IGF2 mRNA-binding protein 1 promotes mesenchymal cell properties by enhancing the expression of LEF1

Dissertation

zur Erlangung des
Doktorgrades der Naturwissenschaften (Dr. rer. nat.)

der

Naturwissenschaftlichen Fakultät I – Biowissenschaften –

der Martin-Luther-Universität
Halle-Wittenberg,

vorgelegt

von Frau Anne Marie Zirkel

geb. am 30.11.1982 in Ludwigsfelde

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Die Arbeit wurde am 04.09.2013 verteidigt.

In memory of my aunt,

Antje Zirkel (*19.08.1961 - †18.09.1990)

» Doctors are men who prescribe medicines of which they know little, to cure diseases of which they know less, in human beings of whom they know nothing. «

Voltaire

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1 Introduction

1.1 Insights in eukaryotic gene regulation

Gene expression in eukaryotic cells is regulated at different levels. A tight control is essential for complex developmental and differentiation processes as well as maintaining cell or tissue homoeostasis. Changes in gene expression are implemented by transcriptional and/or post-transcriptional control.

Transcriptional control defines which and how much of an mRNA is synthesized. The promoter which is driving the gene specific mRNA production may be silenced or activated depending on the conditions of the respective cell types. In order to adapt to variable environmental or metabolic conditions promoter activity can be regulated for example by Chromatin methylation or Histone acetylation and is specifically modulated by DNA binding proteins, called transcription factors, either repressing or promoting transcription in response to cell signaling. For example, in the context of developmental and malignant processes like the epithelial-mesenchymal-transition a switch of transcription factors leads to the suppression of epithelial genes and the activation of genes important for mesenchymal cell properties. This variation in transcriptional activity allows the cell to acquire new cellular features to pursue different functions.

The post-transcriptional control of gene expression defines if and how much of the transcribed RNA is finally translated into functional protein. The mRNA turnover is characterized by intramolecular determinants as well as trans-acting factors. Besides other regulatory steps, the post-transcriptional fine tuning by RNA-binding proteins (RBPs) and microRNAs results in a defined ratio of protein synthesis and mRNA degradation. Accordingly, an abnormal regulation of RBPs and microRNAs in malignancies results in an altered turnover and subsequent translation of their target mRNAs. The translated protein itself can be modified in various ways, e.g. phosphorylation, acetylation or ubiquitylation, thereby representing additional levels in controlling the functional outcome of the gene. A highly interrelated cross-talk of transcriptional, post-transcriptional and post-translational control of gene expression thereby allows specialization and adaptation of cells. However, the escape from a

tightly regulated expression of genes by mutations and epigenetic modifications can lead to malignancies like cancer. The pathological consequences resulting from defects in the control of gene expression will be first described in the context of epithelial-mesenchymal-transition followed by introducing an oncofetal group of RNA-binding proteins and their role in post-transriptional gene regulation.

1.2 Epithelial-mesenchymal-transition in development and disease

In the process of tumor progression many cancer types develop metastases derived from the primary tumor. The capacity to invade the surrounding tissue requires the transformation of tumor cells with changes in morphology/ de-differentiation, migration and adhesion. At the invasive front of a solid tumor of epithelial origin (carcinoma) cells undergo a trans-differentiation process called epithelial-mesenchymal-transition (EMT). This transitory process was first characterized by Elizabeth Hay in the context of embryonic development where it is essential during gastrulation and for the formation of several organs and tissues including the neural crest (reviewed in (Gavert and Ben-Ze'ev, 2008; Hay, 2005)). EMT is characterized by the loss of epithelial characteristics like cell-cell adhesion (tight junctions, adherens junctions, desmosomes and gap junctions), an apical-basal polarity and lack of single cell movement. On the other hand mesenchymal features like spindle shape, no or few cell-cell contacts, high motility and a front-back polarity are acquired.

In general, there are three types of EMT (Kalluri and Weinberg, 2009). Type I EMT occurs during implantation, embryogenesis and organ development. Programmed changes are essential for the proper development of the organism. The main signaling pathway involved in gastrulation is the canonical WNT pathway. A lack of WNT3 leads to improper EMT associated gastrulation in the embryo (Liu et al., 1999; Skromne and Stern, 2001). The TGFβ-family members Nodal and Vg1 coordinate the WNT activity and are necessary for a functional EMT in mesodermal development (Chea et al., 2005; Collignon et al., 1996; Shah et al., 1997; Skromne and Stern, 2002; Varlet et al., 1997). A cooperation of FGF receptors with the WNT proteins has also been reported to regulate gastrulation (Ciruna and Rossant, 2001; Perea-Gomez et al., 2002; Rossant et al., 1997; Zhao et al., 2006). Gastrulation

associated EMT is furthermore coordinated by the transcription factors Snail, Eomes and Mesps (Arnold et al., 2008; Lindsley et al., 2008; Nieto, 2002). In addition, EMT is essential for the development of migratory neural crest cells. Epithelial cells from the neuroectoderm start as pre-migratory neural crest cells. They dissociate from the neural fold upon EMT-driven changes, become motile and finally differentiate at distant parts of the organism into for example melanocytes (Kalluri and Weinberg, 2009). This reprogramming is achieved by WNT, FGF, BMP, c-Myb, and msh homeobox 1 (Msx-1) mediated signaling pathways (Karafiat et al., 2007; Liem et al., 2000; Villanueva et al., 2002).

In contrast, type II EMT is associated with tissue regeneration and organ fibrosis. Epithelial-mesenchymal-transition related to organ fibrosis occurs in the liver, kidney, lung and intestine (Kim et al., 2006; Potenta et al., 2008; Zeisberg et al., 2008; Zeisberg et al., 2007a; Zeisberg et al., 2007b). It is characterized by the release of inflammatory signals and components of the complex extracellular matrix by inflammatory cells and fibroblasts (Kalluri and Weinberg, 2009). Several marker proteins like the fibroblast-specific protein 1 (FSP1), α-SMA and Collagen I, generated during process of EMT, are used to characterize the progression of an EMT in the development of fibrosis in various organs (Okada et al., 1997; Strutz et al., 1995; Zeisberg et al., 2003b). Intermediate phenotypical appearance with both epithelial- as well as mesenchymal-like morphological characteristics and markers (epithelial: E-cadherin (CDH1) and cytokeratins (KRT) , mesenchymal: FSP1 and α-SMA) are frequently observed with this EMT type (Kalluri and Weinberg, 2009). As a result of inflammation, individual cells eventually leave the epithelial layer and fully transform into cells with a fibroblastic phenotype before accumulating in the interstitium of the tissue (Okada et al., 1996). The recruitment of a diversity of cells to the inflammatory injury of mice kidney has been reported. These cells, mostly macrophages and activated resident fibroblasts, release a series of growth factors that trigger EMT e.g. TGF-β, PDGF, EGF and FGF-2 at the side of injury (Strutz et al., 2002). They furthermore produce chemokines and matrix metalloproteases (MMPs), namely MMP-2, MMP-3 and MMP-9. This helps the epithelial cells to degrade the basement membrane, type IV collagen and laminins, to finally infiltrate the surrounding microenvironment (Strutz et al., 2002). The role of TGFβ-induced EMT in organ fibrosis was demonstrated using BMP-7, a TGFβ antagonist, in mouse models of kidney, liver, billiard tract, lung and interstitial fibrosis (Zeisberg et al.,

2003a; Zeisberg et al., 2003b). The systemic administration of recombinant BMP-7 to mice with severe fibrosis led to a reversal of EMT and a reduction of damaged epithelial structures (Zeisberg et al., 2003a).

Type III EMT is the epithelial-mesenchymal-transition associated with cancer progression and metastasis formation. Complex genetic and biochemical processes are required for the acquisition of invasiveness and the subsequent formation of metastasis. The activation of an EMT program has been proposed to be an essential turning point of epithelial cells to attain a malignant phenotype (Thiery, 2002). Cells typically observed at the invasive front of primary tumors have acquired a mesenchymal-like phenotype and express mesenchymal markers like FSP1, α-SMA, vimentin and desmin (Yang and Weinberg, 2008). Eventually these cells enter stages of the invasion-metastasis-cascade including intravasation, transport through the circulation, extravasation, formation of micrometastases, and ultimately colonization (Kalluri and Weinberg, 2009). The final formation of metastasis may involve mesenchymal-epithelial-transition (MET) processes, the reversal of EMT. The idea of a MET at the side of secondary colonies is supported by the frequent observation that metastasizing cells that have successfully colonized at distant sites no longer exhibit a mesenchymal phenotype. They resemble histopathologically the original primary tumor (Kalluri and Weinberg, 2009). Note that, all three types of EMT share common characteristics and do not necessarily present independent cellular processes.

Activation of EMT in cancer can be facilitated via various signaling pathways. In order to obtain mesenchymal cell features like single cell motility the cancer cells need to undergo changes of cell-cell as well as cell-matrix adhesion. A disruption of functional cell-cell contacts is mainly achieved by the reduction of E-cadherin levels (Peinado et al., 2004). E-cadherin (CDH1) belongs to the Cadherins, a family of transmembrane adhesion receptors. The homophilic binding of CDH1 molecules on the surface of adjacent cells is required for the formation of adherens junctions (Gumbiner, 2005). The cytoplasmic domain of CDH1 is bound by β-catenin (CTNNB1) or plakoglobin (γ-catenin, JUP). These catenins bind the adapter molecule α-Catenin (CTNNA1) which in turn is associated with F-actin bundles thereby linking the protein complex to the actin cytoskeleton (Ben-Ze'ev and Geiger, 1998; Yamada et al., 2005). The loss of CDH1 was reported to correlate with tumor invasiveness, metastasis and patient mortality (Birchmeier and Behrens, 1994). Furthermore it was

demonstrated that during the transition from adenoma to carcinoma in gastric tumors, CDH1 is lost at the invasive front (Perl et al., 1998). The transcriptional control of CDH1 has been extensively studied in the past decades. Several families of transcription factors were shown to bind to the consensus E-box sequences in the CDH1 promoter repressing its transcription. The Snail family members Snail (SNAI1) and Slug (SNAI2), the ZEBs (ZEB1 and 2) and the basic helix-loop-helix (bHLH) members Twist (TWIST1) and E47 (TCF3) are reported repressors of CDH1 (Peinado et al., 2007). The disruption of the adherens junction complex also contributes to changes in signal transduction in the context of EMT. For example, a reduction of CDH1 from adherens junctions leads to the release of CTNNB1, a major co-regulator of the WNT pathway. In many cancer cells mutations of the CTNNB1 degrading machinery, a multi protein complex consisting of AXIN1, APC, GSK3B and CK1, mediate nuclear accumulation of CTNNB1 and the subsequent activation of target genes via the TCF/LEF1 transcription complex (Clevers, 2006; Conacci-Sorrell et al., 2002; Polakis, 2000).

The transcriptional activation complex of CTNNB1-TCF/LEF1 was shown to induce the expression of EMT related genes including SNAI2, fibronectin (FN1) and Vimentin (VIM) (Conacci-Sorrell et al., 2003; Gilles et al., 2003; Gradl et al., 1999; Jesse et al., 2010; Lambertini et al., 2010). Therefore besides the transcriptional repression of epithelial genes a transcriptional activation of mesenchymal genes also takes place in order to mediate the EMT process. Moreover, matrix metalloproteases (MMPs) are necessary for the disruption of cell-cell contacts by proteolytic cleavage as well as for the digestion of extracellular matrix components to facilitate invasion of the cells (Egeblad and Werb, 2002). Members of this group of proteases were shown to be up-regulated in various cancer types and were demonstrated to stimulate EMT during tumor development (Orlichenko and Radisky, 2008). The composition of extracellular matrix (ECM) and their membrane bound receptors also undergoes remodeling upon EMT. An increased expression of FN1, vitronectin (VTN) and type I collagen, for example, has been reported in context of EMT and was correlated to integrin switches in favor of a mesenchymal phenotype (Boudreau and Jones, 1999; Imamichi et al., 2007; Imamichi and Menke, 2007).

1.3 The convergence of signaling pathways promoting EMT

It has been quite confusing to understand the mechanisms involved in EMT when focusing on single signaling pathways. Numerous pathways can induce and promote EMT mainly via the modulation of transcriptional regulators. However, emerging evidence indicates that these pathways interact and cooperate with each other. Therefore, the next chapter will shortly describe the main signaling pathways involved in EMT and their interconnections. The scheme shown in Figure 1.1 illustrates components of these pathways in a simplified manner.

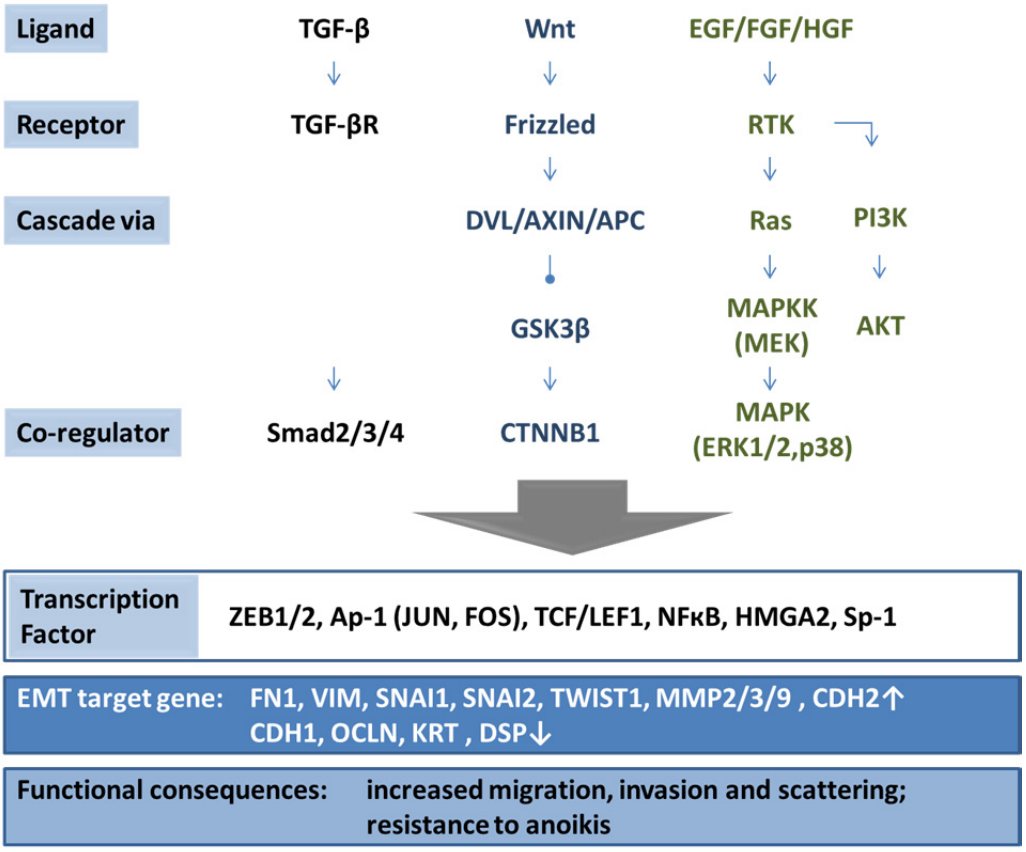


Figure 1.1: Schematic overview of the major signaling pathways involved in EMT.

1.3.1 TGF-β/ SMAD pathway

The TGF-β pathway is one of the major signaling pathways to induce EMT in embryogenesis and plays an important role in tumor progression and metastasis formation (Nawshad et al., 2005; Oft et al., 1998). TGF-β is involved in tumor progression at every stage for example by inhibiting cell proliferation via induction of cyclin kinase inhibitors (Blobe et al., 2000; Elliott and Blobe, 2005). Frequent mutations within this pathway are mainly due to defects in the SMAD proteins or TGF-β receptors that have been observed in many cancer types like pancreatic and colon cancer (Elliott and Blobe, 2005). Three isoforms of TGF-β (TGFB1-3) and three seronine/threonine kinase receptors (TGFBR1-3) have been described in mammalian cells (Attisano and Wrana, 2002). Binding of TGF-β to the receptors type I and II leads to complex formation and phosphorylation of the type I receptor (TGFBRI) (Massague, 2000). This activates downstream effectors of the SMAD family (Feng and Derynck, 2005; Massague et al., 2005). The activated receptor-regulated SMADs 2 and 3 (R-SMADs) associate with the co-SMAD4 and subsequently translocate into the nucleus to regulate target transcription (Massague, 2000). The inhibitory SMADs 6 and 7 block phosphorylation of SMAD2 (Elliott and Blobe, 2005). Functional SMAD-complexes recognize the DNA sequence CAGAC but their DNA affinity is too low to fulfill the transcriptional activation or repression of specific target genes without the assistance by additional transcription factors (Shi et al., 1998). The expression of multiple variations of transcription cofactors by different cell types enables a complex outcome of the TGF-β pathway (Massague, 2000). TGF-β signaling can also occur SMAD-independently by activation of the phosphoinositide 3-kinase (PI3K), AKT and MAPK. Both SMAD-dependent and –independent pathways overlap and can cooperate to regulate transcription of various EMT master regulators like TWIST1, SNAI1 and SNAI2 (Ahmed and Nawshad, 2007). Another transcription factor, namely HMGA2, is involved in an up-regulation of SNAI1 through transcriptional cooperation with SMADs during induction of an EMT by TGF-β (Thuault et al., 2008). Finally, Sp-1 transcription factor is required for the expression of VIM and the induction of EMT in pancreatic cancer cells in response to TGF-β signaling and seems further to be involved in the induction of MMP9 in MDCK cells (Jorda et al., 2005; Jungert et al., 2007).

