constructs showed no more than 30% of the activity received by the p-ßgal-Lam-CAT plasmid. In conclusion, these data support the presence of an IRES motif in the leader region of LamB1 that allows cap-independent translation of LamB1 in murine carcinoma cells. From the data obtained by deletion analysis, we conclude that the sequence upstream of nucleotide 155 does not significantly contribute to cap-independent translation of the LamB1 5´-UTR.

LamB1 is not translationally upregulated in the human (metastatic) colon cancer cell line SW620.

It was previously reported that human LamB1 5´-UTR is translational upregulated in murine cancer cell lines as well as in a human cancer cell line [110, 112, 159]. Analysis of a bicistronic reporter construct containing the LamB1 5´-UTR revealed a 12-fold upregulation of reporter gene activity compared to a control plasmid in the human colon cancer cell line SW480 [110]. Notably, the increase of reporter gene activity was even higher in SW480 carcinoma cells than in murine MIM-Ras carcinoma cells. Accordingly, these data motivated us to establish a human EMT model in order to study the translational regulation of the LamB1 5´-UTR. Therefore we utilized the differentiated HT-29 colon carcinoma cells, the epitheloid SW480 and the spindle-shaped (metastatic) human SW620 colon carcinoma cells. Monocistronic reporter assays were performed to examine the effect of the LamB1 5´-UTR on general translation in human cancer cells.

We established a construct which contains the 5´-UTR upstream of the Firefly luciferase reporter gene (pLam-F) (Figure 18) [110]. The Firefly luciferase reporter gene is driven by a CMV promoter that is flanked by short intron sequence (IVS). Another construct carrying the encephalomyocarditis virus (EMCV) 5´-UTR was used as a positive control for IRES-mediated translation (pEMCV-F) and an empty plasmid served as negative control (pF) (Figure 18) [110]. The constructs were transfected into the three described colon carcinoma cell lines (Figure 19A) and assayed for the relative Firefly luciferase activity. As shown in Figure 19B, an enhanced reporter activity mediated by the LamB1 5´-UTR was detected in SW480 cells. In contrast, a strong decrease was measured in SW620 cells. As expected, the reporter activity of pLam-F was low in HT-29 cells but still higher compared to the negative control. Unfortunately, the relative Firefly luciferase activities of the positive control were in all transfected cell lines weaker than the empty vector activies. To confirm the obtained results, Western blot analysis was performed (Figure 19C). In accordance with the monocistronic reporter assay, the LamB1 protein level was upregulated in SW480 cells but not in SW620 cells. Taken together, these data confirmed a strong translational upregulation of LamB1 in SW480 colon cancer cells which exhibit increased malignancy. However, SW620 cells that have undergone EMT did only show a moderate increase in translation.

Interference with IRES-mediated translation by the LamB1 3´-UTR

Recent data revealed that the 3´-UTR of the mRNA communicates with the 5´-cap-complex via interaction of poly(A)-binding protein (PABP) with the N-terminal part of eIF4G in order to modulate cap-dependent translation. The importance of the 3´-UTR within the IRES-mediated translation still remains to be investigated [144]. To elucidate a possible role of the LamB1 3´-UTR on translation of LamB1, we performed a monocistronic Firefly luciferase assay. Therefore, vectors containing either the full length LamB1 5´-UTR (pLamF) or no insert (pF) upstream of the open reading frame (ORF) of the Firefly luciferase reporter gene were generated (Figure 20) [110]. Furthermore, the LamB1 3´-UTR was inserted into pLam-F and pF vectors, generating the pLam-F-Lam and pF-Lam constructs (Figure 20). Western blot analsis of LamB1 expression in parental MIM-1-4 hepatocytes, malignant MIM-Ras and metastatic MIM-RT cells showed an upregulation of LamB1 protein with the increase of malignancy (Figure 21A). Therefore, the four established monocistronic reporter constructs were then transiently

co-transfected with ß-galactosidase plasmid into neoplastic MIM-Ras and metastatic MIM-RT hepatocytes (Figure 21B) and relative reporter gene activities were determined (Figure 22A, B). As expected, relative Firefly luciferase activities of the constructs containing the LamB1 5´-UTR showed enhanced values compared to the empty vector construct in MIM-Ras and MIM-RT cells (Figure 22A). Notably, the plasmid that additionally carried the LamB1 3´-UTR (pLam-F-Lam) could not maintain the reporter gene activity provided by the pLam-F construct (Figure 22A). The evalution of the relative Firefly luciferase activites normalized to the empty vector activities revealed that the observed decrease is stronger in MIM-RT cells than in MIM-Ras cells (Figure 22B). Interestingly, in the absence of the LamB1 5´-UTR (pF-Lam), the LamB1 3´-UTR was able to promote the translation of the reporter gene. The received reporter activities normalized to the empty vector activity showed higher translation than the results obtained from pLam-F plasmid (Figure 22B). Additionally, the impact of the LamB1 3´-UTR on the Firefly luciferase activity was stronger in MIM-RT cells than in MIM-Ras cells (Figure 22B). From these data, we concluded that the regulatory role of LamB1 3´-UTR might depend on the sequence located upstream of the reporter gene. In the case of LamB1 5´-UTR, the data suggest that the LamB1 3´-UTR provides an inhibitory function on translation in hepatic carcinoma cells.

Interference of the ILEI 3´-UTR with translation

In order to detect novel EMT regulators, expression profiling was recently performed by employing total versus polysome-bound mRNAs of EpRas and Ep-XT breast carcinoma cells [82]. Within a cluster of genes specific for EMT and metastasis, the protein termed ILEI was revealed to be translational upregulated [82, 112]. ILEI belongs to a group of secreted proteins with largely unknown function (FAM3A-D) [82]. Experiments have recently shown that stable overexpression of ILEI causes EMT, tumor growth and metastasis in the mammary carcinoma EpH4/EpRas model (Figure 23A) [82].

Within our experiments, we focused on the question whether the translational upregulation of ILEI upon EMT is influenced by the 3´-UTR. Therefore, we compared the translation efficiency of a reporter gene in presence or absence of the ILEI 3´-UTR in a murine mammary EpH4/EpRas/Ep-XT tumor model which reflects EMT (Figure 23A). In order to rule out an interference of the ILEI 3´-UTR with translation, we performed a monocistronic reporter assay. We generated a construct where ILEI 3´-UTR is

downstream arranged of a CMV-driven Firefly luciferase reporter gene (Figure 23B). Plasmids with (pF-ILEI) or without the ILEI 3´-UTR (pF) were transiently transfected into nontumorigenic epithelial EpH4 cells, tumorigenic epithelial EpRas cells and metastatic, mesenchymal Ep-XT cells (Figure 23A). Subsequently, Firefly luciferase assays were performed out and the obtained reporter gene activities were normalized to the empty control vector activities. Interestingly, enhanced reporter activities in the presence of the ILEI 3´-UTR were obtained in all three cell lines. As shown in Figure 23C, the highest Firefly luciferase activity was measured in the mesenchymal Ep-XT cells. Additionally, we could observe a continuous increase of reporter gene activity corresponding to the progressive malignancy of the cell lines, from EpH4 cells to EpRas cells towards Ep-XT cells. From these data we concluded that the ILEI 3´-UTR is able to promote translation depending on the malignant stage in mammary carcinoma cells.