1.3.2 Receptor tyrosine kinase signaling (RTK)

Growth factors such as the epidermal growth factor (EGF), HGF, TGF-α or fibroblast growth factors (FGF) transduce their signals via the activation of receptor tyrosine kinases and their downstream effector Ras (Rommel and Hafen, 1998; Shields et al., 2000). Depending on the stage of de-differentiation of the targeted cell these growth factors can stimulate proliferation or modulate cell plasticity. In most cases a single stimulus by a ligand is insufficient to induce proliferation and EMT. However, several studies indicate that synergistic action of activated RTKs can accompany the induction of EMT (Gotzmann et al., 2002; Janda et al., 2002; Oft et al., 1998; Valles et al., 1990). The constitutive activation of RTKs and their downstream activated signaling transducers such as mitogen activated protein kinase (MAPK) and PI3K contribute to hyperplastic/ pre-malignant lesions which enables epithelial cells to increase their rate of proliferation (Hernandez-Alcoceba et al., 2000). Although, the stage of pre-malignancy does not necessarily involve changes in the epithelial phenotype the additional stimulation by cytokines like TGF-β induces and maintains EMT in cooperation with active Ras signaling without changes in proliferation (Gotzmann et al., 2002; Oft et al., 1998; Oft et al., 1996). The TGF-β/Ras collaboration is associated with the acquisition of metastatic behavior along with de-differentiation, resistance of apoptosis and the modulation of transcriptional regulation of junctional proteins (Gotzmann et al., 2004). It has been reported that the synergistic cooperation of TGF-β with both, MAPK and PI3K signaling is necessary for proper EMT regulation in different epithelial cell types (Bakin et al., 2002; Bakin et al., 2000; Gotzmann et al., 2004; Yu et al., 2002).

One effector that becomes activated by several stimuli like TGF-β or EGF is the nuclear factor κB (NFκB). Several groups have demonstrated a constitutive activation of NFκB and NFκB -dependent overexpression of pro-metastatic and anti-apoptotic genes in breast cancer cells (Biswas et al., 2000; Nakshatri et al., 1997; Sovak et al., 1997). NFκB was shown to be sufficient for the induction of an EMT in mammary epithelial cells when overexpressed (Huber et al., 2004). Furthermore, NFκB activates SNAI1 expression in response to AKT signaling (Julien et al., 2007) A repressive function on CDH1 with enhancement of EMT was shown for NFκB in

mammary epithelial cells and was correlated to changes of ZEB-1 and ZEB-2 expression levels (Chua et al., 2007). Furthermore the transcription factor activator protein-1 Ap-1, consisting of c-Jun (JUN) as homodimer or c-Fos/Fra-2 (FOS/FOSL2) (JUN/FOS) as heterodimer, was reported to be involved in the induction of SNAI1 and SNAI2 expression in MAP kinase kinase (MEK1)-induced EMT in intestinal epithelial cells (Lemieux et al., 2009). Additionally, the induction of VIM by Fra-1 was demonstrated in Ha-Ras-induced EMT in colon cancer cells (Andreolas et al., 2008).

1.3.3 WNT/CTNNB1 pathway

The WNT signaling pathway is involved in stem cell renewal and also implicated in the induction of EMT in cancer. The canonical WNT pathway is activated by binding of the WNT ligands (mostly WNT1 or WNT3A) to a trans-membrane receptor complex consisting of frizzled (FZD) and the LDL receptor-related proteins LRP5 or LRP6 (Clevers, 2006). The signal transduction is mediated by a conformational change of the receptor complex. Recent studies showed that binding of AXIN1 to the cytoplasmic tail of LRP6 is a crucial step in signal transduction of the WNT pathway (Mao et al., 2001). This binding is regulated by phosphorylation of the LRP6-AXIN1 complex by the kinases GSK3 and CK1γ (CSNK1G3) (He et al., 2004; Tamai et al., 2004). The cytoplasmic domain of FZD interacts with Dishevelled (DVL) (Chen et al., 2003). In contrast to most other pathways, WNT-induced receptor complex formation titrates away the negative regulator, AXIN1, which is part of the CTNNB1 destruction complex (Clevers, 2006). As a scaffolding protein of this complex AXIN1 is bound by APC and two constitutively active serine-threonine kinases (CK1α/δ (CSNK1A1/ CSNK1D) and GSK3A/B) thereby regulating cytoplasmic levels of CTNNB1 (Clevers, 2006). Phosphorylation of CTNNB1 by CK1 (CSNK1A1) and GSK3 leads to ubiquitinylation mediated by β-TrCP (BTRC) and subsequent proteasomal degradation (Aberle et al., 1997). CTNNB1 plays a central role in this pathway. When stabilized, the protein accumulates in the cytoplasm and translocates into the nucleus. Subsequently, CTNNB1 forms a transcriptional activation complex with the T-cell factor (TCF)/ LEF family of transcription factors. A comprehensive list of genes activated by the WNT pathway can be found on the web page of Roel Nusses lab (http://www.stanford.edu/group/nusselab/cgi-bin/WNT/target_genes). WNT target

genes important in EMT like JUN, MMP7, Nr-Cam, CD44, Claudin-1 or Met were reported to be de-regulated in the context of human colon cancer. Mutations of WNT pathway components are frequently observed in this type of cancer. Germline mutations in the APC gene result in the hereditary cancer syndrome termed familiar adenomatous polyposis (Kinzler et al., 1991; Nishisho et al., 1991). Sporatic colorectal cancer is often caused by the loss of both APC alleles (Kinzler and Vogelstein, 1996). The loss of APC leads to stabilization of cytoplasmic CTNNB1 and therefore enhanced transcriptional activation of WNT target genes. In rare cases mutation in AXIN2 and CTNNB1 itself have been reported (Liu et al., 2000; Morin et al., 1997). At the invasive front of colorectal cancer tissue nuclear CTNNB1 and the loss of CDH1 have been visualized. This correlated to the activation of several CTNNB1-TCF targets supporting its contribution to invasive cancer development by EMT (Brabletz et al., 2001; Gavert et al., 2005; Jung et al., 2001; Schmalhofer et al., 2009). Besides its function in transcriptional regulation, CTNNB1 serves a role in establishing adherens junction complexes, thereby anchoring the actin cytoskeleton to cell-cell contact sides. The stability of adherens junction complexes is regulated by phosphorylation. The complex is stabilized by seronine/threonine phosphorylation of CTNNB1 (Bek and Kemler, 2002) or CDH1 (Lickert et al., 2000). Destabilization of the complex is mediated via tyrosine phosphorylation of CTNNB1 by FER, SRC or EGF-receptor (Piedra et al., 2003; Roura et al., 1999). A cross-regulation was reported for the receptor tyrosine kinase (RTK) c-Met (MET). The receptor becomes activated by its ligand hepatocyte growth factor (HGF) and subsequently phosphorylates CTNNB1 on tyrosine 142. CTNNB1 accumulates, shuttles into the nucleus and TCF/LEF-mediated gene transcription increases (Danilkovitch-Miagkova et al., 2001). Furthermore, HGF was shown to induce the expression of LEF1 through PI3K/AKT and NFκB signaling. HGF-induced LEF1 expression was correlated to tumor migration and invasion in tumor-derived cell lines (Huang et al., 2012). TGF-β and WNT pathways can also enhance their transcriptional potential by synergistic cooperation of components from both pathways. SMAD2/4 complexes were shown to bind to LEF1 independently of CTNNB1 to repress CDH1 expression in response to TGFB3 treatment during mouse palate development (Nawshad et al., 2007). Additionally, a study reported that the complex formation of SMAD3/4-LEF1-CTNNB1 enhanced the expression of the Xtwn promoter synergistically (Labbe et al., 2000; Nishita et al., 2000).

1.3.4 The role of LEF1 in development and disease

The lymphoid-enhancer binding factor 1 (LEF1) belongs to T-cell factor family of transcription factors. There are four members of this family expressed in most vertebrates: TCF1 (TCF7), TCF3 (TCF7L1), TCF4 (TCF7L2) and LEF-1 (LEF1). LEF1 binds the DNA motif (A/T)(A/T)CAA(A/T)G, called WNT-responsive element (WRE) with high affinity via its high-mobility group (HMG) domain (Giese et al., 1995; Gustavson et al., 2004; Haynes et al., 1996). Transcriptional activation or repression by LEF1 is mediated by associating to co-activator or –repressors. Thus, LEF1 serves as DNA-anchored platform to recruit transcriptional and chromatin remodeling complexes. DNA-bending and DNA-looping were shown to be mediated by LEF1 to facilitate transcriptional complex formation (Giese et al., 1995; Haynes et al., 1996; Jash et al., 2012; Love et al., 1995; Yun et al., 2009). Different isoforms created by alternative splicing or alternative promoter usage are described in the literature (Cordray and Satterwhite, 2005; Hoeppner et al., 2009; Hovanes et al., 2001; Hovanes et al., 2000).

Transcriptional activation via LEF1 was mainly reported to involve co-activation by nuclear CTNNB1 (Behrens et al., 1996; Billin et al., 2000; Huber et al., 1996; Tutter et al., 2001). The first 56 amino acids of LEF1 comprise the CTNNB1 binding domain necessary for LEF1-CTNNB1 interaction (Hsu et al., 1998). The recruitment of additional co-activators (e.g. p300, BCL9 and Pygopus) further facilitates gene transcription (Hecht et al., 2000; Kramps et al., 2002; Miyagishi et al., 2000; Sun et al., 2000). In the absence of canonical WNT signals the TCF/LEF transcription factors are bound by co-repressors like TLE/Groucho and histone deacetylases (HDACs) to mediate transcriptional repression (Billin et al., 2000; Brantjes et al., 2001; Levanon et al., 1998). Among the reported target genes activated by LEF1 are factors involved in cell cycle regulation like MYC and cyclin D1 (CCND1) (He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999). In most studies there is only little discrimination among the TCF/LEF1 members. However, the cell-context-dependent expression and function of the TCF/LEF1 leads to highly diverse pattern of activation and repression depending on the respective cell lineage and the cell state (Mao and Byers, 2011). Genetic manipulations of the LEF/TCFs highly mimic the outcome of similar treatments by WNT ligands and their

receptors supporting that the LEF1/TCF factors are the main downstream effectors of this pathway (Arce et al., 2006). The LEF1-specific up-regulation was shown for the fibronectin (FN1) promoter in Xenopus (Gradl et al., 1999). In human osteoblasts LEF1 was found to bind to and regulate the Slug (SNAI2) promoter (Lambertini et al., 2010). A mechanism involving gene activation by gene looping via LEF1 was proposed for collagen II, MMP13 and Cox2 (Jash et al., 2012; Yun et al., 2009). The majority of studies report the transcriptional co-activation of LEF1 by CTNNB1. However additional mechanisms involving LEF1-dependent transcription independently of CTNNB1 have also been described in the literature. Early studies showed that the regulation of the T-cell receptor α enhancer mediated by LEF1 was dependent on the association of ALY to LEF1 but not CTNNB1 (Hsu et al., 1998). The co-regulation of SMADs with LEF1 during palatal fusion was suggested to repress CDH1 expression in response to TGFB3 (Nawshad et al., 2007). Moreover, LEF1 was shown to enhance target gene expression by synergizing with nuclear IGF2R1 (Warsito et al., 2012). Finally, LEF1 was demonstrated to enhance the expression of the matrix-metallopeptidase matrilysin (MMP7) in an AP-1 dependent but CTNNB1-independent manner (Rivat et al., 2003).

During embryonic development LEF1 is expressed in a variety of murine tissues revealed by in situ hybridization (Oosterwegel et al., 1993b). At day 10.5 (E10.5) LEF1 was detected in neural crest, branchial arches, and limb buds. At day E14.5, LEF1 was expressed in the thymus, lung, kidney, tooth germs, brain, and inner ear (Oosterwegel et al., 1993b). However, impairment of several organs in LEF1-deficient mice revealed only partially overlap with tissue expressing LEF1 in normal mice development (van Genderen et al., 1994). Mutant mice not only exhibited higher postnatal lethality. They also lacked teeth, mammary glands, whiskers, and body hair, and showed an absence of the mesencephalic nucleus of the trigeminal nerve (TMN), a specialized cell type in the brain (van Genderen et al., 1994). The study suggested a crucial role of LEF1 in the development of organs that require inductive interactions between epithelial and mesenchymal cells including neural crest cell migration (van Genderen et al., 1994). Shortly after birth LEF1 expression becomes restricted to the thymus and a small set of cells that are undifferentiated and mitotically active stem cells. The best studied examples are differentiating B and T lymphocytes and cells at the base of hair follicles that express LEF1 whereas the mature differentiated B and T lymphocytes and keratinocytes do

not or barely exhibit LEF1 expression (Kratochwil et al., 1996; Oosterwegel et al., 1993a; Travis et al., 1991; Zhou et al., 1995). The proper maturation and proliferation of B lymphocytes was shown to depend on LEF1 (Reya et al., 2000).

In contrast to the low levels of LEF1 expression in adult differentiated tissues LEF1 expression is frequently up-regulated in different types of cancer. Several studies demonstrated LEF1 to be overexpressed in chronic lymphocytic leukemia (Jelinek et al., 2003; Klein et al., 2001; Tandon et al., 2011). At least one study suggested a CTNNB1 independent role of LEF1 in this disease due to the lack of nuclear staining of CTNNB1 in 88% of the investigated lymphoma cases (Tandon et al., 2011). Furthermore an up-regulation of LEF1 was shown in colon cancer and colon cancer–derived cell lines compared to normal colon tissue (Hovanes et al., 2001; Korinek et al., 1997; Porfiri et al., 1997). The consequences of LEF1 overexpression have been controversially discussed. Nuclear LEF1/TCF4 was correlated with poor prognosis in cerebral metastasis of lung adenocarcinoma (Bleckmann et al., 2012). A positive correlation of LEF1 expression with longer overall survival was demonstrated in colon cancer (Kriegl et al., 2010). This was furthermore shown to inversely correlate to TCF4 expression levels, another member of the T-cell factor family. A similar differential expression pattern for LEF1 and TCF4 was reported in melanoma. LEF1 was expressed by differentiated/ proliferative phenotype of melanoma-derived cell lines whereas TCF4 was mainly expressed by the de-differentiated/ invasive phenotype of cells (Eichhoff et al., 2011). It was suggested that melanoma cells switch back and forth between stages of proliferation and invasion thereby driving their metastatic potential (Carreira et al., 2006; Hoek et al., 2008; Hoek et al., 2006). In contrast, enhanced expression levels of LEF1 in highly migrating cells from metastatic melanomas supported the metastatic potential of LEF1 (Murakami et al., 2001). Its pro-invasive potential was demonstrated in various studies revealing that LEF1 regulates matrix metalloproteases (MMPs) in glioblastoma- and breast cancer-derived as well as endothelial-derived cell lines (Bucan et al., 2012; Liu et al., 2012; Planutiene et al., 2011).

1.4 The IGF2 mRNA binding protein family

The members of a highly conserved RNA-binding protein family have been studied in a variety of cell types and are found to be up-regulated in a multitude of malignancies (reviewed in (Bell et al., 2012; Yaniv and Yisraeli, 2002; Yisraeli, 2005)). Terminology in the literature of the three family members in vertebrates has been confusing (ZBP-1, IMP, KOC, CRD-BP, VICKZ). For an easier understanding the gene symbol IGF2BP1-3 is used throughout this study. The name was derived from the ability to bind to Insulin-like growth factor 2-mRNA resulting in IGF2-mRNA-binding proteins. The functions of these related proteins have been implicated in RNA localization, RNA stability as well as translational control. RNA regulation is mediated through binding to target transcripts via canonical RNA-motifs. The highly conserved domain structure of the IGF2BPs includes two RNA-recognition-motifs (RRMs) and four hnRNPK homology domains (KH-domains). RNA-binding was shown to be facilitated via the KH-domains (Farina et al., 2003; Wächter et al., 2013).

Several target mRNAs of the IGF2BPs have been identified in the past decades. The mechanisms by which the IGF2BPs, mainly IGF2BP1, mediate the post-transcriptional control of gene expression differ in terms of bound region within a certain target RNA and the final outcome. In *Xenopus* Vg1-RBP/ Vera, the orthologue of the human IGF2BP3, mediates intracellular localized translation of Vg1 mRNA (a member of the TGFB superfamily) to the vegetal cortex of *Xenopus* oocytes (Deshler et al., 1998; Havin et al., 1998). One of the best studied IGF2BP1 target mRNAs is the β-Actin (ACTB) mRNA. IGF2BP1 binds to a 54 nucleotide stretch in the ACTB 3'UTR, termed the zipcode, and mediates the translational repression of this mRNA (Kislauskis et al., 1994; Ross et al., 1997). In chicken, ZBP-1 (Zipcode binding protein 1) was shown to facilitate localized translation of ACTB mRNA in growth cones of developing neurons (Huttelmaier et al., 2005; Zhang et al., 2001). In addition, IGF2BP1 was revealed to bind to regions within the 5'UTR and the 3'UTR leading to enhanced translation of the HCV RNA (Weinlich et al., 2009). Furthermore binding of IGF2BP1 to a rare codon region within the coding sequence (MYC, PTEN, MDR1) has been described resulting in the stabilization of these transcripts (Doyle et al., 1998; Sparanese and Lee, 2007; Stohr et al., 2012). The protection from microRNA binding of the β-TrCP (BTRC) and MITF mRNAs was another proposed mechanism by which IGF2BP1 regulation is mediated (Elcheva et al., 2009; Goswami et al., 2010). A 3'UTR-dependent control of gene expression was

furthermore shown for CTNNB1, MAPK4 and CD44 (Gu et al., 2008; Stohr et al., 2012; Vikesaa et al., 2006). In contrast to a translational control of the MAPK4 mRNA, IGF2BP1 stabilizes the CTNNB1 and CD44 transcripts. A conserved binding motif, based on the variety of binding sites of different target mRNAs, has not been confirmed so far. It is very likely that in addition to the CAUH (H = A, U, or C) sequence as proposed in recent PAR-CLIP studies (Hafner et al., 2010) supplementary sequences may be essential for proper binding and regulation. Such a model of additive sequences in a defined spacing was previously described for the ACTB zipcode (Chao et al., 2010; Patel et al., 2012). The binding to highly structured 5'UTRs as shown for the HCV- or IGF2-mRNAs would furthermore suggest that motifs created by secondary folding of the RNA may be involved and necessary for the association of IGF2BPs. It also has to be taken into account that homo- or hetero-dimerization on their targets contributes to a proper association. Until now, a simple deduction of binding motifs cannot be performed due to the diverse nature of known target mRNAs.

The IGF2BPs localize mainly in the cytoplasm where they associate with RNA in RNP-complexes with granular like structures. The presence of import and export signals indicates a shuttling from the nucleus into the cytoplasm (Nielsen et al., 2003). The association with target mRNAs at the site of transcription in the nucleus has been suggested by several groups (Huttelmaier et al., 2005; Oleynikov and Singer, 2003; Pan et al., 2007). It was proposed that the IGF2BPs associate with naive RNA transcripts. This was underlined by the findings that IGF2BPs are found in complexes together with components of the exon-junction complex and do not co-precipitate with eIF4E (Jonson et al., 2007; Weidensdorfer et al., 2009). It is therefore assumed that IGF2BP1, and presumably also the other IGF2BP family members, regulate their target mRNAs by 'caging' them inaccessible in RNP granules to prevent either mRNA degradation or translation. This model is consistent with findings that localized translation of ACTB mRNA in neurons is mediated by IGF2BP1. The binding of the protein leads to the translational repression of ACTB mRNA until neuronal growth cones are reached. Here the localized translation is facilitated by tyrosine (Y396) phosphorylation of IGF2BP1 mediated by SRC which releases the ACTB mRNA from the protein (Huttelmaier et al., 2005). Phosphorylation was furthermore reported to regulate localized translation of Vg1 mRNA in *Xenopus* oocytes. Mitogen activated protein kinases (MAPKs) were shown

to mediate the phosphorylation of Vg1-RBP which results in the release of Vg1 mRNA to the vegetal cortical during meiotic maturation (Git et al., 2009).

All three IGF2BP members are phosphor-acceptors for the mTOR kinase. The phosphorylation of IGF2BP2 (S162/164) by mTORC1 enhances binding to the IGF2 mRNA and increases its translation (Dai et al., 2011). Interestingly, phosphorylation of IGF2BP1 (S181) and IGF2BP3 (S183) by mTORC2 is required to enable IGF2-leader 3 mRNA translational initiation by internal ribosomal entry and is essential for proper splicing (Dai et al., 2013).

A complex network of the transcriptional control of IGF2BP1 expression has been proposed via two feedback mechanisms in the literature. In HEK293 cells the transcriptional up-regulation of IGF2BP1 was shown by ectopically expressed CTNNB1/TCF4 (Noubissi et al., 2006). In response to CTNNB1 signaling this modulates the expression of BTRC and MYC. In addition BTRC mRNA was shown to be prevented from miRNA degradation by IGF2BP1 itself. BTRC on the other hand is involved in the degradation of CTNNB1 as part of the so called Skp1-Cul1-Fbox proteins (SCFs), an ubiquitin protein ligase complex, creating a negative feedback loop. This regulation additionally involves a positive feedback regulation via MYC which is stabilized by IGF2BP1 and also enhances IGF2BP1 transcription (Noubissi et al., 2010). Controversially, CTNNB1 was also demonstrated to regulate IGF2BP1 expression by a positive feedback mechanism in breast-cancer derived cells (Gu et al., 2008). It remains unclear whether a positive or negative feedback mechanism is finally essential for IGF2BP1 expression.

1.5 The IGF2BPs in development

The IGF2BPs are described in the literature as oncofetal proteins. High expression levels in the stages between zygote and embryonic stages are characteristics of the IGF2BPs (Hansen et al., 2004). An expression peak can be detected at embryonic day 12.5 (E12.5) in mice followed by a decline towards birth (Hansen et al., 2004). Gene trap experiments of β-Galactosidase under control of the IGF2BP1 promoter in mice revealed an expression at day E10.5 mainly in the fore- and hindbrain, the snout, the branchial arches, the developing limb buds and the tail (Hansen et al., 2004). The expression in the fore-, hindbrain and neural tract was increased at day E12.5. At day E17.5 IGF2BP1 expression showed an overall

decrease but remained at high levels in the intestine, kidney and liver. A similar expression pattern of IGF2BP3 at day E12.5 has been reported in the literature (Mori et al., 2001; Mueller-Pillasch et al., 1999). Vg1RBP/ Vera is also expressed in the neural tube and neural crest cells in developing Xenopus (Yaniv et al., 2003).

The physiological role of IGF2BP1 in embryonic development was studied in IGF2BP1-deficient mice generated by a gene trap in intron 2. These mice showed impaired gut development, dwarfism and increased perinatal mortality. The about 40% smaller sized animals revealed smaller organs which were caused by hypoplasia rather than apoptosis. A down-regulation of IGF2 mRNA translation was observed whereas the overall expression of other target mRNAs like MYC remained unchanged in the knockout mice (Hansen et al., 2004).

In the adult organism the high expression observed in the embryo is substantially abolished with the exception of reproductive tissues (Hammer et al., 2005). Such an expression pattern was mainly observed for IGF2BP1 and 3. In agreement, more recent studies showed that although low levels of IGF2BP1 mRNA were detected in brain, lung and spleen of 16-week-old male mice its expression was abolished in 80-week-old male mice (Bell et al., 2012). However a largely age-independent modest expression of IGF2BP3 mRNA in lung, spleen, kidney and gut was also shown (Bell et al., 2012). Various studies revealed that IGF2BP2 is expressed in various adult tissues (reviewed in (Christiansen et al., 2009; Yaniv and Yisraeli, 2002; Yisraeli, 2005). In agreement, RT-PCR data from adult mouse tissue collected from mice of different ages showed the expression of IGF2BP2 in all analyzed tissue. Only the pancreatic expression of IGF2BP2 was very low compared to the other tissue samples (Bell et al., 2012). However a more closer look at the islets of Langerhans, a specialized cell type of the pancreas involved in insulin production and glucose homeostasis, by a different group showed a much higher expression of IGF2BP2 in the adult mouse compared to the overall expression level of the pancreas (Dai et al., 2011). These studies indicated a more global role of IGF2BP2 in cellular function like metabolism.

A well characterized feature of the IGF2BPs is their role in the control of spatiotemporal mRNA localization in neuronal development. The spatiotemporal restricted control of the ACTB mRNA by far is the best characterized example (Dahm and Kiebler, 2005). Binding of IGF2BP1 to the ACTB mRNA in relatively stable cytoplasmic mRNP complexes is necessary for the directed transport into developing

axons and dendrites (Eom et al., 2003; Zhang et al., 2001). SRC mediated phosphorylation of IGF2BP1 allows the locally restricted translation of ACTB mRNA which promotes growth cone guidance (Huttelmaier et al., 2005; Leung et al., 2006; Yao et al., 2006). Furthermore it was shown that IGF2BP1 promotes the outgrowth and branching of neurites in hippocampal neurons (Perycz et al., 2011).

In summary the IGF2BPs are important regulators of embryonic development. For IGF2BP1 and 3 the biphasic embryonic expression in different murine tissues is essentially abolished in the adult organism with very few exceptions. The IGF2BP2 expression is maintained at least in adult mice indicating a distinct role different from the other family members. In addition a crucial role in neuronal development has been described among the investigated species.

1.6 The role of IGF2BPs in cancer

Their fundamental role in embryonic development points to their functions when *de novo* synthesized in different tumor entities. However, until now most of the available studies either present descriptive oncology/epidemiology aspects or pure functional *in vitro* findings (reviewed in (Bell et al., 2012)). In various studies an up-regulation of the two family members IGF2BP1 and 3 in different cancer types has been reported.

The commonly accepted idea about the function of IGF2BP1 is that it regulates a subset of target mRNAs and thereby modulates cell fate. Regarding its role in tumorigenesis the function of IGF2BP1 has been mainly studied in tumor derived cell lines. IGF2BP1 was shown to regulate cancer relevant target mRNAs like CD44, BTRC, CTNNB1, MYC and KRAS (Elcheva et al., 2009; Gu et al., 2008; Mongroo et al., 2011; Noubissi et al., 2006; Vikesaa et al., 2006). This was correlated with increased invadopodia formation and proliferation and on the other hand opposes apoptosis in different tumor-derived cell lines. However, it has been controversially discussed whether IGF2BP1 has pro- or anti-migratory and invasive capacities. In breast- and colon-carcinoma derived cell lines IGF2BP1 was shown to antagonize invasiveness and migration. This was correlated with an active CTNNB1 signaling in the respective cell lines (Gu et al., 2009; Gu et al., 2008; Noubissi et al., 2006). On the other hand, in osteosarcoma and ovarian-carcinoma derived cell lines

IGF2BP1 was reported to control directed migration via modulation of HSP27 phosphorylation in a MAPK4 (mitogen-activated protein kinase 4)-dependent manner (Stohr et al., 2012). Therefore IGF2BP1 promoted migration in these cells. Based on immunohistochemical analyses a high expression of IGF2BP1 in ovarian carcinoma was correlated with a poorer overall survival rate of patients (Kobel et al., 2007). This study furthermore showed that the depletion of IGF2BP1 from ovarian-carcinoma derived cell lines decreased proliferation and cell survival implicating IGF2BP1 as a potentially oncogenic factor (Kobel et al., 2007). In agreement, a destabilization and therefore down-regulation of the oncogene MYC was shown in response to the IGF2BP1 depletion.

Many of these in vitro studies provide evidence for a role of IGF2BP1 in regulating target mRNAs essentially involved in tumorigenesis and cancer progression. However, information about a potential role in primary tumors and especially in metastasis is largely missing up to now. Although one study correlated an up-regulation of the IGF2BPs in lymph node metastasis derived from colorectal carcinoma, the study is lacking discrimination between the IGF2BP family members (Vainer et al., 2008). The study of a transgenic mouse model expressing IGF2BP1 in mammary epithelial cells driven by the WAP-promoter (whey acidic protein-promoter) strongly supported the pro-oncogenic capacity of IGF2BP1 (Tessier et al., 2004). Ninety-five percent of female adult mice developed mammary tumors within 60 weeks when highly expressing IGF2BP1. Mice revealing a lower expression of IGF2BP1 still showed tumor incidences of 60 % (Tessier et al., 2004). These tumors were multifocal and capable of metastasizing. Furthermore the study demonstrated that the levels of the IGF2BP1 target RNAs ACTB and MYC remained unaffected by the transgenic overexpression of the protein. On the other hand the H19 and IGF2 transcript levels were significantly elevated although IGF2 protein levels were unchanged at the same time. These observations showed that *in vitro* evidence for the regulation for example of the MYC mRNA by IGF2BP1 cannot necessarily be applied on *in vivo* function and further investigations are needed.

Based on RT-PCR analyses a *de novo* synthesis of IGF2BP1 has been reported in many tumor types like breast, brain, colorectal and mesenchymal cancer (reviewed in (Bell et al., 2012)). Studies using immunohistochemical analyses also revealed an up-regulation of IGF2BP1 in ovarian, testis, colon and lung carcinoma as

well as melanoma and different types of lymphoma (reviewed in (Bell et al., 2012)). Apparently further investigations, combining *in vitro* and *in vivo* analyses, are required to fully elucidate the oncogenic role of IGF2BP1 in tumor development.

Only very few studies have correlated IGF2BP2 with an up-regulation in liposarcoma, liver cancer and endometrial carcinoma (Cleynen et al., 2007; Lu et al., 2001; Zhang et al., 1999). In contrast, consistent with its continuous expression also in adult organisms, the majority of studies suggest the implication of IGF2BP2 in the development of Type 2 Diabetes (T2D) rather than tumorigenesis (Christiansen et al., 2009). A single nucleotide polymorphism (rs4402960) in the second intron of the IGF2BP2 gene has been reported to correlate with T2D in genome-wide association studies (Christiansen et al., 2009).

A multitude of descriptive studies were published over the last few years which indicated that the expression of IGF2BP3 correlates to tumor aggressiveness in many malignancies (reviewed in (Bell et al., 2012; Findeis-Hosey and Xu, 2011). In a broad variety of tumor types an up-regulation or *de novo* synthesis of IGF2BP3 was reported and the protein was therefore suggested as a biomarker for poorer prognosis and metastases formation for different cancer entities (Findeis-Hosey et al., 2010; Kapoor, 2008; Li et al., 2011a; Lu et al., 2009; Walter et al., 2009). However, a single study also reported an improved survival rate in response to IGF2BP3 expression (Noske et al., 2009).

In contrast to IGF2BP1 a role IGF2BP3 in regulating distinct mRNA transcripts remains poorly investigated. The only transcript that was shown to be regulated by IGF2BP3 is the leader3 5'UTR of the IGF2 mRNA. IGF2BP3 binds to the highly structured 5'UTR and enhances the translation of the IGF2 mRNA in K562 leukemia cells and glioblastoma (Liao et al., 2005; Suvasini et al., 2011). Another mRNA regulated by IGF2BP1 and 3 is the CD44 mRNA which is stabilized by both proteins in a 3'UTR-dependend manner (Vikesaa et al., 2006). However, this study only provided evidence for the binding of IGF2BP1 to the CD44 mRNA and not IGF2BP3. The pro-oncogenic potential of IGF2BP3 was demonstrated by a transgenic mouse model. The overexpression of IGF2BP3 driven by the metallothionein promoter induced neoplastic transformation of the exocrine pancreas (Wagner et al., 2003). An extensive remodeling of the organ was observed with increased production of extracellular matrix components like collagen type I and fibronectin (Wagner et al.,

2003). Although not directly related to tumorigenesis, the transformation of healthy tissue induced by the overexpression of IGF2BP3 in the transgenic mouse models emphasizes its role in developing malignancies (Wagner et al., 2003).

In conclusion, the vast amount of correlative studies provides evidence for a role of IGF2BP1 and IGF2BP3 in malignancies supporting their function in tumor development in addition to a role in embryogenesis. Future studies may shed light in the functions of all three IGF2BP family members with regard to their re-expression or mis-expression in malignancies and diseases.

1.7 Aim of the study

The post-transcriptional control of gene expression involved in EMT processes has been mainly studied in the context of microRNA (miRNA) dependent regulation. The miR-200 family was shown to interfere with TGF-β induced EMT by antagonizing the expression of the CDH1 repressors ZEB1 and ZEB2 involving a feedback mechanism (reviewed in (Brabletz and Brabletz, 2010). Another miRNA family, namely the miR-34 family, was reported to modulate cell plasticity via double-negative feedback loop which links p53 signaling and negative regulation of SNAIL expression, another 'EMT-driving' transcriptional regulator (Brabletz, 2012; Siemens et al., 2011). Although frequently overexpressed in various cancer types, little information is available about the role of RNA-binding proteins (RBPs) in the regulation of EMT-like processes. At least two RBPs were recently shown to be involved in EMT. Sam68, essentially involved in splicing, was demonstrated to control EMT alternative splicing-activated nonsense-mediated mRNA decay of the proto-oncogene SF2/ASF (Valacca et al., 2010). In addition, the IRES transacting factor La was reported to enhance the translation of the ECM lamininB1 during malignant EMT stimulated by PDGF signaling (Petz et al., 2012a; Petz et al., 2012b).

IGF2 mRNA binding proteins (IGF2BPs) comprise a group of three proteins two of which, IGF2BP1 and IGF2BP3, were proposed to serve essential functions during embryogenesis and in cancer [reviewed in: (Bell et al., 2012; Stohr and Huttelmaier, 2012; Yaniv and Yisraeli, 2002; Yisraeli, 2005)]. In contrast to IGF2BP2 which appears to be the main or even exclusive IGF2BP member expressed in non-neoplastic adult tissue, IGF2BP1 and IGF2BP3 were found to be severely up-regulated in various cancers [reviewed in: (Bell et al., 2012; Findeis-Hosey and Xu,

2011)]. However, in view of the multitude of descriptive studies indicating elevated expression of IGF2BP1/3 to correlate with tumor aggressiveness, their role in cancer cells remains poorly understood. Despite various studies indicating a regulatory role of IGF2BPs, particularly IGF2BP1, in directing the migration and invasive potential of tumor-derived cells it remains elusive, if and how IGF2BP1 modulates gene expression signature. The potential of metastasis formation increases with the enhanced expression of mesenchymal genes thereby promoting migration, invasiveness and aggressiveness of tumor cells. One key aspect that remains to be addressed in this respect is a putative function of the IGF2BP protein family member in modulating mesenchymal versus epithelial properties of cancer-derived cells. The aim of this study was to investigate the role of IGF2BP1 in tumor cell dissemination by regulating distinct target mRNA transcripts at the post-transcriptional level thereby modulating cell properties of non-tumorigenic and tumor-derived cell lines.

Although a frequent up-regulation of IGF2BP1 and 3 has been reported in a variety of malignancies, up to now only few expression studies of the IGF2BPs are available in tissue samples derived from patients suffering from squamous cell carcinoma of the head and neck. Therefore the second goal of the study was to investigate whether an abnormal expression of the IGF2BPs can be determined in this type of cancer.

2 Material and Methods

2.1 Material

2.1.1 Chemicals and reagents

Chemicals were obtained from Sigma Aldrich and Roth unless otherwise stated. Enzymes, PCR master mixes and DNA-markers were purchased from Promega and NEB. Transfection reagents, qPCR master mixes as well as protein markers were obtained from Life technologies. Cell culture dishes were received from TPP, cell culture solutions (DMEM, trypsin, Hanks, FBS) from PAA.