Signaling during hepatocellular EMT

The LamB1 mRNA is considered to harbor an IRES motif that mediates cap-independent translation [110]. Importantly, IRES-dependent translation of LamB1 mRNA is of particular interest as LamB1 has been reported to have severe implications in tumor progression [110, 161]. Therefore, we aimed to highlight signaling events which are involved in the regulation of IRES-mediated translation of LamB1. In our experimental setting, we showed that LamB1 is translational upregulated during hepatocellular EMT, dependent on the collaboration of constitutive active Ras and TGF-ß [42]. It has been reported that TGF-ß signaling cooperates with Ras through activation of ERK/MAPK and PI3K/AKT pathways [94]. Noteworthy, these pathways are frequently activated in tumors and play an important role in translation control, for example by regulating the availability of ribosomal proteins and eukaryotic initiation factors [148]. In order to gain insight into the translational regulation during hepatocellular EMT model, we aimed to determine the activation of PI3K and MAPK pathways. Therefore, we performed Western blot analysis of MIM-1-4, MIM-Ras and MIM-RT cell lysates (Figure 24A). To investigate MAPK signaling, we determined the unphosphorylated and phosphorylated protein levels of ERK and eIF4E. With regard to the PI3K signaling, we examined the phosphorylation status of AKT and 4E-BP1 proteins. As shown in Figure 24B, Western blot analysis revealed that constitutive active Ras signaling provided an increase of phosphorylated ERK protein which correlates with the progression in malignancy from MIM-Ras to MIM-

RT cells. As expected, unphosphorylated ERK protein levels kept constant in MIM-1-4 and MIM-Ras cells as well as in fibroblastoid MIM-RT cells. The anti-(phospho)-ERK antibody detected two ERK proteins, one with 42 kDa (p42) and a second one with 44 kDa (p44) molecular weight. Due to the fact that eIF4E-phosphorylation is mainly regulated by activated ERK, we expected a similar phosphorylation pattern of ERK and eIF4E proteins. As assumed, Western blot analysis revealed enhanced phosphorylation of eIF4E protein in MIM-RT cells as compared to MIM-14 and MIM-Ras cells. Levels of phosphorylated eIF4E in parental MIM-1-4 and neoplastic MIM-Ras cells were equal. Additionally, a slight increase of unphosphorylated eIF4E protein was detected in neoplastic MIM-Ras and MIM-RT cells compared to MIM-1-4. These data suggest that the availability of functional ERK and eIF4E is mainly provided by phosphorylation. Constitutive active Ras signaling might switch on translation via phosphorylation of ERK and eIF4E proteins, further leading to malignant transformation during EMT. Western blot analysis of phospho-AKT and AKT protein did not allow a proper interpretation but propose a slight increase for both proteins in MIM-RT cells. These data partially correlate with 4E-BP1 protein phosphorylation. Taken together, the eIF4E, AKT and 4E-BP1 protein synthesis is upregulated in MIM-Ras cells, which show tumorigenic features as well as in MIM-RT cells, which have undergone EMT. In conclusion, Western blot analysis revealed that signal effectors of the MAPK and PI3K pathway, namely ERK, eIF4E and 4E-BP1 protein are activated by enhanced phosphorylation in our murine hepatocellular EMT model, suggesting that these signaling cascades might have a crucial role in cap- and cap-independent translation of LamB1.

5. Discussion

As a subunit of the heterotrimeric Laminin, LamB1 belongs to a group of ECM proteins that are able to interact with surrounding cells [158]. These ECM-cell interactions affect multiple cellular processes such as cell adhesion, migration, proliferation and differentiation with the aim to maintain a physiological order within tissues [159]. All these processes are known to be crucial for the fate of malignant cells during tumor progression, suggesting an important role of Laminin in tumorigenesis. [160]. In addition, it is known that neoplastic epithelial cells frequently express aberrant Laminin receptors, directly correlating with enhanced invasiveness [110, 159]. Laminin signaling is mediated by laminin and integrin receptors which activate regulatory pathways involved in metastasis such as the MAPK and PI3K pathway [159-160]. Expression profiling of total versus polysome-bound mRNAs revealed LamB1 to be translational upregulated upon hepatocellular EMT [110, 161]. Actually, little is known about the translational regulation of LamB1 but some efforts have been performed to gain insight into the underlying molecular mechanisms. In this context, first evidence were recently presented for a cap-independent translation initiation of LamB1 which allows cells to immediately respond to changes under (patho)-physiological conditions [110]. In particular, LamB1 5´-UTR was capable to direct IRES-driven translation of a bicistronic reporter assay. Moreover the LamB1 expression increased under conditions of impaired cap-dependent translation by expression of human rhinovirus 2A protease or heat shock of cells [110]. In order to respond to the common criticism of the bicistronic reporter assay that the downstream reporter activity can arise from the presence of cryptic promoter or splice sites rather than from a *bona fide* IRES, additional experiments confirmed that the activity was indeed mediated by IRES [110, 165].

Finding the minimal sequence responsible for LamB1 IRES activity

While the IRES-mediated translation mechanism is commonly accepted as an alternative mode of translation in situations of attenuated cap-dependent translation, little is known about the nature and the molecular details of cellular IRESs [126, 166]. Sequence analysis could not yet identify any similarities among known cellular IRESs [167]. Thus, IRESs form complex and stable secondary structures allowing them to interact with components important for translation initiation [119]. Recently, a strong correlation

between IRES activity and structural stability of several yeast and fruit fly IRESs was detected. The highest IRES activities were found in RNA segments harboring the weakest secondary structures [119]. However, in our study we focused on the human LamB1 IRES element with the aim to identify the region upstream of the initiation codon that retains full IRES activity and therefore is responsible for cap-independent translation initiation. Our experimental analysis of the full length LamB1 5´-UTR provided evidence for an initiation mechanism alternative to cap-dependent translation. In our bicistronic reporter assays, the LamB1 5´-UTR was able to direct translation in murine neoplastic hepatocytes. Notably, the bicistronic reporter construct containing the LamB1 5´-UTR showed a 30-fold upregulation compared to the control construct (Figure 17). In order to describe a possible *in vivo* structure of the 335 nt long and GC rich (68%) LamB1 5´-UTR, Zuker algorithm has previously been performed and predicted a strong secondary structure with a minimal free energy of -154 kcal/mol [110]. Such a proposed stem-loop motif is likely to negatively interfere with the cap-dependent translation, as it has already been shown for secondary structures with a free energy up to -50 kcal/mol [163, 168-169]. Interestingly, the predicted secondary structure lies within the nucleotide position 180 to 335, the sequence which is highly conserved between different species (Figure 14). In accordance with these data we assumed that the nucleotide sequence 180 to 335 harbors the IRES activity. To determine the sequence responsible for translation initiation, we generated bicistronic reporter constructs containing different deletions of the LamB1 5´-UTR sequence. Unfortunately, none of the three tested fragments, 235 to 335; 200 to 335 and 155 to 335 of the 5´-UTR retained full IRES activity. The sequence region 155 to 335 reached 60% of the full length LamB1 5´-UTR activity, whereas the activity of the two shorter constructs significantly decreased ($P<0,008$), reaching only 30% of the full length LamB1 5´-UTR activity (Figure 17). Interestingly, the nucleotide segment 200 to 335 and 253 to 335 showed similar activities, thus supposing that the sequence directly upstream of the initiation codon contains elements important for IRES activity. These data further suggest that the sequence from nucleotide 200 to 253 may be irrelevant for IRES activity. However, the assumption that the 30% activity from the sequence 200 to 335 could be the result of aberrant expressed transcripts rather than from IRES-driven translation, can be partly refused by the fact that the activity levels are comparable with the one from the XIAP positive control. The IRES motif of the human XIAP 5´-UTR is a well described example for a cellular IRES which directs strong cap-independent translation and thus serves as optimal positive control to study the human

LamB1 5´-UTR [141]. Besides the unusual long 5´-UTR (>1,6 kb for human XIAP transcript) and the predicted complex secondary structure of the 5´-UTR, a polypyrimidine tract (PPT) located 34 nucleotides upstream of the initiation codon has been described. PPT deletion experiments revealed the need for the functional PPT in order to retain full XIAP activity. Therefore a specific sequence within the PPT may be criticial for XIAP activity, an observation firstly described in cellular IRESs [141]. Investigations on IRES of picornaviruses have shown that their translation activities are stimulated by noncanonical IRES trans-acting factors (ITAFs) such as polypyrimidine tract binding protein (PTB) or La autoantigen, presumably by stabilizing their active conformation [170]. These observations became relevant to us, as preliminary *in silico* analysis revealed as well PPTs (PPT-1, position -177 to -161; PPT-2, -204 to -194; PPT-3, -297 to -289) within the LamB1 5´-UTR. Accordingly, the detected PPTs sequences may be indispensable for full LamB1 IRES function [141]. However, one must take into consideration that past efforts in searching for conserved structures or general mechanisms among cellular IRESs were unable to identify common features of cellular IRESs [167]. It remains to be determined whether the PPTs within the 5´-UTR may have relevance for LamB1 IRES activity. Furthermore, the functionality of the LamB1 IRES could be provided by a combined effect of short modules that promote internal initiation, as it is the case for c-myc [171]. The main future project involves the identification of ITAFs by using RNA affinity chromatography in order to examine the molecular mechanisms underlying the LamB1 IRES translation. To reveal other sequence elements involved into the cap-independent translation initiation process, future experiments should include the characterization of the yet not examined LamB1 5´-UTR sequence from nucleotide position 1 to 155. In this way, each PPT should be deleted to test whether the specific PPT sequence is required for internal translation by reduction of IRES activity. Further analysis will be required to analyze whether the PPT elements are sufficient to mediate IRES activity by simply testing just possible PPTs and by including experiments with mutated PPT versions. Our data together with results of future experiments are considered to determine the minimal sequence required for LamB1 IRES-mediated translation. Previously identified and structural described cellular and viral IRESs may help us to interpret results of our LamB1 5´-UTR investigations. In this context we always have to keep aware that secondary structures of RNA generated and fold *in vitro* may differ from *in vivo* co-transcriptionally folded RNA [134].

The IRES-driven translation of LamB1 is of particular interest, as this ECM protein has severe implications in cancer progression [110]. Therefore the determination of the minimal IRES sequence for LamB1 biosynthesis may provide new insight into structural features important for the function of cellular IRESs in a neoplastic background.

Human EMT model?

EMT provides tumorigenic cells with mesenchymal features, thus enabling cancer cells to locally invade and metastasize throughout the human body [6, 46, 58, 63, 167]. In order to develop therapeutic strategies which help to hinder metastasis, various *in vitro* and *in vivo* EMT models have been established to study the molecular and cellular mechanisms and their regulatory components that drive this phenotypic conversion. In this regard, the ECM protein LamB1 was revealed to be involved within the EMT process [110]. LamB1 is translationally upregulated during hepatocellular EMT, what might be at least partially provided by an IRES motif in the leader region of LamB1 [110, 161]. The results from the present study support this observation as discussed. Beside the examination of the LamB1 gene expression in murine hepatic cell lines (Figure 17 and 21), we aimed to employ a human *in vitro* EMT model corresponding to our murine model (MIM-1-4, MIM-Ras and MIM-RT cell lines) to investigate LamB1 translation. Therefore, we performed Western blot analysis of three colon cancer cell lines isolated from primary adenocarcinomas (HT-29 and SW480) and from a lymph node metastasis (SW620) derived from the same patient as the previously isolated SW480 cell line. Our analysis results demonstrate that only the primary tumor cell line SW480 expresses a prominent LamB1 level. Unfortunately, the assumed increase of LamB1 expression, as observed in the murine metastatic MIM-RT cell line, could not be detected in human metastatic SW620 cells (Figure 19 and 21). Consequently, these colon cancer cell lines are not suitable to study LamB1 translation during human EMT. However, there are possible interpretations to explain the differences of LamB1 expression in the examined human and murine cell lines. Firstly, the cells originate from primary tumors and from lymph node metastasis and therefore are adapted to different microenviroments. Accordingly, the cancer cells might increase or decrease specific protein levels such as those from LamB1, in order to adapt themselves to their actual environment. Secondly, the previous study concerning the translational regulation of LamB1 was perfomed in a murine EMT model, established by cancer cell lines that were in part genetically

manipulated *in vitro*. Therefore, one may assume that the features of the examined murine and human cancer cell lines, including LamB1 expression, basically differ.

The regulatory impact of LamB1 and ILEI 3´-UTRs on translation

Translational regulation is mainly directed by the interaction of RNA-binding proteins with the 5´- and/or 3´-UTR of the mRNA [126, 154]. Besides the various features of the 5´-UTR that allow the regulation of the translation initiation process, the 3´-UTR has also other regulatory capacities such as regulation of mRNA stability [155-156, 172-173]. The complete regulatory potential of the mRNA UTRs is still unclear but so far investigations have already revealed some mechanisms for translational control, presenting another complex and well-organized level of gene expression regulation [113].

An effective mechanism to modulate cap-dependent translation is mediated by the communication of the 3´-UTR with the cap-complex via the interaction of poly(A)-binding protein (PABP) with the N-terminal part of eIF4G protein [113]. Little is known about the involvement of the 3´-UTR on IRES-mediated translation [144]. Within our study, we aimed to analyze the impact of the LamB1 3´-UTR and its associated regulatory components on the IRES-dependent translation control. So far, we tested whether the LamB1 3´-UTR effects general translation efficiency in a monocistronic Luciferase assay in the presence or absence of the LamB1 IRES-containing 5´-UTR. Therefore, we compared the impact of LamB1 3´-UTR on translational activity in neoplastic MIM-Ras and metastatic MIM-RT cell lines (Figure 22). Our results indicate an inhibitory effect of the LamB1 3´-UTR on translation in the presence of the IRES-containing LamB1 5´-UTR. In particular, the inhibition of translation mediated by the LamB1 3´-UTR was stronger in MIM-RT cells than in MIM-Ras cells. In contrast, in the absence of the LamB1 5´-UTR, LamB1 3´-UTR is able to increase translation. Enhanced translation activity was measured in both cell lines but again the effect was slightly stronger in MIM-RT cells. Notably, the employed monocistronic experimental setting allows no differentiation between cap-dependent or cap-independent translation. To complete our analysis, we additionally tested the influence of the LamB1 5´-UTR on translation in the absence of the LamB1 3´-UTR. Interestingly, LamB1 5´-UTR enhances translation efficiency in the LamB1 3´-UTR absence (Figure 22). In accordance with our actual knowledge about the 3´-UTR influence on translational control, the obtained data do not allow us a clear conclusion. Our data neither suggest a general enhancing nor a general inhibitory effect

on cap-dependent translation. Results of several investigations have led to the concept of a functional interaction between the “head” and the “tail” of mRNA transcripts. The existence of a closed-loop or circular structure between the poly(A) tail and the 5´cap, mediated by PABP, has been described [113]. This transcript circularization is thought to promote translation initiation because of its ability to stimulate mRNA binding to the preinitiation complex (PIC) as well as to facilitate reinitiation by the post-termination ribosomes [113, 150]. Besides the important function of the poly(A) tail for cap-dependent translation initiation, an indispensable role of the poly(A) tail for the IRES-mediated translation is also proposed [113, 154]. For example, IRES-driven translation of c-myc and BiP mRNA was enhanced by the poly(A) tail in the absence of intact eIF4G and PABP [144]. Therefore, it is evident that the cap-independent translation is influenced by the poly(A) tail, whereas the impact of the remaining 3´-UTR sequence is still unclear. With respect to our data, we can detect a regulatory effect of LamB1 3´-UTR on both cap-dependent and cap-independent translation. However, further experiments will be needed to rule out underlying mechanisms. Together, our data suggest that the impact of the LamB1 3´-UTR to enhance or to inhibit translation might depend on the sequence or secondary structure present upstream of the initiation codon. However, there is no doubt that the 3´-UTR with its multiple binding sites for several regulatory factors possesses an eminent regulatory potential to alter gene expression [113]. Various factors such as microRNAs (miRNA) are able to target specific sites within the 3´-UTR, thus influencing different aspects of the mRNA such as mRNA metabolism, conformation, stability, localization or translational efficiency [113, 115, 174]. Therefore, sequence and structural analysis of the LamB1 3´-UTR has to be performed to reveal important regulatory elements which may help us to interpret our data. Furthermore, one must take into consideration that the 135 bp long LamB1 3´-UTR can be considered as a rather short 3´-UTR since quantitative analysis of UTRs suggest a mean 3´-UTR length of human transcripts > 500 bp [175-176]. In addition, it is hypothesized that the 3´-UTR length increases with evolution and with the complexity of organisms [113]. Therefore, one should address the question of what is the general consequence of shorter 3´-UTR as in the case of LamB1. In this context, it was recently reported that mRNAs with shorter 3´-UTRs, generated by alternative cleavage and polyadenylation (APA), exhibit increased stability and produce more protein, partly due to the loss of miRNA-mediated repression. Importantly, the incidence for APA is high in cancer cells, supposing a consequent loss of 3´-UTR repressive elements leading to oncogene activation [177].

Within our examinations concerning the regulatory role of 3´-UTRs on translation efficiency, we aimed to analyze the regulatory capacity of the 3´-UTR of ILEI on translation. This cytokine-like protein was recently shown to be both necessary and sufficient for EMT, tumorigenesis, and metastasis of normal epithelial cells [82]. In our experimental setting, we wanted to rule out an interference of the ILEI 3´-UTR with translation by using a monocistronic reporter assay. The presence of the ILEI 3´-UTR was able to enhance the translational efficiency of a reporter gene in murine breast cancer cell lines (Figure 23). Additionally, we show that the positive influence of the ILEI 3´-UTR on translation progressively increases with the malignancy of cell lines. Therefore, the ILEI 3´-UTR is able to stabilize and enhance translational efficiency, an observation that goes together with the assumption that ILEI is upregulated exclusively at the translational level [82]. From previously performed *in silico* analysis, we found that the 1620 bp long ILEI 3´-UTR contains three polyadenylation sites, four miRNA binding site and several other regulatory elements. In this context, we suggest further investigations in order to highlight the existence of ILEI 3´-UTR isoforms and in particular to detect a possibly interference of one ILEI 3´-UTR isoform with a cellular state of malignancy.