2.1.2 Bacteria

Strain	Genotype
DH5alpha	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1
LB (Luria Bertani)	1% (w/v) Trypton, 0.5% (w/v) yeast-extract, 1% (w/v) NaCl, supplemented with 1.5% (w/v) Agar for production of LB-Agar

2.1.3 Cell lines

Cell line	No.	Original tissue	Publication
HEK293A	ACC-305	human embryonic kidney	(Graham et al., 1977b)
HCT116	CCL-247	human colorectal carcinoma	(Brattain et al., 1981)
U2OS	HTB-96	human osteosarcoma-cell line	(Heldin et al., 1986)
FaDu	HTB-43	human pharynx squamous cell carcinoma	(Giard et al., 1973)
CAL-33	ACC 447	human tongue squamous cell carcinoma	(Gioanni et al., 1988)
SAS	TKG0470	human tongue squamous cell carcinoma	

ES2	CRL-1978	human clear cell ovarian carcinoma
HT-144	HTB-63	human melanoma

2.1.4 Antibodies

Table 2.1: Summary of primary and secondary antibodies used

primary antibody	produced in	company/provider
anti-LEF1 (C18A7)	rabbit	Cell Signaling
ChIPAb+ anti-LEF1	mouse	Millipore
anti-IgG	mouse	Millipore
anti-CDH1	rabbit	Sigma Aldrich
anti-VCL	mouse	Sigma Aldrich
anti-ACTB	mouse	Sigma Aldrich
anti-CTNNB1	rabbit	Cell Signaling
anti-IGF2BP1	mouse	BSBS AB facility
anti-IGF2BP2	mouse	BSBS AB facility
anti-IGF2BP3	mouse	BSBS AB facility
anti-TUBA4A,DM1α	mouse	Sigma Aldrich
anti-Fibronectin	mouse	Santa Cruz
anti-GFP	mouse	Roche
anti-Flag	mouse	Sigma Aldrich
anti-SNAI2	rabbit	Cell Signaling
anti-CTNND1	mouse	BD Transductions
anti-SNAI1	mouse	Cell Signaling
anti-HSP27 (HSPB1)	goat	Santa Cruz
anti-Vimentin	mouse	BD Transductions
anti-ZEB1	rabbit	Santa Cruz
anti-KRT8	rat	Kind gift of Prof.Magin, University of Leipzig
secondary antibodies		
IRDye® 700 anti-IgG-mouse-infrared-dye	donkey	LI-COR Biosciences GmbH
IRDye® 700 anti-IgG-rabbit-infrared-dye	donkey	LI-COR Biosciences GmbH
IRDye® 800CW anti-IgG-mouse-infrared-dye	donkey	LI-COR Biosciences GmbH
IRDye® 800CW anti-IgG-rabbit-infrared-dye	donkey	LI-COR Biosciences GmbH
IRDye® 800CW anti-IgG-rat-infrared-dye	donkey	LI-COR Biosciences GmbH
IRDye® 800CW anti-IgG-goat-infrared-dye	donkey	LI-COR Biosciences GmbH
dylight488™-conjugated anti-mouse-IgG F(ab)2	donkey	Jackson ImmunoResearch
dylight488™-conjugated anti-rabbit IgG F(ab)2	donkey	Jackson ImmunoResearch
Cy™3-conjugated anti-mouse-IgG F(ab)2	donkey	Jackson ImmunoResearch
Cy™3-conjugated anti-rabbit-IgG F(ab)2	donkey	Jackson ImmunoResearch
dylight649™-conjugated anti-mouse-IgG F(ab)2	donkey	Jackson ImmunoResearch
dylight649™-conjugated anti-rabbit IgG F(ab)2	donkey	Jackson ImmunoResearch

2.1.5 Plasmids

pCR®-blunt	Invitrogen
pcDNA3.1 (zeo)	Invitrogen
pGEMTeasy	Promega
pGL4.12 [*luc2CP*]	Promega
pRL-CMV Vector	Promega
pLVX-puro	Clontech
pLVX-shRNA2	Clontech

The following plasmids were obtained from Addgene:

pcDNA3 -CTNNB1-S33Y (ID19286)
Topflash (ID12456)
Fopflash (ID12457)
pGL2-Snail (ID31694)
shS2-1 (ID10905)

2.1.6 Oligonucleotides

Oligonucleotides for cloning and qRT-PCR were obtained from Life Technologies. SiRNAs were purchased from MWG.

Table 2.2 Oligonucleotides used to generate constructs

primer name	primer sequence (5' to 3')
Flag-Lef1wt s	ccGGATCCatgccccaactctccggaggaggtggc
Flag-Lef1wt as	ccGAATTCtcagatgtaggcagctgtcattcttggac
pLVX-Lef1wt s	ccGGATCCatgccccaactctccggaggaggtggc
pLVX-Lef1wt as	ccGAATTCtcagatgtaggcagctgtcattcttggac
Flag-TCF4 s	ggGGATCCatgccgcagctgaacggcggtggag
Flag-TCF4 as	ggGAATTCctattctaaagacttggtgacgagc
Luc-LEF1-(A) s	ccGAATTCaacatggtggaaaacgaagctcattcc
Luc-LEF1-(A) as	ccCTCGAGaaatgacaatttttaaaaatgttttattacaaagc
Luc-LEF1-(B) s	ctGAATTCaaacccagactgtctccacggcc
Luc-LEF1-(B) as	ccCTCGAGaaatgacaatttttaaaaatgttttattacaaagc
FN1-839 s	ccttCTCGAGaaaaagtaaactgttactttgtcc
FN1-839 as	ggAGATCTgttgagacggtgggggagag
FN1-789 s	ccttCTCGAGacttccccgggatctgcaaagcgcc
FN1-739 s	ccttCTCGAGaagctcattaaaggtctctgttc
FN1-689 s	ccttCTCGAGcctttctcagagccagacaggcac
FN1-559 s	ggCTCGAGggcagccccgccctgggactg
FN1 ∆LEF4 EcoRV s	ctGATATCagggagcgggatgggggaaaggcag
FN1 ∆LEF4 EcoRV as	ctGATATCaagggtactgactcggactcccttat

SNAI2 s	ccaaCTCGAGtgtcaaaagtgtgagagaat
SNAI2 as	gggcGGATCCcttgccagcgggtctggc
CDH1 s	ccacCTCGAGcacagcgcccccactgcc
CDH1 as	ccacGGATCCggctggccggggacgccg
GFP-SNAI2 s	agcGAATTCatgccgcgctccttcctggtcagg
GFP-SNAI2 as	gcaCTCGAGtcagtgtgccacacagcagccagac

primer	primer sequence (5' to 3')
shC s	GATCCGttgtactacacaaaagtactg**TTCAAGAGA**cagtacttttgtgtagtacaaTTTTTTACGCGTG
shC as	AATTCACGCGTAAAAAAttgtactacacaaaagtactg**TCTCTTGAA**cagtacttttgtgtagtacaaCG
shI1-1 s	GATCCGccgggagcagaccaggcaa**TTCAAGAGA**ttgcctggtctgctcccggTTTTTTACGCGTG
shI1-1 as	AATTCACGCGTAAAAAAccgggagcagaccaggcaa**TCTCTTGAA**ttgcctggtctgctcccggCG
shL1-1 s	GATCCGgaaagaaatgagagcgaat**TTCAAGAGA**attcgctctcatttctttcTTTTTTACGCGTG
shL1-1 as	AATTCACGCGTAAAAAAgaaagaaatgagagcgaat**TCTCTTGAA**attcgctctcatttctttcCG

Table 2.3 qRT-PCR primers and Taqman-assays

qRT-PCR	forward (5' to 3')	reverse (5' to 3')
LEF1	CGGGTACATAATGATGCCAA	TCACTGTAAGTGATGAGGGGG
CDH1	GCCGAGAGCTACACGTTCAC	GTCGAGGGAAAAATAGGCTG
FN1	ACCAACCTACGGATGACTCG	GCTCATCATCTGGCCATTTT
VCL	TTACAGTGGCAGAGGTGGTG	TCACGGTGTTCATCGAGTTC
RPLP0	GGCGACCTGGAAGTCCAACT	CCATCAGCACCACAGCCTTC
ACTB	AGAAAATCTGGCACCACACC	AGAGGCGTACAGGGATAGCA
PPIA	GTCAACCCCACCGTGTTCTT	CTGCTGTCTTTGGGACCTTGT
CTNNB1	TCGAAATCTTGCCCTTTGTC	ATCCCGAGCTAGGATGTGAA
IGF2BP1	TAGTACCAAGAGACCAGACCC	GATTTCTGCCCGTTGTTGTC
IGF2BP2	ATCGTCAGAATTATCGGGCA	GCGTTTGGTCTCATTCTGTC
IGF2BP3	AGACACCTGATGAGAATGACC	GTTTCCTGAGCCTTTACTTCC
MYC	AGCGACTCTGAGGAGGAAC	CGTAGTTGTGCTGATGTGTG
SNAI2	TCGGACCCACACATTACCTT	TTGGAGCAGTTTTTGCACTG

ChIP qRT-PCR	forward (5' to 3')	reverse (5' to 3')
FN1 P1&2	TTGTCCTTGCAAAAGAAAACTTC	CCTTTAATGAGCTTCTACTAAGTACCG
FN1 P3	GCGGAACTCCCGGTACTTAG	GCCTGTCTGGCTCTGAGAAA
FN1 P4	GCGCTGAGAAGGGAAGAAGT	CCATCCCGCTCCCTTTCTTT
Intergenic	CGTGCTTGTGCATTTACCCGCC	TGCCTCCATAGTGACTGCGCCT
CDH1	TGGTGGTGTGCACCTGTACT	GGGCTTTTACACTTGGCTGA
SLUG R3	TGCCCCCCTTCTCTGCCAGAGTT	TTCCGCGAAGCCAGGGGCAGCG

Taqman- probes	assay ID
PPIA	Hs99999904_m1
IGF2BP1	Hs00977556_m1
IGF2BP2	Hs01118009_m1
IGF2BP3	Hs00559907_g1
RPL30	Hs00265497_m1

Table 2.4 siRNAs

siRNA	sequence (5' to 3')
Control (siC)	UUGUACUACACAAAAGUACUG
siIGF2BP1-1 (sil1-1)	CCGGGAGCAGACCAGGCAA
siIGF2BP1-2 (sil1-2)	UGAAUGGCCACCAGUUGGA
siIGF2BP1-3 (sil1-3)	CCAUCCGCAACAUCACAAA
siIGF2BP2-1	CCAUAAAGAACAUCACUAA
siIGF2BP3-1	UAAGGAAGCUCAAGAUAUA
siCTNNB1	GGCUUGGAAUGAGACUGCUGAUC
siLEF1-1	GAAAGAAAUGAGAGCGAAU
siLEF1-2	GAUGGAAGCUUGUUGAAAA

2.1.7 Systems

DC Protein Assay	Biorad
Dual-Glo™ Luciferase Assay System	Promega
pCR®-blunt	Invitrogen
GenElute™ HP Plasmid Miniprep Kit	Sigma Aldrich
Plasmid Midi Kit (100)	Qiagen
Fibronectin 1 (FN1) ELISA Kit	antibodies-online.com
SimpleChIP®Enzymatic Chromatin IP kit	Cell Signaling
Wizard® SV Gel and PCR Clean-Up System	Promega

2.1.8 Standard buffers

Phosphate buffered saline (0,01M) (PBS)	137 mM NaCl 2.7 mM KCl 10 mM Na2HPO4 2 mM KH2PO4
Phosphate buffered saline-Tween (PBS-T)	0.01 M PBS 1% Tween-20
Tris/ Acetate/ EDTA (TAE)	40 mM Tris 20 mM glacial acetic acid 1 mM EDTA

2.2 Methods

2.2.1 Cell biological techniques

2.2.1.1 Cell culture

All cell lines were cultured in Dulbecco's modified Eagle's medium DMEM supplemented with 10% fetal bovine serum (FBS) and L-alanyl-L-glutamine (GlutaMAX, Life Technologies) at 37°C and 5% CO_2.

2.2.1.2 Transfection

Cells were transfected with siRNAs by RNAiMax or plasmids by Lipofectamine 2000. For RNA interference studies $2x10^5$ HEK293 or $5x10^5$ U2OS cells were seeded per well of a 6 well plate 16 h prior to transfection. The respective cell line was transfected with 200 pmol siRNA (target or control siRNA) and 6 µl of RNAiMAX for 72 h. The siRNA sequences used are summarized in Table 2.4.

For plasmid transfection cells were again seeded in 6well plates ($4x10^5$ HEK293 or $5x10^5$ U2OS) 16 h prior to transfection. Transient overexpression was performed by transfection of 3 µg of the indicated plasmids with 4 µl Lipofectamine 2000 for 48 h. For RNAi-recovery studies, cells were co-transfected with indicated shRNA-encoding and Flag-tagged protein encoding plasmids for 72 h.

2.2.1.3 Inhibition of RNA synthesis

HEK293 cells were transfected with IGF2BP1-directed or control siRNAs for 72h. RNA transcription was blocked by treatment with actinomycin D [5 µM] for the indicated time points and RNA abundance was monitored by quantitative real-time PCR.

2.2.1.4 Lentiviral transduction

The stable overexpression and knockdown of LEF1 and SNAI2 was carried out by lentiviral transduction. Cloning of the constructs into the pLVX- puro or pLVX-shRNA2 vector was performed as described in 2.2.2.1. GFP-or shC expressing viruses were used as control. The production of lentiviral particles was accomplished by transfection of HEK293T cells with the following plasmids. A 10 cm plate with

$2x10^6$ HEK293T cells was transfected with 20 µg of the pLVX-plasmids, 6 µg of pMD2G and 15 µg psPAX2. The latter ones were needed for packaging of the virus. The DNA was delivered into the cells using calcium-phosphate precipitation. The total volume of the transfection solution was 1 ml containing the DNA diluted in 500 µl of bi-distilled water, 500 µl of 2x HBS and drop-wise added 50 µl 2.5M $CaCl_2$. The virus containing supernatant was collected on days 2 and 3. Finally the virus was concentrated using Lenti-X™ Concentrator (Clontech).

For lentiviral transduction U2OS or HT-144 cells ($1x10^5$ cells) were seeded in a 6 well plate and 100 µl of the virus solution was added for 48 hours. The cells were washed 4 times with Hanks and transferred to a 6 cm plate. Puromycin (2µg/ ml) was added for 3 days to select for the virus positive cells. Cells were tested for stable expression of LEF1 or GFP by Western blotting. The stable cell population was grown further in medium containing 1 µg/ ml puromycin.

2x HEPES-buffered saline (HBS)

280 mM NaCl
10 mM KCl
1.5 mM Na_2HPO_4
12 mM Glucose
50 mM HEPES
pH 7.05

2.2.2 Molecular biological methods

2.2.2.1 Cloning

The transient or stable expression of different recombinant proteins required cloning of different constructs which are presented in detail in the following paragraph and summarized in Table 2.5. Cloning was standardized carried out by amplification of the sequence of interest using Pfx polymerase (Invitrogen) or Phusion® High-Fidelity DNA Polymerase (Thermo Scientific). The purified PCR product (kit) was inserted in pCR®- Blunt or pGEMT easy vectors. All PCR-amplified products were sequenced upon successful insertion by Seqlab Göttingen.

The coding sequences of the full length LEF1 (NM_016269) and the shorter variant lacking Exon VI (NM_001130713) were generated by RT-PCR from total

HEK293 RNA. Also the two alternative 3'-UTRs of LEF1 isoforms (**A**: NM_016269; NM_001130713; NM_001166119; **B**: NM_001130714) were generated by RT-PCR from total HEK293 RNA. The LEF1 coding sequences were inserted via BamHI/ EcoRI in pcDNA3.1zeo-Flag and pLVX-puro plasmids, respectively. The LEF1 3'UTRs were inserted via EcoRI/ XhoI into pcDNA3.1neo-LUC. The pcDNA3.1neo-LUC vector contained the coding sequence of a firefly luciferase (LUC) inserted via HindIII/ EcoRI and the vector encoded BGH-3'UTR. The coding sequence of TCF4 (TCF7L2; NM_030756) was also generated by RT-PCR and inserted via BamHI/ EcoRI in pcDNA3.1zeo-Flag.The GFP-SNAI2 construct was based on PCR amplification from the Addgene plasmid; pTK-SLUG (ID: 36986) and inserted into pLVX-puro via EcoRI/XhoI.

For lentiviral transduction shPlasmids were generated by direct cloning of annealed oligos (for oligo sequence see table 2.2) into pLVX-shRNA2 via BamHI/EcoRI.

Fibronectin (FN1) minimal promoter sequences were PCR-amplified from HEK293 genomic DNA and transferred into pGL4.21 (Promega) via XhoI/ BglII sites. Subsequent shortening of this sequence for the indicated constructs was carried out by PCR amplification, using the respective primers, followed by the insertion into pGL4.21 via XhoI/ BglII. The recently published promoter sequences of SNAI2 and CDH1 important for LEF1 regulation were generated by PCR amplification from genomic DNA and inserted into pGL4.21 via XhoI/ BamHI in BglII (Jesse et al., 2010; Lambertini et al., 2010). The SNAI1 promoter sequence was purchased as pGL2 construct from Addgene and inserted into pGL4.21 via KpnI/HindIII sites.

The FN1 promoter (FN-839) lacking the LEF1-binding site 4 (FN1ΔLEF4) was generated by PCR amplification of two products, by which the binding site was replaced by an EcoRV restriction site (Figure 2.1). The two PCR products were fused by 3-point-ligation. All constructs used in this study are presented in Table 2.2.

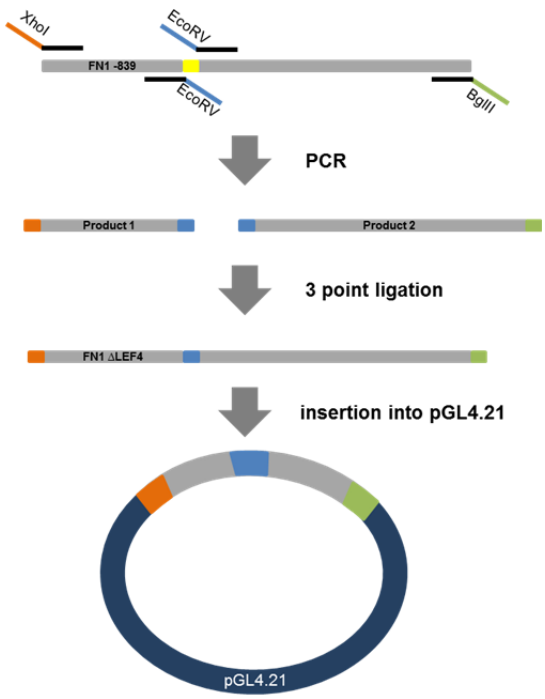


Figure 2.1: Scheme displaying the deletion of the LEF1 binding site 4 (indicated in yellow) in the FN1 minimal promoter sequence by PCR amplification.