In conclusion, it remains unclear how those 3´-UTRs enhance or inhibit translation. So far, only few *trans*-acting proteins have been identified. Together with the low degree of conservation between 3´-UTR sequences, this knowledge makes functional predictions very difficult [174]. However, investigation of the extremely diverse translational control mechanisms hold promise for the future development of highly specific RNA-based therapies that may enable to target the expression of individual genes.

MAPK and PI3K signaling during hepatocellular EMT

We demonstrate the cap-independent translation of LamB1 during hepatocellular EMT, a process featuring several hallmarks of liver carcinoma progression [38]. The employed murine EMT model is represented by MIM hepatocytes that undergo EMT, caused by the synergy between oncogenic Ras and TGF-ß1 signaling [42, 178]. TGF-ß signaling cooperates with Ras through the activation of ERK/MAPK and PI3K/AKT signaling pathways [94]. Ras and PI3K/AKT are frequently activated in tumors and are known to play a crucial role in translation control and malignant transformation [148]. In this context, the PI3K/AKT/mTOR pathway regulates protein translation by activating the

ribosomal protein S6 and various translation initiation factors important for the recruitment of the ribosome to the mRNA [149, 179]. To gain insight into translational regulation of hepatocellular EMT, we focused our investigation on the activation of MAPK and PI3K pathways. In general, the PI3K/AKT/mTOR and Ras/MAPK cascades signal towards the components of the translation machinery, thus regulating translation initiation process. mTOR, a downstream kinase of PI3K, directly phosphorylates 4E-BPs and S6 kinases (S6Ks), which are components of the translation machinery, and indirectly activates the eukaryotic initiation factor (eIF) 4B and 4G [123, 180]. On the other hand, the Ras/MAPK pathway drives the phosphorylation of eIF4E and eIF4B. An important regulatory step within the cap-dependent translation initiation is the mRNA 5´cap recognition process by eIF4F complex [115, 121]. A number of different translation initiation factors, including the 5´cap-binding protein eIF4E, are essential in order to establish the eIF4F complex. The eIF4E antagonists, the unphosphorylated translation repressor protein 4E-BPs, compete with eIF4G for a common eIF4E-binding site. In the case of phosphorylation, the interaction of 4E-BPs with eIF4E is disrupted, thus leading to the recruitment of eIF4G to the 5´-cap by eIF4E and further activate cap-dependent translation [115]. Additionally, mitogenic and/or stress stimuli induce phosphorylation of eIF4E via MAPK/Erk, while the role of eIF4E phosphorylation concerning translation initiation remains controversial.

In our Western blot analysis, the expression of LamB1 was enhanced in Ras-transformed hepatocytes and again increased upon TGF-ß treatment (Figure 24). The translational upregulation of LamB1 might be provided by Ras-induced enhancement of cap-dependent translation. In this context, it has recently been shown that the upregulation of LamB1 correlates with enhanced IRES activity in a bicistronic reporter assay [110]. The authors point out that the IRES-driven translation appears to be relevant in situation of cellular stress which is provided by TGF-ß signaling upon the induction of EMT [94]. It is hypothesized that MAPK/ERK signaling generates resistance against TGF-ß mediated effects such as cell cycle arrest and apoptosis during the early phase of hepatocellular EMT, whereas PI3K/AKT signaling is activated by TGF-ß autocrine production and designate a maintenance phase [62, 94]. However, our Western blot analysis reveal elevated levels of phosphorylated ERK and phosphorylated eIF4E protein in Ras-transformed hepatocytes and further increases during TGF-ß mediated EMT, thus indicating an activation of the MAPK pathway. The analysis of the activation of the PI3K pathway are difficult to interpret but we suppose a slight increase

of phosphorylated AKT protein upon TGF-ß induced EMT. In contrast elevated (phospho)-4E-BP1 protein levels are considerably apparent in cells with constitutive active Ras signaling. Therefore we conclude that translational repressor protein 4E-BP1 is partly deactivated upon hepatocellular EMT by AKT/PI3K mediated phosphorylation, thus leading to the release of the 5´-cap binding protein eIF4E. Finally, ERK/MAPK signaling mediates phosphorylation of liberated eIF4E protein, which in turn enables cap-dependent translation initiation. However, further experiments have to confirm these data. In addition, we aim to highlight the activation status of other regulators involved in the regulation of cap-dependent and IRES-mediated translation. The knowledge about the regulation of translation in (de)differentiated cells may help to better understand human diseases such as cancer.

6. References

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Figure 14

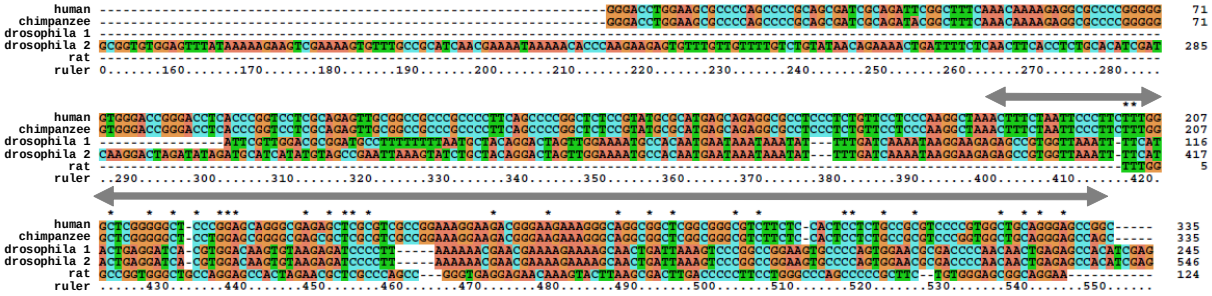


Figure 14. Alignment of human Laminin B1 5´-UTR with different species revealed a highly conserved region between nucleotide position 180 to 335. Arrow indicates assumed IRES localization.