Table 2.5 constructs

	vector	cloning	insertion via
Flag-LEF1	pcDNA3.1-Flag zeo	RT-PCR, Zero Blunt (Life Technologies)	BamHI EcoRI
pLVX-LEF1	pLVX puro- new MCS	RT-PCR, Zero Blunt (Life Technologies)	BamHI EcoRI
Flag-LEF1ΔExonVI	pcDNA3.1-Flag zeo	RT-PCR, Zero Blunt (Life Technologies)	BamHI EcoRI
Flag-TCF4 (TCF7L2)	pcDNA3.1-Flag zeo	RT-PCR, Zero Blunt (Life Technologies)	BamHI EcoRI
Luc-LEF1-(A)	pcDNA3.1 FFL	RT-PCR from HEK293 cells, Zero Blunt (Life Technologies)	EcoRI XhoI
Luc-LEF1-(B)	pcDNA3.1 FFL	RT-PCR from HEK293 cells, Zero Blunt (Life Technologies)	EcoRI XhoI
FN1-839	pGL4.21	PCR from HEK293 genomic DNA, pGEMTeasy (Promega)	XhoI BglII
FN1-789	pGL4.21	PCR from FN-839, pGEMTeasy	XhoI BglII
FN1-739	pGL4.21	PCR from FN-839, pGEMTeasy	XhoI BglII
FN1-689	pGL4.21	PCR from FN-839, pGEMTeasy	XhoI BglII
FN1 -559	pGL4.21	PCR from FN-839, pGEMTeasy	XhoI BglII
FN1 ΔLEF4	pGL4.21	PCR from FN-839, pGEMTeasy	XhoI BglII
FN1+1	pGL4.21	PCR from FN-839, pGEMTeasy	XhoI BglII
SNAI2	pGL4.21	PCR from HEK293 genomic DNA, pGEMTeasy (Promega)	XhoI BamHI in BglII
CDH1	pGL4.21	PCR from HEK293 genomic DNA, pGEMTeasy (Promega)	XhoI BamHI in BglII

SNAI1	pGL4.21	subcloned from pGL2 Snail	KpnI HindIII
pLVX puro-new MCS		previously described (Stohr et al. Genes Dev. 2012)	
GFP-chZBP1	pLVX puro- new MCS	previously described (Stohr et al. Genes Dev. 2012)	
GFP-SNAI2	pLVX puro- new MCS	PCR based on Addgene; pTK-SLUG (ID: 36986)	EcoRI XhoI
shC	pLVX-shRNA2	direct cloning of annealed oligos	BamHI EcoRI
shI1-1	pLVX-shRNA2	direct cloning of annealed oligos	BamHI EcoRI
shL1-1	pLVX-shRNA2	direct cloning of annealed oligos	BamHI EcoRI

2.2.2.2 DNA Isolation

Preparation of small scale plasmid DNA was carried out using the GenElute™ HP Plasmid Miniprep Kit (Sigma Aldrich) according to the manufacturer´s instructions. For large scale plasmid DNA preparations the Plasmid Midi Kit (100) from Qiagen was used following the manufacturer´s instructions.

2.2.2.3 RNA isolation and RT-PCR

Total RNA was isolated from cells by Trizol reagent followed by Chloroform extraction. In detail, cells were harvested in 1 ml Trizol per well from a 6-well plate. The lysed cells were transferred to a 1.5 ml reaction tube. After pelleting of cell debris by centrifugation for 5 min at 13000 rpm RNA was extracted by adding 200 µl of chloroform. The aqueous phase was transferred to a fresh reaction tube with subsequent precipitation of the RNA with 500 µl Isopropanol for 25 min at room temperature. The precipitated RNA was pelleted by centrifugation for 10 min at 13000 rpm followed by two washing steps with 80% ethanol. The final RNA pellet was diluted in an appropriate volume of RNase free water. RNA quality and concentration was determined using an infinite M200pro spectrometer (TECAN). The isolated RNA was used for a reverse transcription reaction. Therefore 2 µg RNA were incubated with 1 µl oligo-dT primer (500 ng/µl) for 5 min at 65°C and cooled down to 4°C for additional 5 min to allow denaturation and primer annealing. Afterwards the master mix including the reverse transcriptase was added and reaction was executed at 42°C for 2 h followed by an inactivation step of the reverse transcriptase at 75°C for 15 min.

Trizol	Master Mix
0.8 M Guanidiniumthiocyanat	4 µl 5xRT-buffer
0.4 M Ammoniumthiocyanat	1 µl dNTPs (10mM)
0.1 M Natriumacetat, pH 5.0	0.25 µl RNasin
5% (w/v) Glycerol	0.5 µl M-MLV-RT
48% Roti-Aqua-Phenol	
for RNA isolation	

2.2.2.4 Quantitative real-time PCR (qRT-PCR)

RNA abundance was determined by quantitative real-time PCR (qRT-PCR). This technique was used to precisely obtain and discriminate RNA levels of different samples e.g. knockdown studies. Furthermore the abundance of specific DNA sequences co-immunopurified by chromatin immunoprecipitation studies was determined by qRT-PCR.

Two common methods were used, SYBR Green I and taqman. Both are based on polymerase-chain-reaction coupled to fluorescence emitting processes correlating to the increase of the PCR product. PCR products are detected and quantified while running the reaction in real time. The SYBR Green I system was mainly used in this study. The SYBR Green I dye intercalates in double-stranded DNA, which represents the newly synthesized PCR product. The subsequently emitted fluorescence signal is detected and employed for quantification. Finally a melting curve represents a tool for quality control to ensure the existence of one specific PCR product.

Second of all the taqman technique is based on a transcript-specific fluorescently-labeled probe in addition to two target-specific primers. The probe is coupled to a fluorescence reporter dye and a quenching molecule in close proximity. The probe hybridizes to the target RNA and is cleaved off during the process of primer elongation by the exonuclease activity of the taq-polymerase. The fluorescence signal of the reporter is no longer quenched and the fluorescence signal can be detected. This method was used for the quantitative assessment of RNA levels from tumor samples of squamous cell carcinoma.

The qRT-PCR measurements were performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems) for 384well-format and iCycler iQ5 (Biorad) for 96well-format. Accordingly two different master mixes were used for the detection of RNA abundance, the SYBR® Select Master Mix (Life Technologies) and the

taqMan universal PCR Master Mix (Life Technologies). Primers for the SYBR Green I method were designed using qPrimerDepot, Primer3 and Primer-BLAST software. All primers, except for ChIP-studies, ideally span exon-exon borders to specifically detect cDNA. Taqman-probes were obtained as a 20x ready-to-use mix including probes and primers from Applied Biosystems. The DNA levels subsequently measured from ChIP studies were exclusively measured in 96well-format using a 2x PCR master mix (Promega) with addition of SYBR Green I and fluorescein (passive reference). Primers for ChIP- studies were designed based on the limited positions within the genomic sequence of interest of the FN1 and CDH1 promoters or the location in an intergenic region as a control. Sequences of primers and assay ID`s of probes used for qRT-PCR are listed in Table 2.3.

The reaction was carried out in a volume of 10 µl (384well-format) or 15 µl (96-well format), respectively. The cDNA obtained in 2.2.2.3 was diluted 1:6. Thereof 5 µl were used per reaction. All samples were analyzed in triplicates.

Reaction set up to determine RNA abundance: 384well-plate

SYBR Green I:	**Taqman:**
5 µl 2x Master Mix	5 µl 2x Master Mix
0.02 µl each primer (100µM)	0.5 µl of 20x Probe Mix

The PCR reaction was performed according to the manufacturer's instructions with an initial denaturation step at 95°C to activate the hot-start taq polymerase followed by 40-50 cycles of repeated denaturation (95°C, 15 s) and annealing/amplification (60°C, 1 min) steps. In addition melting curve analyses were performed for SYBR Green I reactions to evaluate the specificity of the PCR product.

Reaction set up to determine DNA levels from ChIP studies: 96well-plate

SYBR Green I: 7.5 µl 2x Master Mix (Promega)
0.03 µl each primer (100 µM)
0.3 µl Fluorescein (passive reference, 750nM)
0.3 µl SYBR Green I (SIGMA, 86205, 1:1000)
1.9 µl bi-distilled H_2O

The PCR reaction was carried out with an initial denaturation step at 95°C for 3 min followed by 50 cycles of denaturation (95°C, 30 s), annealing (60°C, 30 s) and amplification (72°C, 30 s). In addition melting curve analyses were performed to evaluate the specificity of the PCR product.

Data were analyzed by the ΔC_t- or the $\Delta\Delta C_t$- method described by Livak and Schmittgen (Livak and Schmittgen, 2001). RNA levels were equivalent to measured cDNA values. Therefore relative transcript levels (RNA ratio) were obtained in comparison of two populations (e.g. control vs. knockdown) and normalization to an endogenous control RNA which was unperturbed throughout all measurements independently of different condition (e.g. knockdown of a gene). Standardized used in this study was cyclophilin A (PPIA) as endogenous controls if not stated elsewhere.

The following formula was used for $\Delta\Delta C_t$ calculation:

$$\boldsymbol{RNA\ ratio} = \mathbf{2}^{(-((\boldsymbol{2T}-\boldsymbol{1T})-(\boldsymbol{2EC}-\boldsymbol{1EC})))}$$

$2T$ – target RNA in explorative population
$1T$ – target RNA in control population
$2EC$ – endogenous control RNA in explorative population
$1EC$ – endogenous control RNA in control population

The enrichment of RNA (cDNA) or DNA from immunoprecipitation experiments was determined by calculating the ΔC_t using the following equation:

$$\boldsymbol{RNA\ /\ DNA\ enrichment} = \mathbf{2}^{(-((\boldsymbol{target}-\boldsymbol{input})))}$$

2.2.2.5 SDS-PAGE and Western blotting

The abundance of cellular proteins was determined by Western blotting. Therefore the cell culture medium was aspirated and cells were washed with PBS. HEK293 cells were rinsed off the plate and U2OS cells were scraped with a rubber policeman from the plate as a PBS suspension and transferred into a 1.5 ml reaction

tube. Note that trypsin can digest cell surface and cell contact proteins. After centrifugation the supernatant was removed. The cell pellet was stored at -80°C until further use or protein extraction was carried out immediately. Extraction of whole cell lysate was achieved using RIPA buffer supplemented with protease inhibitor cocktail (Sigma Aldrich). The extraction process was enforced by pipetting the cell-buffer-solution up and down several times before incubation on ice for 10-15 min. Lysate was cleared by centrifugation for 10 min at 13000 rpm, transferred to a fresh tube and kept on ice. Protein concentration was determined using DC Protein Assay (Biorad). Depending on the protein levels, 10 or 25µg of total protein extracts were prepared for separation by SDS-PAGE. SDS polyacrylamide gel electrophoresis was carried out using the NuPAGE® Novex 4-12% Bis-Tris Gel system (Life Technologies) with NuPAGE® MOPS SDS Running Buffer. Proteins were transferred onto a nitrocellulose membrane by wet-blotting with freshly prepared NuPAGE blotting buffer containing 20% methanol. Successful protein transfer was verified by Ponceau S staining before blocking the membrane with 10% (w/v) skimmed milk / PBS-T to minimize unspecific binding. The membrane was incubated with the indicated primary antibodies (see Table 2.1) in 5% (w/v) skimmed milk overnight at 4°C with constant agitation. After washing with PBS-T the membrane was incubated with IRDye-labeled secondary antibodies (see also Table 2.1) in a 5% (w/v) skimmed milk / PBS-T solution for 1 h at room temperature. Protein abundance was monitored using Odyssey *Infrared Imaging System* (LI-COR Biosciences) at 680 nm or 800 nm. Quantification of the protein bands was carried out with the Odyssey software. Quantified protein levels were determined relative to the control population normalized to Vinculin (VCL) which served as loading control in all analyses.

RIPA- buffer:
20mM Tris-HCl (pH 7.5)
150mM NaCl, 1mM EDTA
1mM EGTA, 1% NP-40
1% sodium deoxycholate (DOC)
2.5mM sodium pyrophosphate
1mM beta-glycerophosphate
1mM Na_3VO_4

NuPAGE® blotting buffer:
50 mM Tris pH 8.5
40 mM Glycin
20% Methanol
0.04% SDS

Ponceau S solution:
0.1% (w/v) Ponceau S
5% Essigsäure

2.2.2.6 Immunoprecipitation techniques

2.2.2.6.1 RNA-co-immunoprecipitation (RIP)

RNA co-immunoprecipitation (RIP) was used to determine an association of IGF2BP1 with target-RNAs. Therefore HEK293 cells were counted (5×10^6) and plated on a 15 cm dish. The next day cells were washed with PBS, rinsed off the plate and pelleted in a 15 ml tube (Falcon). To stabilize the RNA-protein complexes formaldehyde cross-linking was performed prior to the protein extraction. Cells were treated with 1% formaldehyde/ PBS in a volume of 1 ml for 10 min at room temperature. The reactivity of formaldehyde was quenched with 0.1 M Tris-HCl for 5 min. The cross-linked cells were pelleted by centrifugation at 3000 rpm and washed once with 1 ml PBS. Cells were then extracted in 1100 µl ice-cold RIP-buffer supplemented with protease inhibitor cocktail (Sigma Aldrich) and RNasin (Promega) on ice for 15 min. A volume of 10% (50 µl) of the lysate used per IP served as the input fraction. Antibodies for control-IP (IgG mouse) or the IGF2BP1-IP were coupled to proteinG Dynabeads (Life Technologies) in 500 µl ice-cold wash buffer supplemented with yeast tRNA (20 µg/ml). After antibody coupling to beads cell lysates were added in a 1:1 (v/v) ratio and incubated at 4°C overnight with constant agitation. The total volume of each IP reaction was 1 mL consisting of 500 µl pre-coupled beads solution plus 500 µl lysate. Beads were washed once with the washing buffer (WB) and three times with WB additionally containing 0.5 M urea. The washing steps were performed at room temperature with pre-chilled buffers. Protein-RNA complexes were eluted in WB supplemented with 1% SDS at 65°C for 10 min. Reversal of the cross-link was achieved by proteinase K (Roche) digestion for 1h at 65°C. RNA was purified by phenol-chloroform extraction and precipitated by isopropanol. The RNA pellet was finally diluted in 24 µl of RNase free water and treated with RQ1-DNase to remove all traces of DNA contamination. A volume of 12.75 µl of the RNA was used for the reverse transcription with M-MLV reverse transcriptase and random hexamer primers. RNA abundance was assessed by semi-quantitative and quantitative PCR using primers listed in Table 2.3.

RIP-buffer:
10 mM HEPES (pH7.2)
150 mM KCl
5 mM $MgCl_2$
0.5% NP40

Washing buffer (WB):
50 mM Tris-HCl (pH7.4)
300 mM NaCl
5 mM $MgCl_2$
0.01% NP40

Reaction set up for DNAse digestion:
24 µl RNA
3 µl RQ1-DNase
3 µl RQ1-DNase buffer
→ 37°C for 30 min
→ Addition of 3 µl DNase stop solution
→ DNase inactivation at 65°C for 30 min

Reaction set up for reverse transcription:
12.75 µl of DNase treated RNA
1 µl of hexamer primer
→65°C for 5 min
4 µl of 5x RT buffer
1 µl dNTPs
0.25 µl RNasin
1 µl M-MLV RT

2.2.2.6.2 Chromatin immunoprecipitation (ChIP)

A second application for immunoprecipitation was used to co-immunopurify DNA specifically with DNA-binding proteins. Using the SimpleChIP™ Eynzymatic Chromatin IP Kit (Cell Signaling) it was analyzed whether the transcription factor LEF1 binds *in vivo* to the human fibronectin promoter of HEK293 cells. The assay was essentially carried out as described by the manufacturer´s instructions. A total of $4x10^7$ HEK293 cells were used per experiment. The fragmentation of DNA by enzymatic digestion using Micrococcal Nuclease was evaluated by separation on a 1% agarose gel with 100 bp DNA-marker in 1x TAE buffer. The majority of the DNA was digested to a size of 150-900 bp which is approximately the length of 1-5 nucleosomes. Mouse IgG-antibody was used as negative control. Immunopurification of Histon-H3 served as positive control. The LEF1 immunopurification was performed with an antibody validated for ChIP assays obtained from Millipore. The enrichment of the genomic DNA fragments was determined relative to the input fraction for each IP and finally normalized to the IgG control by the ΔC_t-method using quantitative PCR. The PCR products were additionally semi-quantitatively analyzed on a 2% agarose gel containing ethidium bromide.

2.2.2.7 Enzyme-linked immunosorbent assay (ELISA)

Fibronectin (FN1) produced by HEK293 cells is secreted into the cell culture medium. To evaluate an altered expression pattern upon knockdown of different proteins, FN1 levels were determined by ELISA. For this purpose a commercially available FN1 ELISA from Boster Biological Technology kit was obtained. HEK293 cells were transfected with IGF2BP1-, LEF1-, CTNNB1-directed or control siRNAs for 72h. The cells were starved with FBS free DMEM for 16h prior to the collection of the cell culture medium to avoid interference of FN1 from the FBS. The instructions according to the manufacturer were essentially followed to determine FN1 levels of the cell culture supernatant calculated based on a standard curve. Protein amounts were finally normalized to cell number which was determined by flow cytometry (2.2.3.1).