Figure 15

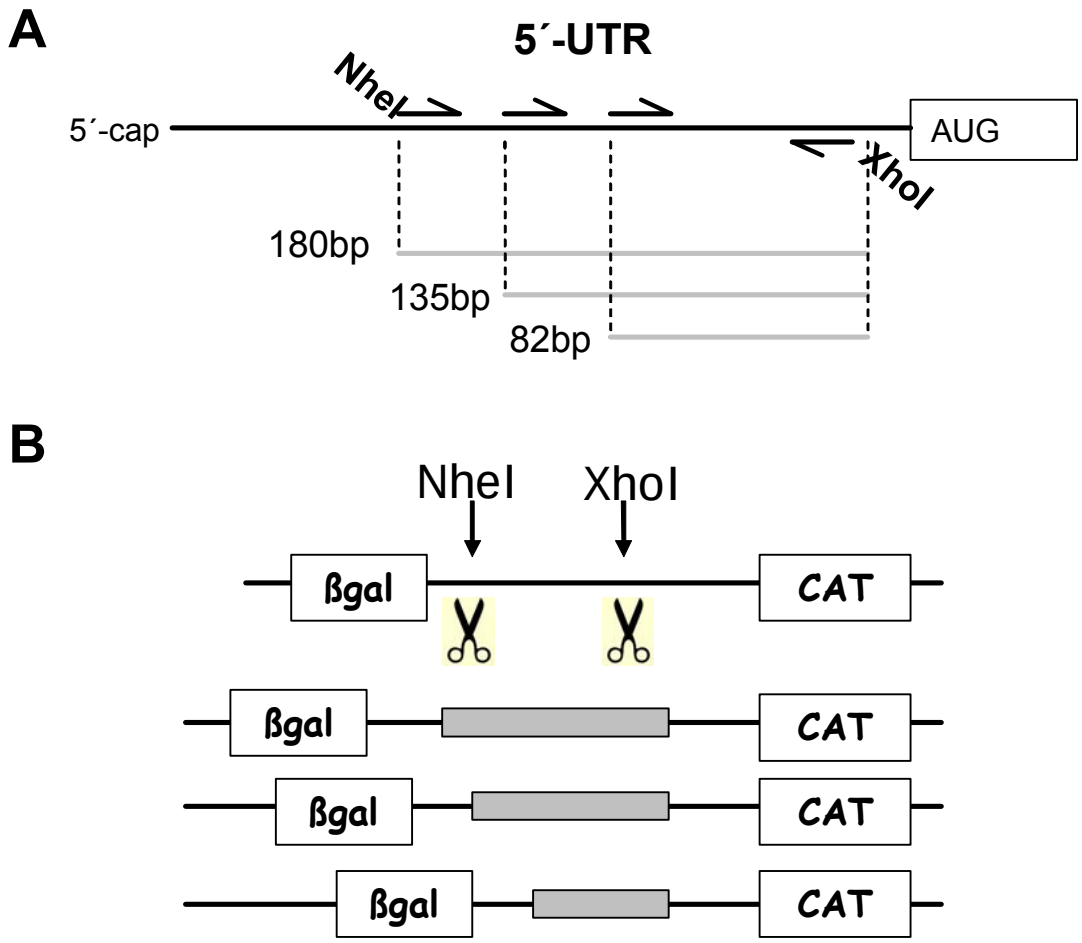
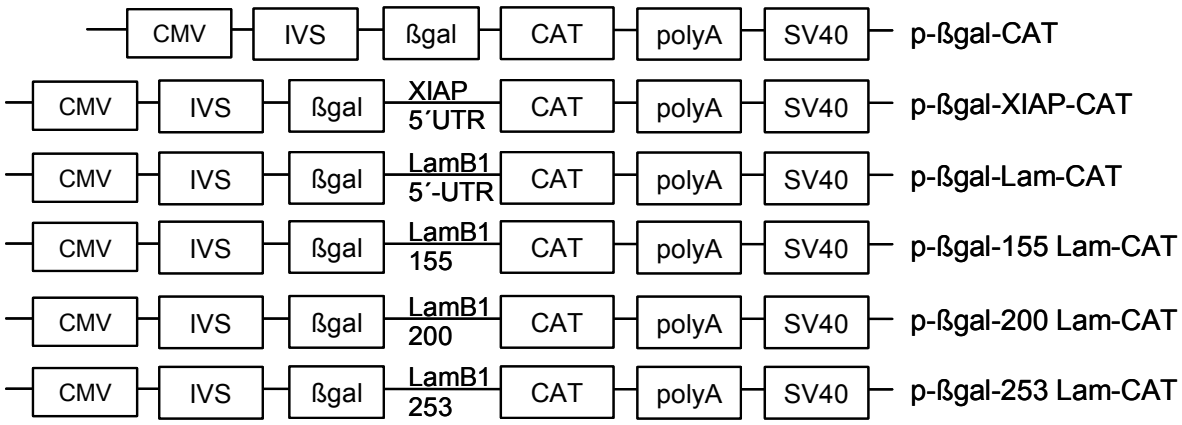


Figure 15. Cloning of the ß-gal/CAT bicistronic reporter constructs containing LamB1 5´-UTR deletions. **A**, Preparation of fragments of the Laminin B1 5´-UTR. Three deletion fragments with a length of 82, 135 and 180 base pairs from the LamB1 5´-UTR were generated by PCR and subsequent enzymatic digestion, generates NheI and XhoI restriction site for cloning. **B**, Construction of bicistronic reporter constructs. Three different deletion fragments of the LamB1 5´-UTR were ligated into a linearized bicistronic reporter plasmid. To investigate translation of reporter constructs, the three 5´-UTR deletions were inserted between the ß-galactosidase and CAT reporter gene. UTR, untranslated region, ßgal, ß-galactosidase reporter gene, CAT, chloramphenicol acetyltransferase.

Figure 16

A



B

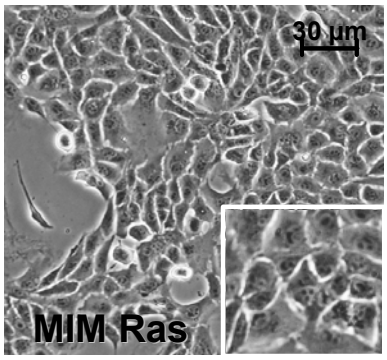


Figure 16. A, Schematic diagram of vectors employed in this study. The bicistronic vectors contain a XIAP, the full length or deletion fragments of the LamB1 5´-UTR in the linker region between ß-galactosidase and CAT reporter. **B**, Phase contrast image of murine malignant MIM-Ras hepatocytes which were transfected with bicistronic reporter constructs containing deletions of the human LamB1 5´-UTR. Insert shows cells at higher magnification. CMV, cytomegalovirus promotor; IVS, intervening sequence; ßgal, ß-galactosidase reporter gene; CAT, chloramphenicol acetlytransferase; polyA, polyadenylation site; SV40, Simian virus 40; LamB1, Laminin B1; XIAP, X-linked Inhibitor of Apoptosis Protein.

Figure 17

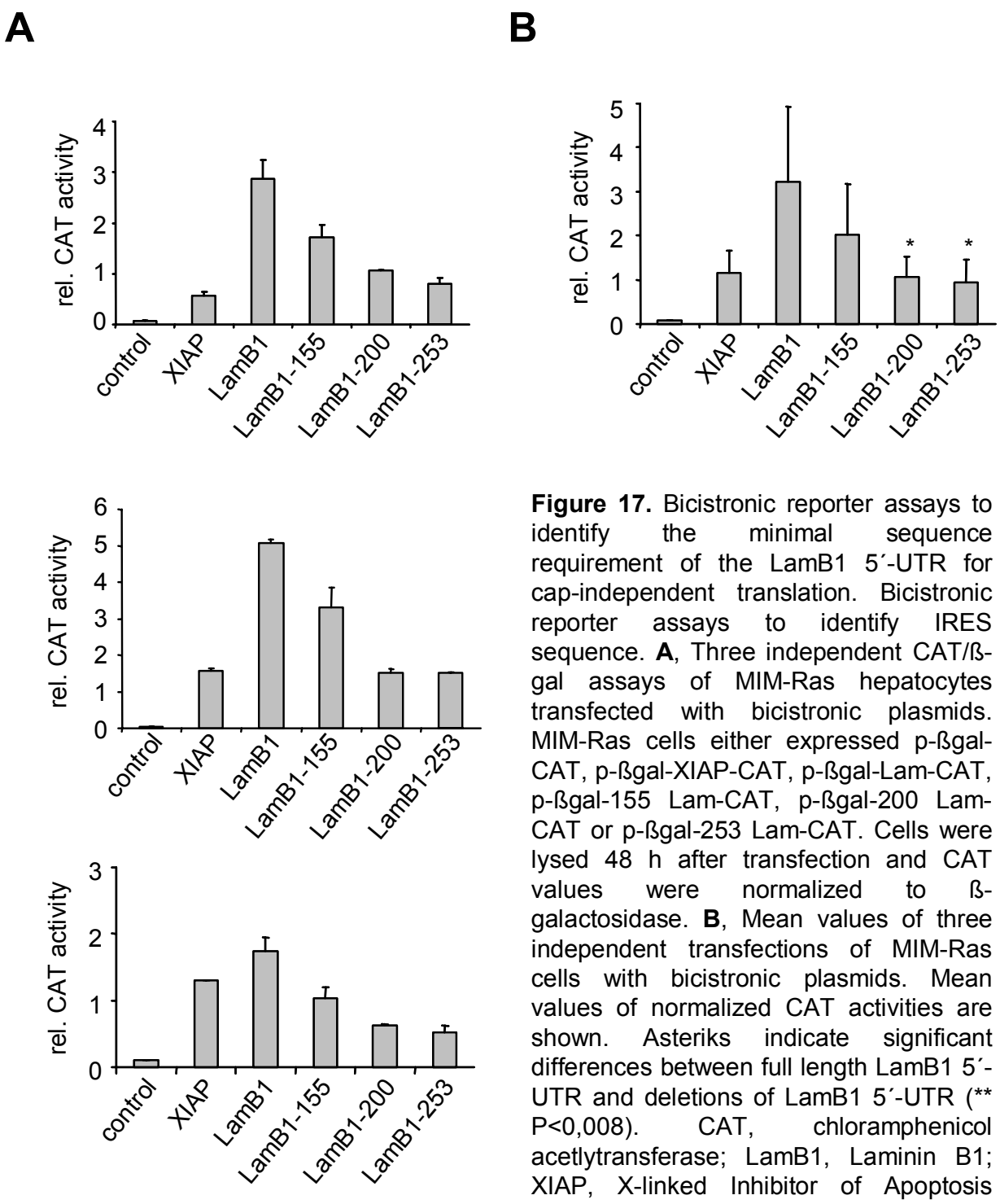


Figure 17. Bicistronic reporter assays to identify the minimal sequence requirement of the LamB1 5´-UTR for cap-independent translation. Bicistronic reporter assays to identify IRES sequence. **A**, Three independent CAT/ß-gal assays of MIM-Ras hepatocytes transfected with bicistronic plasmids. MIM-Ras cells either expressed p-ßgal-CAT, p-ßgal-XIAP-CAT, p-ßgal-Lam-CAT, p-ßgal-155 Lam-CAT, p-ßgal-200 Lam-CAT or p-ßgal-253 Lam-CAT. Cells were lysed 48 h after transfection and CAT values were normalized to ß-galactosidase. **B**, Mean values of three independent transfections of MIM-Ras cells with bicistronic plasmids. Mean values of normalized CAT activities are shown. Asteriks indicate significant differences between full length LamB1 5´-UTR and deletions of LamB1 5´-UTR (** P<0,008). CAT, chloramphenicol acetlytransferase; LamB1, Laminin B1; XIAP, X-linked Inhibitor of Apoptosis Protein.

Figure 18

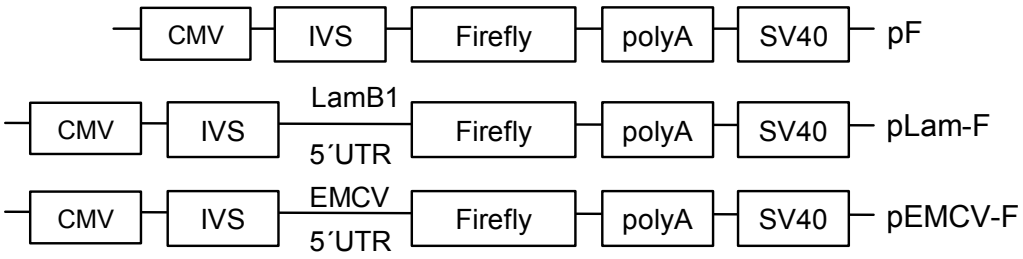


Figure 18. Schematic diagram of monocistronic vectors used to analyze LamB1 translation in human colon cancer cells. The monocistronic vectors contain an EMCV or LamB1 5´-UTR upstream of the Firefly luciferase reporter gene. CMV, cytomegalovirus promotor; EMCV, encephalomyocarditis virus; IVS, intervening sequence; LamB1, Laminin B1; polyA, polyadenylation site; SV40, Simian virus 40.

Figure 19

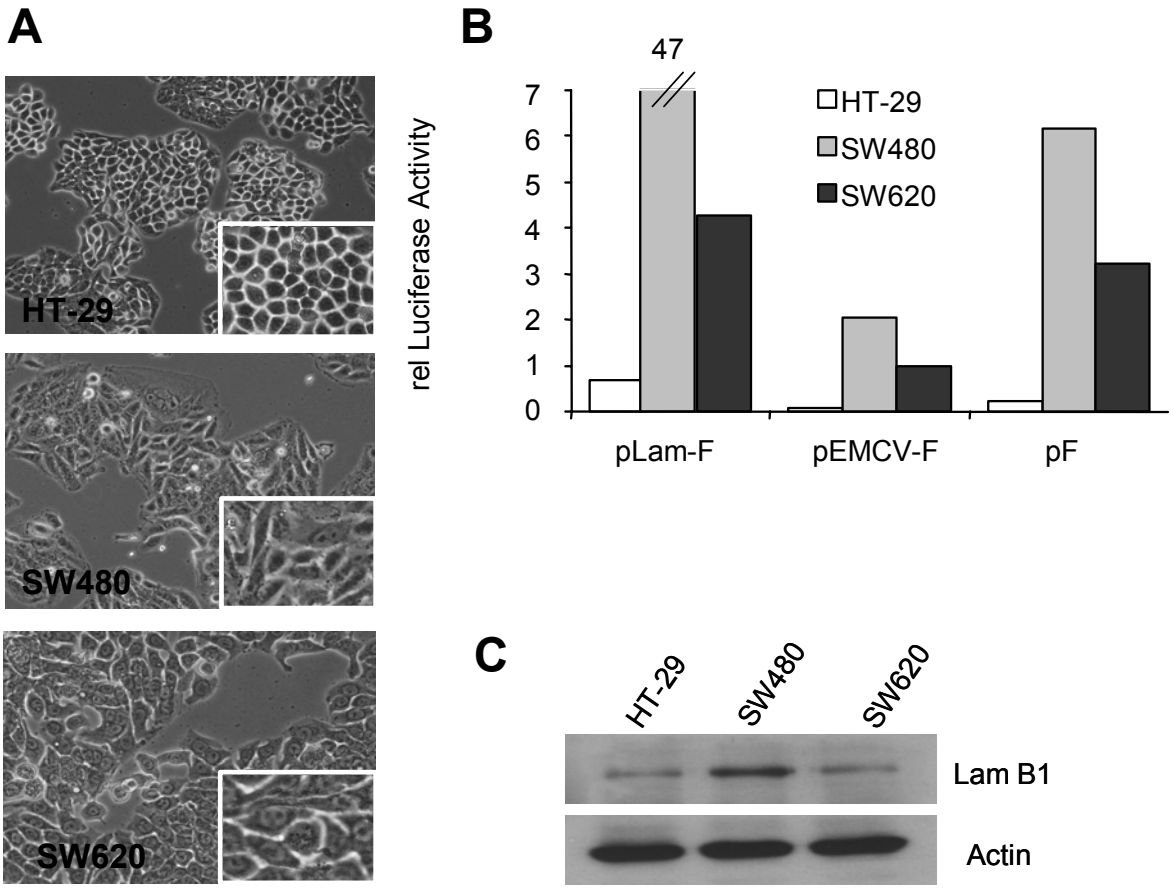


Figure 19. Analysis of LamB1 5´-UTR-dependent translation in human colon cancer cells. **A**, Phase contrast microscopy of polarized HT-29, epitheloid SW480 and fibroblastoid SW620 human colon cancer cells. Inserts show cells at higher magnification. **B**, Firefly luciferase assay of human colon cancer cells, transfected with monocistronic pF, pEMCV-F or pLamF plasmids. Cells were lysed 48 h post-transfection and the firefly activity was normalized to ß-galactosidase levels. **C**, Western Blot analysis of LamB1 in colon cancer cell lines. Actin is shown as loading control. EMCV, encephalomyocarditis virus; LamB1, Laminin B1.

Figure 20

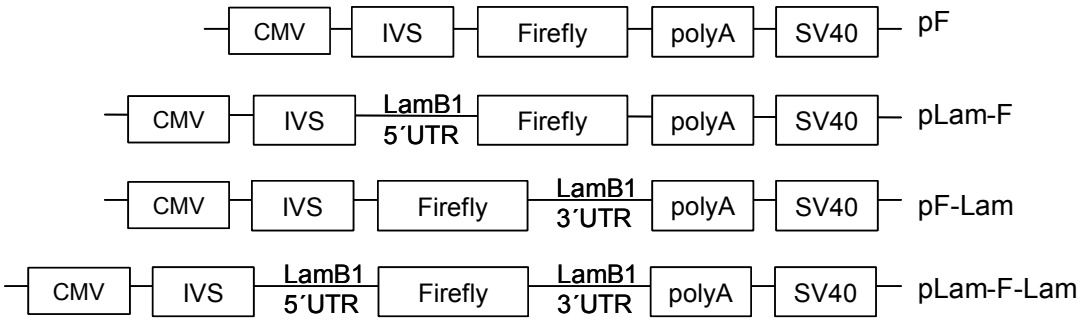


Figure 20. Schematic diagram of vectors used to study the impact of the LamB1 3´-UTR on LamB1 translation. Monocistronic vectors contain either the LamB1 5´-UTR upstream or the 3´-UTR downstream of the reporter gene. Additionally, a monocistronic vector containing both LamB1 5´- and 3´-UTR was employed. CMV, cytomegalovirus promotor; IVS, intervening sequence; LamB1, Laminin B1; polyA, polyadenylation site; SV40, Simian virus 40.