2.2.2.8 Luciferase reporter assay

IGF2BPs regulate their target mRNAs by binding to specific *cis*-elements within the transcripts. Thus it was analyzed whether the siRNA-directed depletion of the IGF2BPs has an impact on the turnover of a firefly luciferase fused to the LEF1-3`UTR. Two alternative LEF1 3' UTRs, both expressed in HEK293 cells, were used for the analysis (A: Acc.No. NM_016269/ 001130713/ 001166119; B: Acc.No. NM_001130714). The BGH-3'UTR was utilized as control firefly reporter. HEK293 cells were counted (2x 10^5) and seeded per well of a 6 well plate (day 1). Cells were transfected with 200 pmol IGF2BP-directed or control siRNAs (day 2) and split into wells of a 12 well plate (day 3). Co-transfection of luciferase reporter was carried out by calcium- phosphate precipitation (see also 2.2.1.4) on day 4. In detail, per 12 well 1 µg of DNA (100 ng firefly luciferase reporter, 100 ng renilla luciferase reporter, 800 ng pcDNA3.1) was diluted in 50 µl H_2O. Volumes of 50 µl 2xHBS and 5 µl of 2.5 M $CaCl_2$ were carefully added and incubated for 20 min at room temperature before added to the cells. To enhance transfection efficiency a glycerol shock (250 µl per 12 well: 125 µl 2xHBS, 75 µl 50% glycerol, 50 µl H_2O) was applied 6 h post transfection. Cells were incubated with the shock buffer for 2 min at room temperature. Cell culture medium was removed, cells were washed and fresh medium was added to the cells. The luciferase reporter assay was finally carried out

72 h post siRNA transfection (day 5) using the Dual-Glo® assay (Promega) according to the manufacturer´s instructions.

Promoter reporter studies were performed to investigate the role of LEF1 on transcriptional activation of the promoter region of FN1, CDH1, SNAI2 and SNAI1. The promoterless pGL4.21 was used as a control reporter for background activity. HEK293 cells were counted ($4x10^4$) and seeded into one well of a 24 well plate per transfection. The next day cells were transfected with firefly (100 ng) and renilla (100 ng) luciferase reporter plasmids as well as pcDNA3.1 vector (300 ng) and 500 ng of Flag-RFP or Flag-LEF1 for 30 h using Lipofectamine 2000. Luciferase activity was determined using the Dual-Glo® assay (Promega). Promoter studies in response to LEF1 and IGF2BP1 depletion were carried out the same way with the replacement of Flag-plasmids by shRNA-plasmids (shC, shI1-1 and shL1-1). Luciferase activity was determined 48 h post transfection.

HEK293 and HCT116 cells were transfected as described above with 250 ng Topflash or Fopflash reporter (firefly reporter) and Flag-tagged RFP, CTNNB1-S33Y, LEF1 or TCF4 for 30 h to analyze transcriptional activity. Renilla luciferase activity served as an internal normalization control on all analyses.

2.2.3 Assays of cell phenotype

2.2.3.1 Flow cytometry

Flow cytometry was used to determine the cell volume of detached cells. HEK293 cells were transfected with IGF2BP1-, LEF1 or control siRNAs for 72 h. Cell culture medium was removed and cells were washed twice with Hanks. The cells were separated by tryptic digestion and subjected to flow cytometry analyses by forward scattering using MACSQuant (Miltenyi). The forward scatter is proportional to the diameter of the cell and is therefore used to determine cell volume.

Furthermore this method was used for cell counting. Cells were prepared for flow cytometry as described above. The cell culture medium was collected and analyzed by ELISA. Propidium iodide treatment was used to determine apoptotic cells and exclude them from measurements in both applications.

2.2.3.2 Immunofluorescence staining

The subcellular localization of different proteins was investigated by immunofluorescence staining and subsequent microscopic analyses. The indicated cell lines were transfected with siRNAs or plasmids depending on the experimental background. Approximately 24 h post transfection cells were split and grown on coverslips for at least another 24 h. Cells were fixed using 4% PFA solution for 20 min and subsequently permeabilized with 0.5% Triton-X in PBS for 5 min. Unspecific binding was blocked with 1% BSA in PBS for 1 h. Primary antibodies were diluted in 1% BSA/PBS and added to the blocked cells for 1 h (antibodies listed in Table 2.1). After washing with PBS cells were incubated with fluorescently labeled secondary antibodies and/or phalloidin-TRITC in 1% BSA/PBS solution for 1 h. Washing steps with PBS were again performed to remove access antibody. Finally DNA was labeled using DAPI for 5 min. The cells were washed twice with distilled water, dehydrated with 80% ethanol and conserved with ProLong® Gold Antifade (Invitrogen) on object slides. All steps were carried out at room temperature. Images of the immunostained cells were acquired by confocal microscopy.

2.2.4 Microscopy

2.2.4.1 Brightfield microscopy

Besides regular examination of viability and confluence of growing cells brightfield microscopy was used to analyze cell morphology upon knockdown of different proteins. Representative images were acquired using a Nikon Coolpix 990 camera attached to a Nikon Eclipse TS100 microscope.

2.2.4.2 LSM microscopy

Confocal laser scanning microscopy was used for the acquisition of images from immunostained cells. Image acquisition was performed on a Leica SP5 microscope with a 63x Plan Apo objective, a white light laser (WLL) and LAS AF software. This technique was employed to investigate changes of protein localization in subcellular structures upon knockdown of IGF2BP1 or LEF1. Furthermore the F-actin cytoskeleton and overall morphology changes were monitored. For wound closure

experiments cells ($1x10^5$/well) were cultured in a 24well plate for 24h and scratched before time lapse microscopy using a Leica LSM-SP5 microscope equipped with a Ludin Cube live cell chamber and a 20x Plan Fluor objective. Images were acquired every 15 minutes. Note that movies of all cell populations were analyzed simultaneously using automated cell segmentation and wound closure algorithms recently described (Glaß et al., 2012).

2.2.5 Statistics

If not noted elsewhere all experiments were performed at least three times independently of each other. The data shown are mean values. Error bars indicate standard deviation for all performed experiments. Statistical significance was determined by Student´s t-testing to evaluate variances of two independent populations expressed as p-values. This p-value indicates the probability of two groups to overlap, in other words the actual difference of two populations in relation to variances of the data. A p-value of 0.05% equals a probability of 5% of one population to overlap with the second tested. P-values were indicated as asterisk in the diagrams as follows: (*) $p<0.05$; (**) $p<0.005$ and (***) $p< 0.0005$.

2.2.6 Nomeclature

Proteins, RNAs and genes are presented as gene symbols throughout this study. They are initially depicted as the most commonly used name and gene symbols are put in parentheses.

3 Results

3.1 IGF2BP1 sustains a mesenchymal phenotype

The oncofetal Insulin like growth factor 2 mRNA-binding protein 1 serves essential functions in embryogenesis and has been reported to be frequently overexpressed in various cancers [reviewed in: (Bell et al., 2012; Yaniv and Yisraeli, 2002; Yisraeli, 2005). Although many studies identified IGF2BP1 to be up-regulated in different tumor entities it remains poorly investigated how the protein modulates cellular functions. However, recent studies suggested its impact in proliferation, migration and invasiveness. This was correlated to the regulation of distinct target mRNAs like MAPK4, PTEN, CD44, CTNNB1 or MYC on the post-transcriptional level (Gu et al., 2008; Kobel et al., 2007; Lemm and Ross, 2002; Noubissi et al., 2006; Stohr et al., 2012; Vikesaa et al., 2006). To evaluate its impact on cellular mRNA fate and thereby controlling processes like epithelial-mesenchymal-transition (EMT), a hallmark of tumor progression, different human cell lines of non-malignant and tumor-derived origin were used for analyses. HEK293A cells (HEK293A) were originally primary cultures of human embryonic kidney cells and transformed with sheared adenovirus (Ad)5 DNA (Graham et al., 1977a) and therefore are not derived from cancerous tissue. The osteosarcoma derived cell line U2OS, ovarian carcinoma-derived ES-2 and melanoma –derived HT-144 cells were used to study the role of IGF2BP1 function in a tumor-derived context which frequently coincide with mutations in a multitude of genes. U2OS cells express only low levels whereas ES-2 and HT-144 cells exhibit high amounts of IGF2BP1. Based on different cellular backgrounds it was of great interest whether a universal functional mechanism can be observed in more than one cell type. In this study loss of function as well as gain of function analyses were used for a detailed evaluation of the role of IGF2BP1 in the regulation of distinct target mRNAs. Results from visual as well as experimentally determined observations were combined to get a more comprehensive understanding of the IGF2BP1-mediated control of post-transcriptional gene expression.

3.1.1 IGF2BP1 sustains mesenchymal cell properties in HEK293 cells

The cell line HEK293 A (HEK293) expressed all IGF2BPs including high levels of IGF2BP1. Three distinct IGF2BP1-directed siRNAs were used to investigate its role in the regulation of target genes and its impact on cellular functions. First of all the specificity of the IGF2BP1-targeting siRNAs was analyzed. The first siRNA (siI1-1) efficiently depleted IGF2BP1 but additionally moderately down-regulated the two family members IGF2BP2 and 3 on protein (Figure 3.1A) as well as on mRNA levels (Figure 3.1B). Two of the siRNAs (siI1-2, siI1-3) showed a high specificity for IGF2BP1 on both protein and RNA levels with good knockdown efficiencies and only minor changes in IGF2BP2 and 3 levels. (Figure 3.1A, B). Note that two peptides of IGF2BP2 were detected by Western blotting (Figure 3.1A). The longest variant (upper band) Acc.no.: NM006548) represents IGF2BP2 isoform A. The shorter variant (lower band) is a mixture of two peptides derived from isoform A by alternative splicing of exon 10 (Acc. no.:NM001007225.1) or by leaky ribosomal scanning (refer to (Bell et al., 2012)).

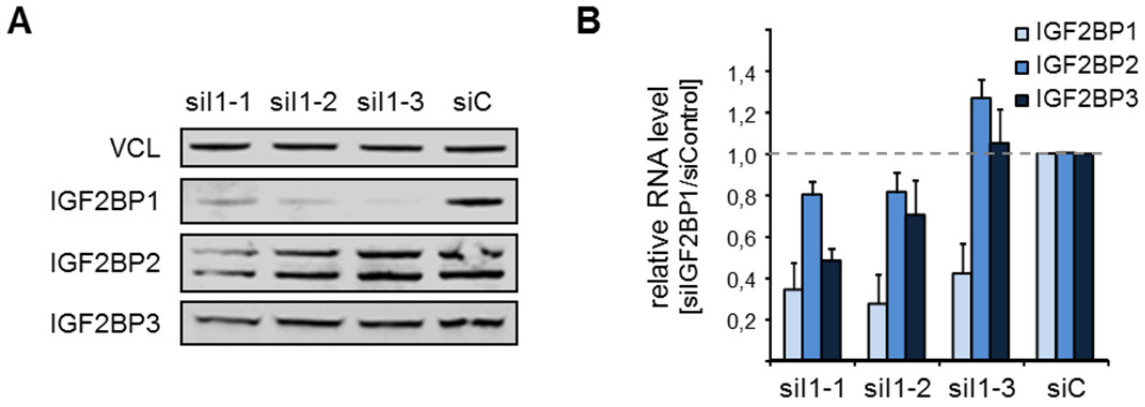
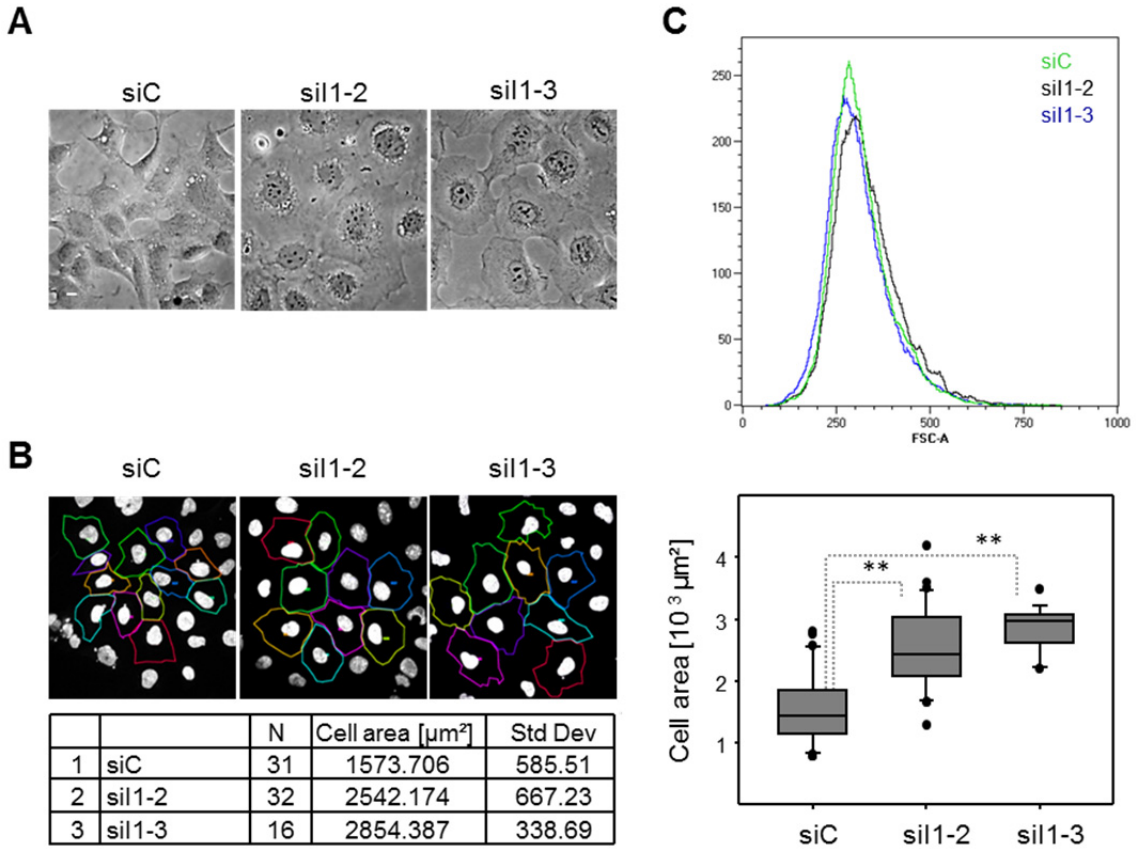


Figure 3.1: Efficiency and specificity of IGF2BP1-directed depletion using three different siRNA. (A, B) HEK293 cells were transfected with control (siC) or IGF2BP1-directed (siI1-1, siI1-2 or siI1-3) siRNAs for 72 h. **(A)** Protein abundance was monitored by Western blotting using monoclonal antibodies specific for IGF2BP1, 2 or 3. Vinculin (VCL) served as a loading control. Due to different expression levels in HEK293 cells 10 µg of total cell extract was subjected to Western blot analyses for the detection of IGF2BP1 and 25 µg for IGF2BP2 and 3. **(B)** mRNA levels of the IGF2BPs upon IGF2BP1 knockdown were determined by qRT-PCR. Changes in RNA abundance was analyzed relatively to controls (siC) using the $\Delta\Delta C_t$-method. Cyclophilin A (PPIA) served as control for normalization. Error bars indicate standard deviation of at least three independent experiments.

Surprisingly, the siRNA-mediated depletion of IGF2BP1 induced morphological changes reminiscent of a mesenchymal-epithelial-transition (EMT) with at least two different siRNAs compared to the control siRNA transfected cells. The cells appeared flattened with increased cell-cell contacts monitored by light microscopy (Figure 3.2A). Furthermore the cell size (area) increased significantly (Figure 3.2B). On the other hand, the volume of detached cells determined by flow cytometry using forward scattering revealed no apparent differences between the IGF2BP1 knockdown and the control cell population (Figure 3.2C). These findings suggested that IGF2BP1 depletion led to changes in cell the morphology of HEK293 cells rather than to an overall increase in cell mass.



		N	Cell area [µm²]	Std Dev
1	siC	31	1573.706	585.51
2	sil1-2	32	2542.174	667.23
3	sil1-3	16	2854.387	338.69

Figure 3.2: IGF2BP1 depletion induces MET-like changes. (**A-C**) HEK293 cells were transfected with control (siC) or IGF2BP1-directed (sil1-2 or sil1-3) siRNAs for 72 h. **(A)** Morphology of the cells was monitored by light microscopy. Bar indicates 10 µm. **(B)** The size of adherent cells was analyzed upon immunostaining of β-catenin (CTNNB1) as well as F-actin labeling by phalloidin. Adherent cells were traced by manual labeling using CTNNB1-defined borders. Image acquisition was performed by LSM-microscopy. The cell area (µm²) was determined using the Leica-SP5 software. The number of analyzed cells (N), mean cell area (μm^2) and standard deviation of cell size (μm^2) are presented in the table. Cell areas of each siRNA treated cell population are presented as box plots. **(C)** The volume of cells was determined by flow cytometry using forward scattering by the MACSQuant (Miltenyi) software. Statistical significance was validated by Student's t-test: ** $p < 0.005$.

The morphological changes observed upon IGF2BP1 depletion were additionally characterized in terms of expression and localization of typical marker proteins. In agreement with a more flattened phenotype intercellular junctions were affected upon IGF2BP1 knockdown revealed by immunostaining of adherens junction components. The two factors β-catenin (CTNNB1) and E-cadherin (CDH1), both essential for proper adherens junctions maintenance, showed enhanced recruitment to cell-cell-contact sites in response to IGF2BP1 depletion (Figure 3.3A (CTNNB1); B (CDH1)). Adherens junction complexes link the F-actin cytoskeleton to the cell membrane. Therefore the distribution of F-actin cytoskeletal fibers was analyzed as well. A re-distribution of the F-actin fibers with an enrichment of F-actin at the cortical periphery was observed. Fibers appeared thickened rather than shortened as previously described in osteosarcoma-derived cells (Stohr et al., 2012). In agreement with its function in interfering with β-Actin (ACTB) mRNA translation an increase of ACTB protein but not mRNA levels was observed upon IGF2BP1 depletion with two different siRNAs (Figure 3.3C). In summary, the data suggested that the knockdown of IGF2BP1 led to an increased formation of cell-cell contacts (adherens junctions) in HEK293 cells and alterations of the F-actin cytoskeleton with enhanced levels of ACTB protein compared to control siRNA treated cells.