Figure 21

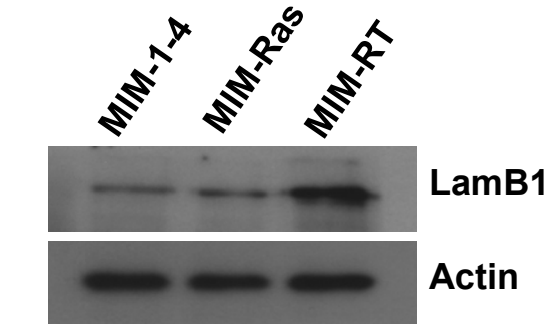


Figure 21. A, Western Blot analysis of LamB1 in parental MIM-1-4, neoplastic MIM-Ras and fibroblastoid-MIM RT cells. Actin is shown as loading control. **B**, Phase contrast microscopy of polarized tumorigenic MIM-Ras and unpolarized metastatic MIM-RT treated with 1 ng/ml TGF-ß 1. Inserts show cells at higher magnification. LamB1, Laminin B1.

Figure 22

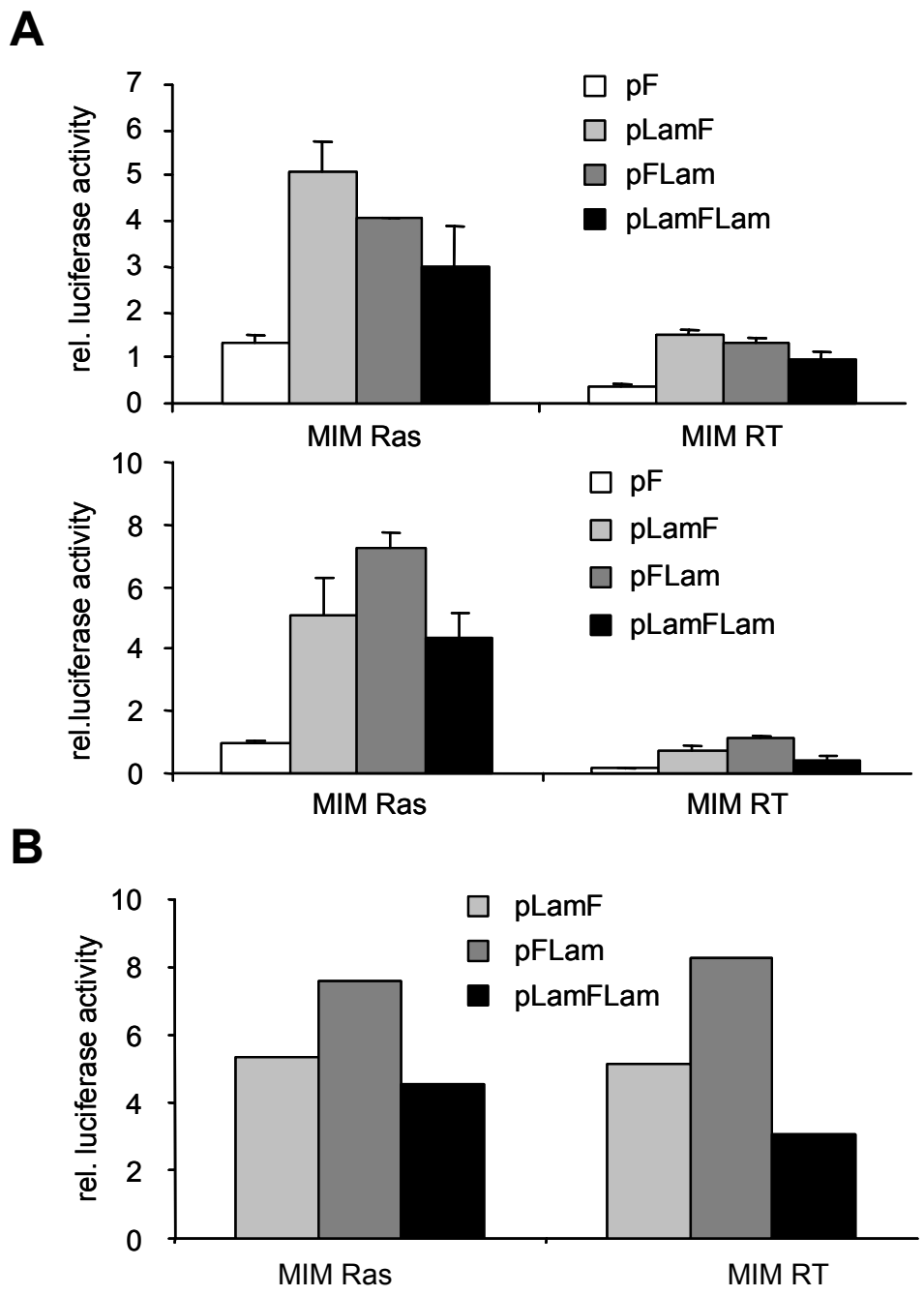


Figure 22. Monocistronic reporter assays to detect regulatory capacities of the 3´- or 5´-UTR of LamB1. **A**, Results of two independent reporter assays are shown. Firefly luciferase activity of MIM-Ras and MIM-RT hepatocytes transfected with either monocistronic pF, pLam-F, pF-Lam or pLam-F-Lam. Cells were lysed 48 h after transfection and Firefly luciferase activity was normalized to ß-galactosidase levels. **B**, Diagram presents mean values of two independent performed transfections normalized to pF values (**A**). LamB1, Laminin B1.

Figure 23

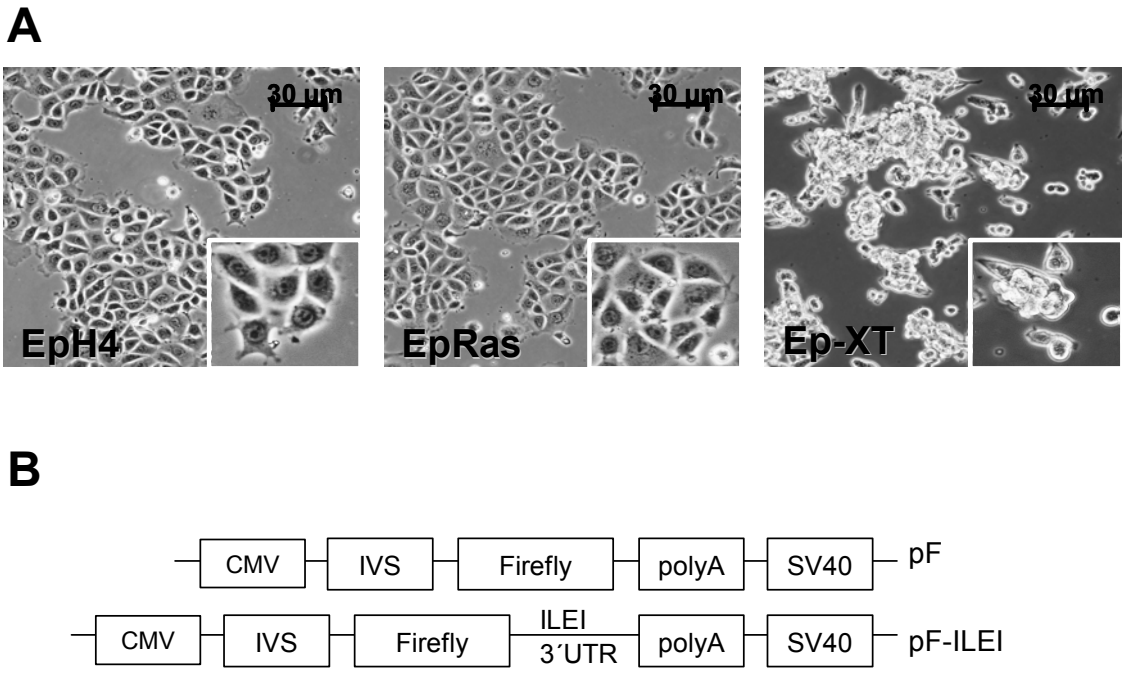


Figure 23. A, Phase contrast microscopy of epithelial EpH4, polarized tumorigenic EpRas and the fibroblastoid Ep-XT cells. Inserts show cells at higher magnification. **B**, Schematic diagram of employed transfection vectors. The monocistronic reporter construct in the absence or presence of ILEI 3´-UTR downstream of the Firefly reporter gene. **C**, Firefly luciferase assay of murine EpH4, EpRas and Ep-XT mammary cells transfected either with monocistronic pF or pF-ILEI. Cells were lysed 48 h post-tranfection and the Firefly luciferase activity was normalized to ß-galactosidase. Diagram presents mean values of three independent performed transfections normalized to pF values. CMV, cytomegalovirus promotor; IVS, intervening sequence; ILEI, Interleukin-like EMT inducer; polyA, polyadenylation site; SV40, Simian virus 40.