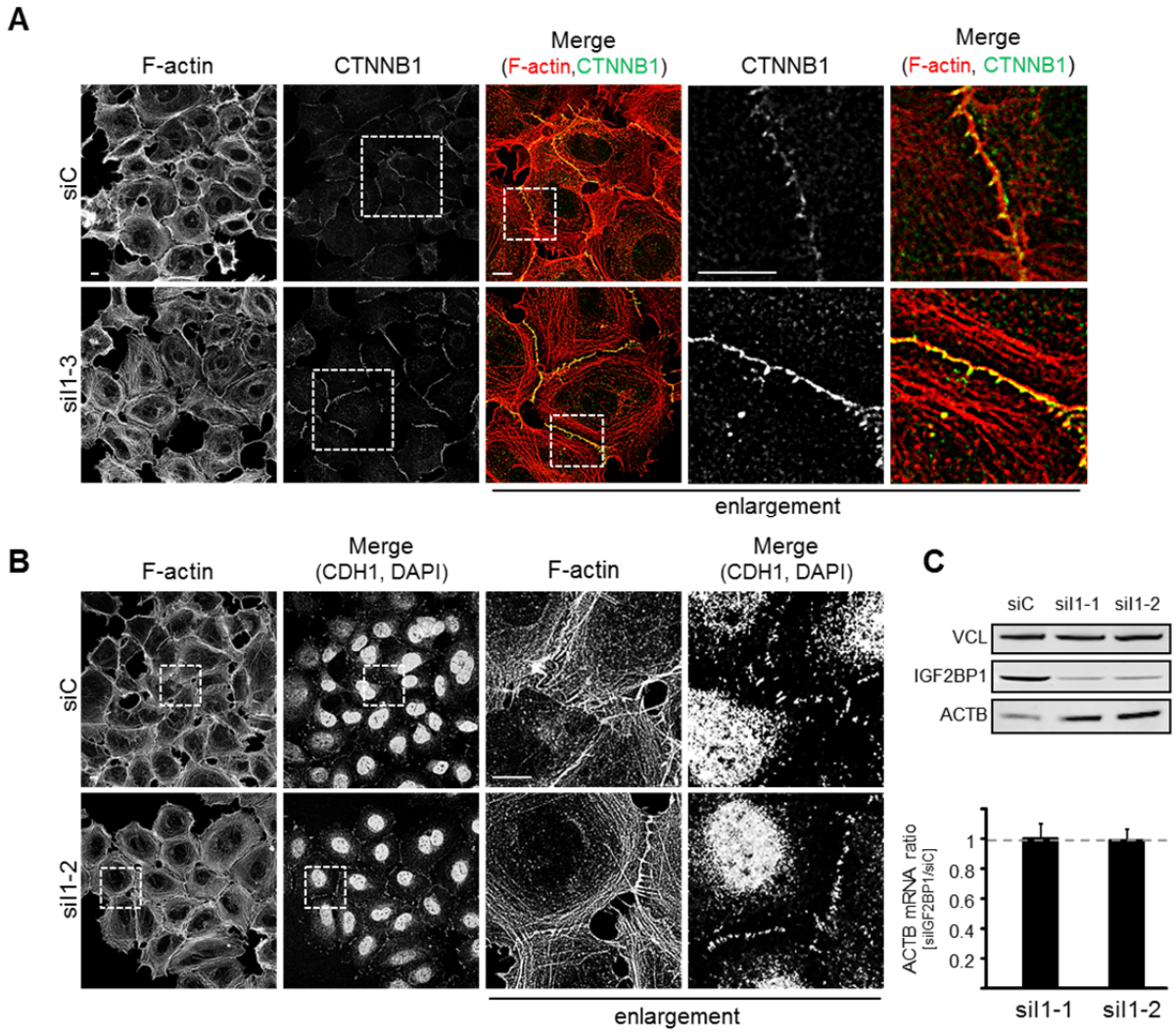


Figure 3.3: Cell-cell junction formation and ACTB protein synthesis are increased upon IGF2BP1 knockdown. (**A**, **B**) HEK293 cells were transfected with IGF2BP1-directed (siI1-2 or siI1-3) or control (siC) siRNAs for 72 h. The F-actin cytoskeleton and cell-cell contacts were analyzed by phalloidin labeling and immunostaining for CTNNB1 **(A)** or CDH1 **(B).** Where indicated nuclei were stained by DAPI. Enlargements of the boxed regions are shown in the right panels. Images were acquired using LSM-microscopy. Bars indicate 10 µm. **(C)** HEK293 cells were transfected with siRNAs targeting IGF2BP1 (siI1-1, siI1-2) or control siRNAs (siC) for 72 h. Protein abundance of ACTB upon IGF2BP1 depletion was monitored by Western blotting, mRNA levels by qRT-PCR. Error bars indicate standard deviation of at least three independent analyses.

Although CTNNB1 and CDH1 were recruited to cell contact sites only a modest increase of CDH1 mRNA and protein was observed (Figure 3.4A, B). In contrast, levels of CTNNB1 protein remained unchanged (Figure 3.4A). CTNNB1 mRNA level even decreased upon IGF2BP1 depletion (Figure 3.4B). This was surprising since previous reports indicated a role of IGF2BP1 in promoting CDH1 expression (Gu et al., 2012). Taken together these findings suggested that the siRNA mediated depletion of IGF2BP1 led to an increased expression of the epithelial

marker CDH1 and its recruitment to adherens junctions correlating to phenotypic changes reminiscent of a MET.

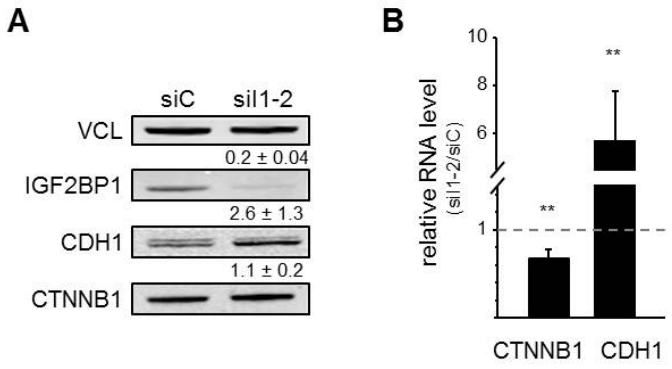


Figure 3.4: IGF2BP1 represses the expression of the epithelial marker CDH1 and promotes CTNNB1 RNA expression. (A, B) HEK293 cells were transfected with siRNAs targeting IGF2BP1 (sil1-2) or a control (siC). **(A)** Protein levels upon IGF2BP1 knockdown were determined by Western blotting using CDH1 and CTNNB1 directed antibodies relative to the control (siC) and normalized to VCL. **(B)** Relative RNA levels were analyzed by qRT-PCR with primers specific for CTNNB1 or CDH1. Error bars indicate standard deviation of at least three independent analyses. Statistical significance by Student's t-test: ** $p < 0.005$.

It remained to be investigated whether mesenchymal marker were also affected by the knockdown of IGF2BP1. In agreement with an enhancement of epithelial-like cell morphology upon IGF2BP1 depletion, the mesenchymal marker fibronectin (FN1) was significantly down-regulated on both, the protein and mRNA level compared to cells transfected with control siRNAs (Figure 3.5A, B). Note that FN1 protein is secreted by HEK293 cells into the cell culture medium and its abundance was therefore determined by ELISA. FN1, an extracellular matrix protein, is important for the migratory capacity, differentiation and adhesion of cells.

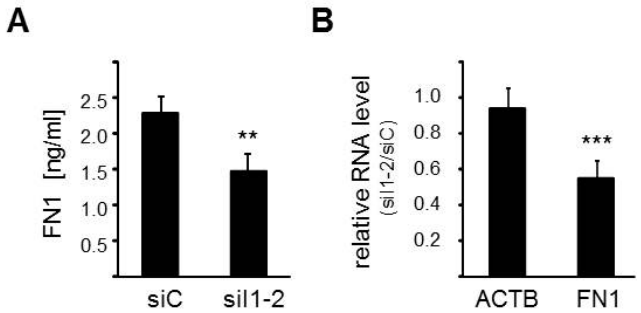


Figure 3.5: IGF2BP1 promotes the expression of the mesenchymal marker FN1. (A) Protein levels were analyzed by ELISA from HEK293 cell culture medium upon transfection of with IGF2BP1-directed or control siRNAs for 72 h. Cells were starved for 16 h in serum-free medium prior to the analyses. The cell number of each transfection was determined by flow cytometry and used for normalization. **(B)** RNA abundance of FN1 was monitored by qRT-PCR. Error bars indicate standard deviation of at least three independent analyses. Statistical significance was validated by Student's t-test: ** $p < 0.005$; *** $p < 0.0005$.

In summary the presented results indicated that IGF2BP1 sustained a mesenchymal character of HEK293 cells by promoting the expression of FN1 and on the other hand interfering with CDH1 synthesis. In consequence, cell-cell junction and cortical F-actin cytoskeleton formation was enhanced upon IGF2BP1 depletion correlating to an epithelial-like phenotype at least *in vitro* in HEK293 cells.

3.1.2 IGF2BP1 promotes mesenchymal gene expression in U2OS and ES-2 cells

IGF2BP1 was previously shown to regulate cell migration and F-actin cytoskeletal integrity in osteosarcoma-derived U2OS cells (Stohr et al., 2012). This was demonstrated to be mediated by a direct regulation of MAPK4 and PTEN by IGF2BP1 (Stohr et al., 2012). IGF2BP1 knockdown induced the cytoplasmic accumulation of MK5, induced by altered expression of MAPK4, and up-regulated phosphorylation of HSP27 at S78 and S82. This was correlated to a MAPK4/MK5-dependent increase of cellular G-/F-actin ratio which is sequestered by the heat shock protein HSP27 (Stohr et al., 2012). Cell motility is a key feature of mesenchymal cells. In contrast, epithelial cells due to an apical-basal rather than a front-back polarity and stronger inter-cellular junctions have less migratory potential. It was analyzed if changes in gene expression and cell morphology, as seen in HEK293 cells, were also observed in U2OS cells. In agreement with previously published observations the siRNA-directed depletion of IGF2BP1 led to a disturbance of the F-actin integrity resulting in a shortening of F-actin fibers analyzed by immunofluorescence studies (Figure 3.6A). In contrast, changes of the overall morphological of U2OS cells were only slightly observed. Again immunofluorescence staining showed an enrichment of CTNNB1 at cell borders but the overall protein abundance remained unaffected upon IGF2BP1 depletion (Figure 3.6A, B). CTNNB1 mRNA levels were moderately but significantly reduced upon IGF2BP1 knockdown correlating to previous findings suggesting IGF2BP1 regulates CTNNB1 mRNA stability (Figure 3.6B). Despite the insignificant impact on cell morphology the depletion of IGF2BP1 led to a reduction of FN1 protein as well as mRNA levels by approximately two-fold (Figure 3.6B). Vinculin (VCL, protein) and RPLP0 (mRNA) remained unaffected upon IGF2BP1 knockdown. In U2OS cells, in contrast to

HEK293 cells, FN1 protein abundance was monitored by Western blotting since U2OS cells express cell-associated FN1. Due to its low abundance CDH1 levels could not be evaluated in these cells.

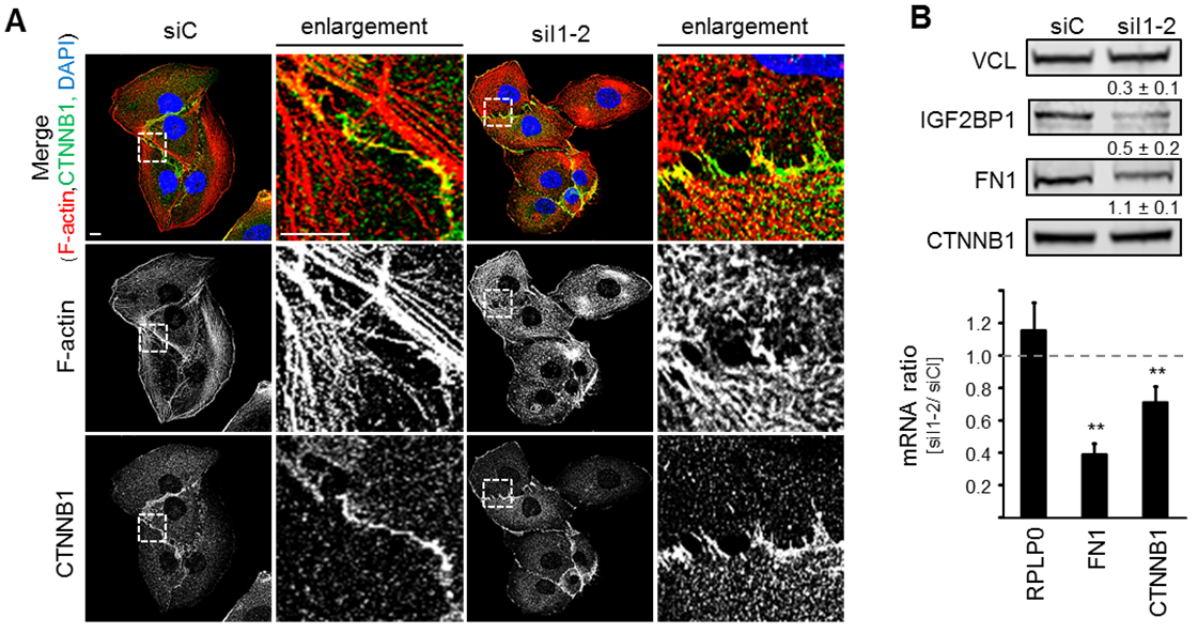


Figure 3.6: IGF2BP1 promotes FN1 expression in U2OS cells. U2OS cells were transfected with IGF2BP1-directed (sil1-2) or control (siC) siRNAs for 72 h. **(A)** CTNNB1 localization and F-actin architecture was analyzed by immunostaining for CTNNB1 and phalloidin labeling. Nuclei were stained by DAPI. Enlargements of boxed regions are shown in the right panels. Image acquisition was performed by LSM-microscopy. Representative images are shown. Bars indicate 10µm. **(B)** FN1 and CTNNB1 protein and RNA abundance was determined by Western blotting and qRT-PCR relative to the controls (siC). RPLP0 remained unaffected upon IGF2BP1 knockdown and served as a control RNA. A representative Western blot is shown. Error bars indicate standard deviation of at least three independent analyses. Statistical significance was validated by Student's t-test: ** $p < 0.005$.

IGF2BP1 was previously shown to regulate migration in the ovarian carcinoma-derived cell line ES-2 (Stohr et al., 2012). These cells have a fibroblastic morphology with few cell-cell contacts typical for mesenchymal cells and express high levels of IGF2BP1. In agreement with observation from HEK293 cells, the siRNA-mediated depletion of IGF2BP1 induced morphological changes with increased cell-cell contacts in these cells (Figure 3.7A). This correlated with a reduction of FN1 (RNA) levels. On the other hand CDH1 (RNA) increased significantly upon IGF2BP1 knockdown (Figure 3.7B). The data strongly supported the role of IGF2BP1 in sustaining a mesenchymal phenotype in different cell types.

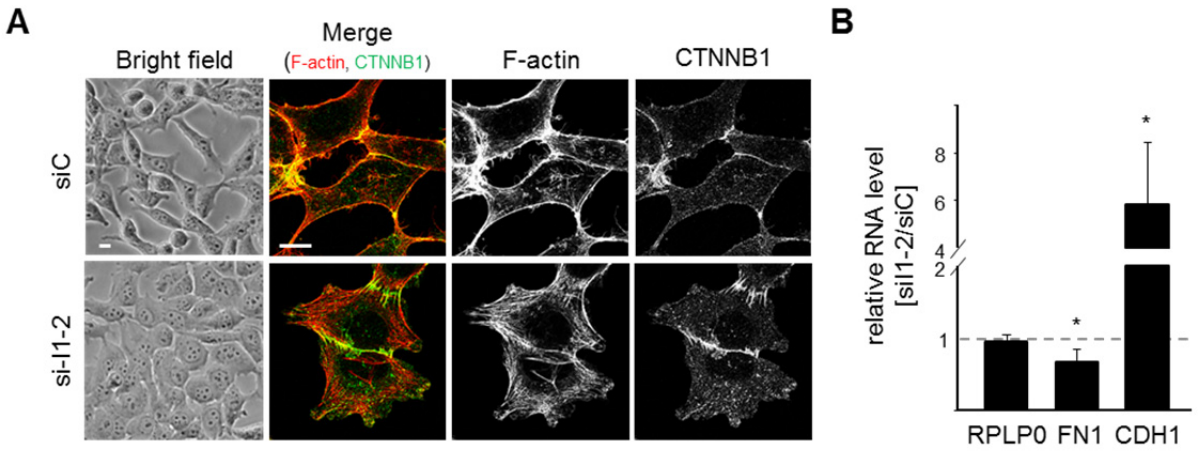


Figure 3.7: Depletion of IGF2BP1 induces morphological changes and altered marker expression in ES-2 cells. (A, B) ES-2 cells were transfected with siRNAs targeting IGF2BP1 (sil1-2) or controls (siC) for 72 h. **(A)** Morphology of the cells and subcellular protein localization was monitored by light microscopy, immunostaining for CTNNB1 and phalloidin labeling of the F-actin cytoskeleton. Bar indicates 10 µm. **(B)** mRNA levels were monitored by qRT-PCR. Statistical significance was determined by Student's t-test: * $p < 0.05$.

Taken together the data suggested that IGF2BP1 enhanced the expression of FN1 cell type independently indicating a similar mechanism in all analyzed cell lines. On the contrary IGF2BP1 interfered with CDH1 expression at least in HEK293 and ES-2 cells suggesting that the protein antagonized epithelial-like cell properties.