Figure 24

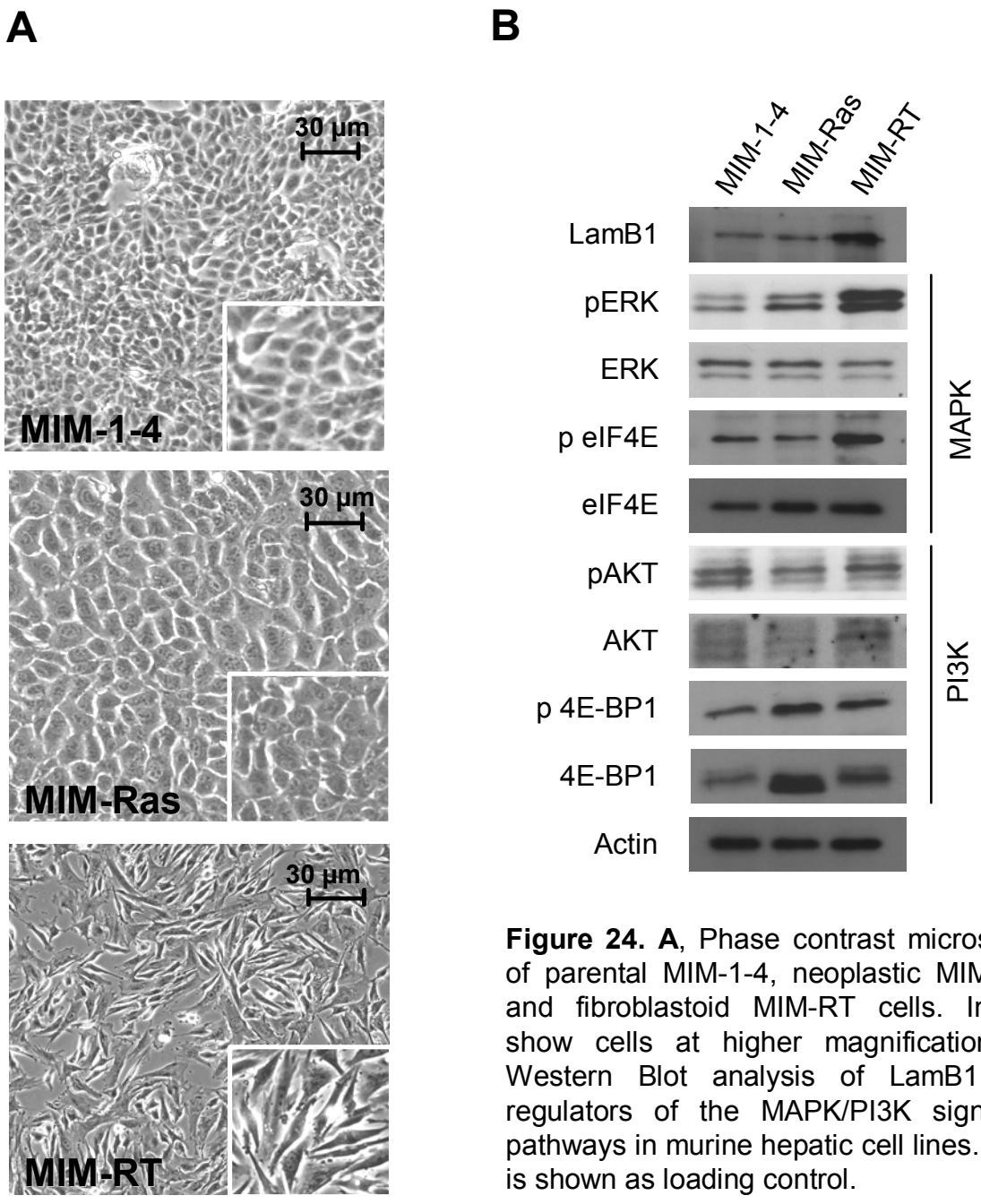


Figure 24. A, Phase contrast microscopy of parental MIM-1-4, neoplastic MIM-Ras and fibroblastoid MIM-RT cells. Inserts show cells at higher magnification. **B**, Western Blot analysis of LamB1 and regulators of the MAPK/PI3K signalling pathways in murine hepatic cell lines. Actin is shown as loading control.

7. Abbreviations

APC	adenomatous polyposis coli
Bcl	B-cell lymphoma
ß–cat	ß-catenin
CAM	cellular adhesion molecule
CBP	Creb-binding protein
ECM	extracellular matrix
EGF	epidermal growth factor
eIF	eukaryotic initiation factor
ELISA	enzyme linked immunosorbent assay
EMCV	encephalomyocarditis virus
EMT	epithelial to mesenchymal transition
ERK	extracellular-signal-regulated kinase
FAK	focal adhesion kinase
FGF	fibroblast growth factor
Fas-L	Fas-ligand
GF	growth factor
GFP	green fluorescent protein
GS	growth signal
GSK-3ß	glycogen synthase kinase ß
Ha-Ras	Harvey rat sarcoma viral oncogene
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HGF	hepatocyte growth factor
IGF	insulin-like growth factor
IGR	ionotropic glutamate receptor
IL	interleukin
ILEI	interleukin-like EMT inducer
IRES	internal ribosome entry site
ITAF	IRES-trans acting factor
JAK	Janus kinase
Jnk	c-Jun amino-terminal kinase
kDa	kilodalton

LamB1	Laminin B1
MAPK	mitogen activated protein kinase
MET	mesenchymal to epithelial transition
MMP	matrix metallo protease
mTOR	mammalian Target of Rapamycin
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NIC	notch intracellular domain
ORF	open reading frame
PDGF	platelet derived growth factor
PABP	poly(A)-binding protein
PI3K	phosphatidylinositol-3’-kinase
PKB	protein kinase B
PKR	protein kinase RNA
PPT	polypurine tract
pRb	retinoblastoma protein
PTB	polypyrimidine tract binding
PTEN	Phosphatase and Tensin homolog
Raf	rapidly growing fibrosarcoma
Ras	rat sarcoma viral oncogene
RhoA	Ras homolog gene family member A
RISC	RNA-induced silencing complex
RNP	ribonucleoprotein
R-Smad	receptor-regulated Smad
RTK	receptor tyrosine kinases
SARA	Smad anchor for receptor activation
SCID	severe combined immuno deficient mice
SMURF	Smad-specific E3 ubiquitin protein ligase
Src	avian sarcoma (schmidt-ruppin A-2) viral oncogene
STAT	signal transducer and activator of transcription
TACE	trans-arterial percutaneous chemo-embolisation
TF	transcription factor
TGF-ß	transforming growth factor
TGIF	TGF-ß induced factor
TNFα	Tumor necrosis factor α

TßR	transforming growth factor receptor
uPA	urokinase-Typ Plasminogen Aktivator
UTR	untranslated region
VEGF	vascular endothelial growth factor
VEGF-R	vascular endothelial growth factor receptor
Wnt	Wingless-type MMTV (murine mammary tumor virus) integration site
XIAP	X-linked inducer of apoptosis

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First of all, I want to thank Wolfgang for giving me the opportunity to do my diploma thesis and to learn so much during this year in his laboratory, as well as for his support and patience when experiments did not work so well. Furthermore, I want to thank Michi for giving me technical support, for all the interesting discussions, the hours she spent to correct this script and for supplying me with cups of hot coffee at the right moments. Thanks to all my great colleagues, Doris, Franziska, Georg, Gudrun, Heidi, Markus, Michi, Pips, Rebeca and Sabine, who gave me technical support and explanations, made me laugh every day and took me like I am. I am especially indebted to Franziska, Heidi, Michi and Wolfgang who motivated me with empathetic words at hard times. Finally, I want to thank Harold for his love and support and for being next to me during all the good and hard times.

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French: basic knowledge

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Wien, März 2010

Mara Hau