3.2 CTNNB1 is not a key driver of transcription in HEK293 cells

Previous studies suggested a role of IGF2BP1 in regulating CTNNB1 mRNA stability (Gu et al., 2008). It was furthermore proposed that IGF2BP1 expression itself is positively regulated via feedback regulation by CTNNB1 signaling (Gu et al., 2008; Noubissi et al., 2006). The WNT/CTNNB1 signaling pathway is involved in the regulation of various genes during development and tumorigenesis. In the past decades many studies suggested a role of cooperative functions of different pathways like TGFβ- and/or WNT/CTNNB1 in EMT. Accordingly, the positive regulation of mesenchymal markers like FN1 and on the other hand a negative regulation of epithelial markers like CDH1 have been proposed to be controlled by these signaling pathways (reviewed in: (Fuxe et al., 2010; Heuberger and Birchmeier, 2010; Lee et al., 2006; Nawshad et al., 2005)). Therefore it was of interest whether

IGF2BP1 could facilitate the regulation of EMT markers by modulating CTNNB1 signaling in HEK293 cells. Although the knockdown of IGF2BP1 led to a decrease of CTNNB1 mRNA levels the steady state protein amount remained largely unaffected (see 3.1.1). Moreover immunofluorescence studies indicated a re-localization to cell-cell contacts rather than an expected overall decrease of the protein. This suggested that the increase of CDH1 to adherens junction facilitated a recruitment of CTNNB1 to these sites. However residual endogenous nuclear CTNNB1 could be involved in transcriptional regulation. Thus it was examined whether the siRNA-mediated depletion of CTNNB1 resulted in similar changes of morphology and gene expression as observed upon IGF2BP1 knockdown. The depletion of CTNNB1 led to morphological changes different from those observed upon IGF2BP1 depletion. HEK293 cells became detached with less inter-cellular junctions. The morphology of the knockdown cells resembled a mesenchymal-like phenotype rather than the opposite (Figure 3.8A). Furthermore the abundance of IGF2BP1 as well as CDH1 on protein and mRNA levels remained unchanged upon CTNNB1 knockdown (Figure 3.8B, C). In contrast, FN1 protein levels were even slightly elevated and mRNA levels remained largely unaffected (Figure 3.8B, C).

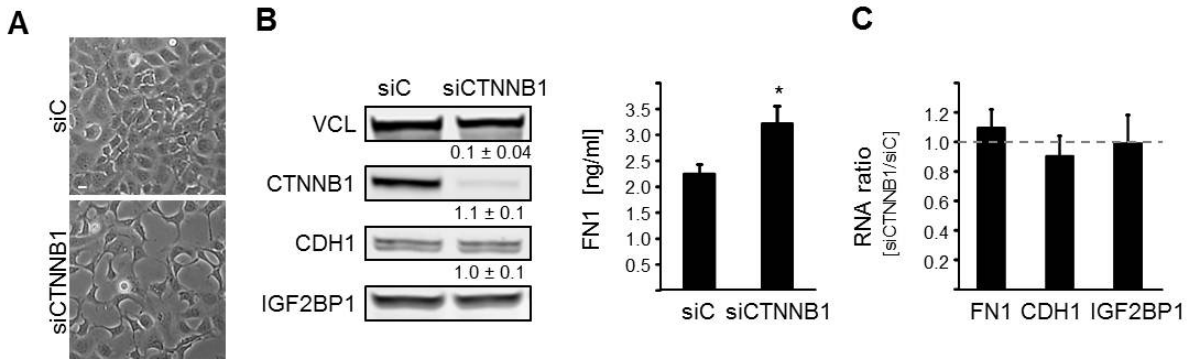


Figure 3.8: CTNNB1-dependent regulation of IGF2BP1, FN1 and CDH1 is insufficient in HEK293 cells. (A-C) HEK293 cells were transfected with CTNNB1-directed (siCTNNB1) or control (siC) siRNAs for 72 h. **(A)** Cellular morphology was monitored by light microscopy. Bar indicates 10 µm. **(B)** Protein abundance of CDH1 and IGF2BP1 was analyzed by Western blotting. FN1 levels were determined by ELISA as described in 3.5. **(C)** The mRNA levels of the indicated genes upon CTNNB1 knockdown were determined relative to control siRNA transfected cells. Statistical significance was validated by Student's t-test: * $p < 0.05$.

These observations were essentially the opposite of what was expected since CTNNB1 has been described, although in different cellular systems, to modulate

transcription of IGF2BP1, FN1 and CDH1 (Gradl et al., 1999; Gu et al., 2008; Huber et al., 1996). Nonetheless these findings did not reveal a conclusive mechanism for the MET-like changes detected upon IGF2BP1 depletion. However, they were in agreement with localization studies of CTNNB1 in HEK293 cells. Immunofluorescence staining exhibited that CTNNB1 was localized at cell-cell contacts in HEK293 cells rather in the nucleus (Figure 3.9A). Notably, the function of CTNNB1 as a transcriptional regulator strongly relies on its re-localization to the cell nucleus in consequence of the cytoplasmic stabilization of the protein. In contrast to HEK293 cells the colorectal carcinoma-derived cell line HCT116 contains N-terminal mutations in CTNNB1 resulting in a stabilization of the protein and thus a constitutive activity. Immunostainings revealed a localization of the protein at the cell membrane, in the cytoplasm as well as in the cell nucleus in these cells (Figure 3.9A). In HEK293 cells only the ectopic overexpression of a stabilized CTNNB1 mutant (CTNNB1-S33Y) resulted in a nuclear localization detectable by immunofluorescence staining compared to endogenous CTNNB1 in non-transfected cells (Figure 3.9A; CTNNB1-S33Y mutant, right panel).

These observations were furthermore supported by luciferase reporter analyses using Topflash and the corresponding mutated Fopflash reporter (Figure 3.9B). The reporters are commonly used to evaluate transcriptional response of CTNNB1 through its binding and activation of transcription factors like the T-cell factor/ LEF1-family members. The Topflash reporter comprises 7 TCF/LEF-binding sites in its promoter region (AGATCAAAGGgggta, TCF/LEF binding site in capital letters) which are mutated in the respective Fopflash plasmid. Endogenous activity measurements revealed an about 10-fold higher luciferase activity ratio of CTNNB1/TCF-LEF (Topflash) in HCT116 cells than in HEK293 cells compared to the corresponding control reporter (Fopflash). The Topflash luciferase activity in HEK293 cells was only enhanced by ectopic expression of the stabilized CTNNB1 mutant (CTNNB1-S33Y) whereas the T-cell factors TCF4 and LEF1 alone remained essentially ineffective. Although comprising binding sites for the TCF/LEF1 family these data indicated that, at least in this cell system, the Topflash reporter displayed CTNNB1 transcriptional activity rather than that of TCF4 and LEF1.

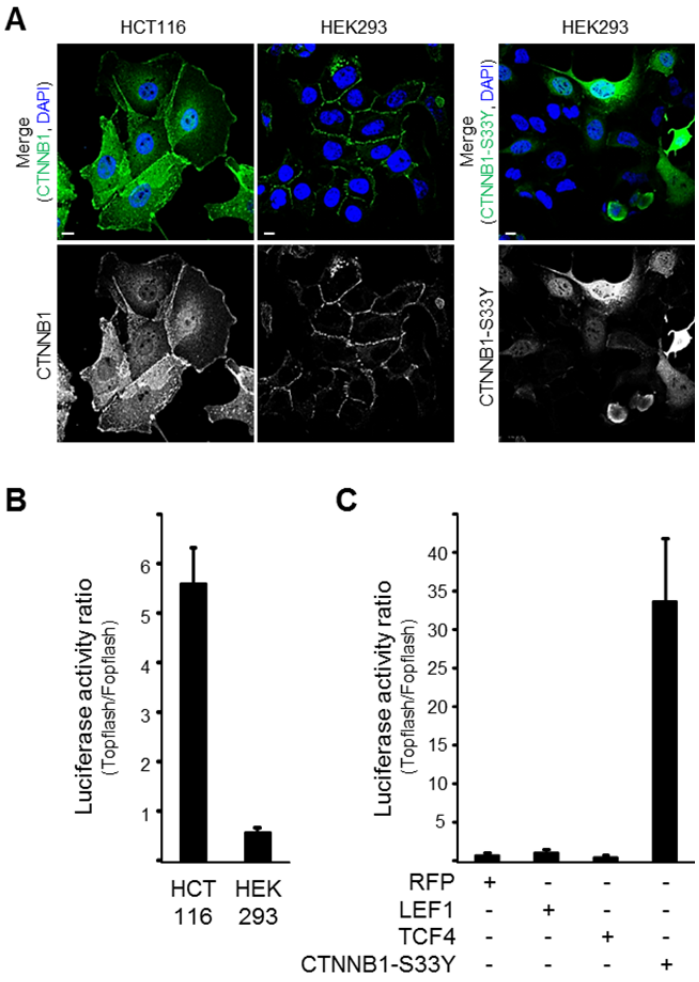


Figure 3.9: CTNNB1-dependent transcriptional activity is dispensable in HEK293 cells. (A) The localization of CTNNB1 was monitored in HCT116 and HEK293 cells by immunostaining and labeling of nuclei by DAPI. HEK293 cells were additionally transfected with a stabilized CTNNB1 mutant (S33Y) for 48 h. Image acquisition was performed by LSM-microscopy. Representative images are shown. Bars indicate 10 µm. **(B)** HCT116 and HEK293 cells were transfected with CTNNB1-responsive Topflash (TF) or the corresponding mutated Fopflash (FF) Firefly luciferase reporter for 30 h. A Renilla luciferase was co-transfected as internal normalization control. The normalized Topflash/Fopflash luciferase activity ratios were determined in each cell population using the DualGlo Luciferase assay. **(C)** To measure the activation of the Topflash reporter HEK293 cells were co-transfected with the aforementioned luciferase reporter in addition to the stabilized mutated CTNNB1 (S33Y) or the TCF-family members TCF4 and LEF1 encoded plasmids. TF/FF ratios were normalized to Renilla luciferase activities. Error bars indicate standard deviation of three independent analyses.

In summary one can conclude that CTNNB1 transcriptional activity was essentially negligible in HEK293 cells. Hence, IGF2BP1 modulated regulatory pathways involved in EMT/MET independently of CTNNB1 at least in this cell type.

3.3 IGF2BP1 regulates the stability of LEF1 mRNA

It remained to be investigated whether IGF2BP1 facilitated its role in promoting pro-mesenchymal cell properties via additional factors or pathways. The role of IGF2BP1 so far has been described in stabilizing its mRNA targets (e.g. MYC, PTEN) or interfering with its translation (e.g. ACTB, MAPK4). Therefore an up-

regulation of CDH1 mRNA and protein upon IGF2BP1 depletion has to occur indirectly via additional regulatory pathways presumably at the transcriptional level. A destabilizing effect on mRNA by IGF2BP1 has not been described, yet. In contrast a direct regulation of FN1 was possible and had to be further analyzed. The down-regulation of FN1 upon IGF2BP1 knockdown indicated that the RBP could directly modulate FN1 mRNA turnover.

In previous studies novel candidate targets of IGF2BP1 were identified by microarray analyses. This screening approach used the feature of selective stabilization of its targets during cellular stress (Stohr et al., 2012)). Among these newly identified targets the transcription factor LEF1 (lymphoid enhancer-binding factor 1) was highly down-regulated upon IGF2BP1 depletion (31.4-fold) and seemed to be a promising candidate to be further analyzed (Table 3.1). These microarray analyses also revealed a decreased expression of FN1 of about 4-fold upon IGF2BP1 knockdown in stressed U2OS cells (Table 3.1). The transcription factor LEF1 was described to be involved in the regulation of CDH1 and FN1 as well as other EMT-targets (Gradl et al., 1999; Jesse et al., 2010; Nawshad et al., 2007). Therefore it was analyzed if and how IGF2BP1 regulates LEF1 and FN1 expression in HEK293 cells.

Probe Set ID	Gene Symbol	fold decreased	Gene Title
243361_at	LEF1	31,4	lymphoid enhancer-binding factor 1
214702_at	FN1	4,4	fibronectin
1558199_at	FN1	4,0	fibronectin
214701_s_at	FN1	3,5	fibronectin

Table 3.1: **FN1 and LEF1 were down-regulated in response to IGF2BP1 depletion upon stress in U2OS cells.** Extracted results from microarray studies by Dr. Nadine Stöhr (Stohr et al., 2012) and unpublished data.

RNA interference (RNAi) was used to analyze if IGF2BP1 regulates LEF1 in HEK293 cells. The siRNA-directed depletion of IGF2BP1 with two different siRNAs led to a significant decrease of steady state protein and mRNA levels of LEF1 (Figure 3.10A). Multiple LEF1 transcripts can be found in databases. These isoforms result from alternative splicing (NM_016269, NM_001130713, NM_001130714, and NM_001166119). In HEK293 cells at least LEF1 isoforms 1-3 are expressed. Preferential expression of LEF1 using an intronic alternative promoter resulting in a CTNNB1-insensitive variant of LEF1 was also reported (Hovanes et al., 2001).

Multiple LEF1 polypeptides detected by Western blotting in HEK293 cells were all down-regulated upon IGF2BP1 knockdown indicating an isoform unspecific regulation. The qRT-PCR primers used in this study detected all isoforms. An association of the indicated mRNAs with IGF2BP1 protein was analyzed by RNA-immunoprecipitation (RIP) techniques. To stabilize RNA-protein complexes and prevent re-assembly in the process of immunoprecipitation a formaldehyde cross-link approach was used. The purified RNAs were analyzed by semi-quantitative- and quantitative RT-PCR. These analyses revealed an association of the two known targets ACTB and MYC (Figure 3.10B). Moreover a co-purification of the LEF1 RNA was observed indicating a direct binding of IGF2BP1 to this mRNA (Figure 3.10B). On the other hand no co-purification of PPIA, VCL and FN1 was observed (Figure 3.10B). Surprisingly, only a very small association with CTNNB1 mRNA was detected in semi-quantitative analyses but could not be conformed using quantitative RT-PCR (Figure 3.10B).

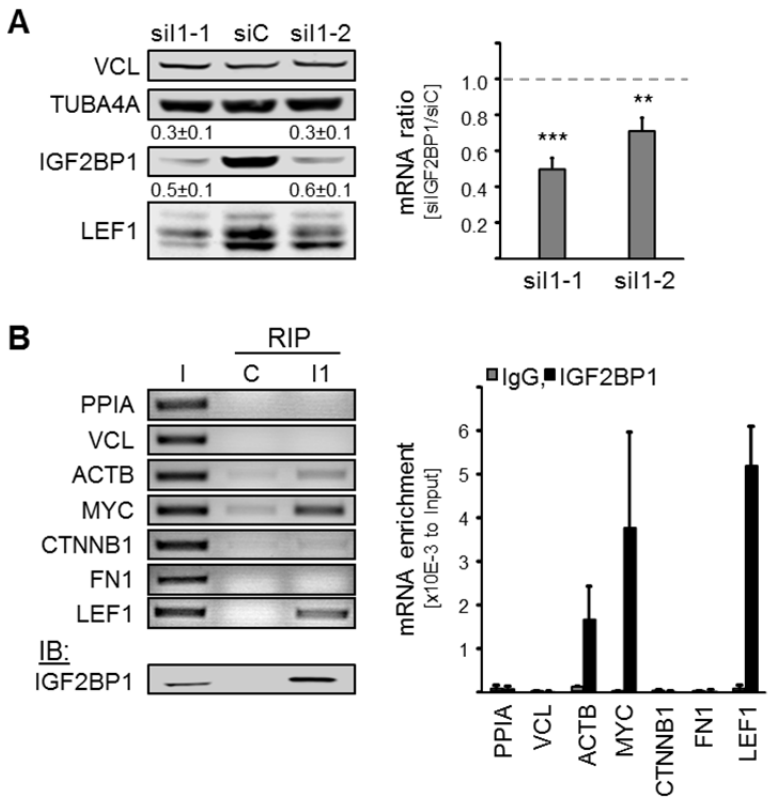


Figure 3.10: IGF2BP1 regulates LEF1. (A) Loss of function analyses were performed by transfecting HEK293 cells with IGF2BP1-directed (siI1-1, siI1-2) or control (siC) siRNAs for 72 h. LEF1 protein and mRNA level were analyzed by Western blotting and qRT-PCR. Protein abundance upon IGF2BP1 knockdown was determined relative to controls (siC) using VCL and TUBA4A for cross-normalization. Relative mRNA abundance was determined by the ΔΔCT-method, as described in 2.2.2.4. **(B)** RIP-studies were used to evaluate the association of IGF2BP1 with the indicated mRNAs. HEK293 cells (10^7) were harvested and subjected to formaldehyde fixation to stabilize *in vivo* RNA-protein interactions. Endogenous IGF2BP1 (I1) was immunopurified by a monoclonal antibody, as indicated by immunoblotting (IB). Co-purified RNAs were analyzed relative to the input fraction (I, equals 10% of the lysate) by semi-quantitative and quantitative real-time-PCR. Semi-quantitative analyses are shown as representative agarose gel, quantitative analyses as bar-diagram determined by the ΔC_t-method. Error bars indicate standard deviation of three independent measurements.

In summary, the data indicated that IGF2BP1 was directly associated with LEF1 mRNAs and regulated its turnover. In contrast, IGF2BP1 did not associate with the FN1 transcript suggesting an indirect regulation of FN1 by IGF2BP1. Furthermore no or only very weak binding of IGF2BP1 to CTNNB1 mRNA was observed providing evidence that endogenous IGF2BP1 is not a main regulator of CTNNB1 in HEK293 cells.

To support these findings, mRNA stability of LEF1 and FN1 transcripts was determined by decay analyses. mRNA turnover was monitored upon blocking transcription by actinomycin D (ActD) in cells transfected with control or IGF2BP1-directed siRNAs. Accordingly, LEF1 mRNA degradation was significantly enhanced upon IGF2BP1 depletion whereas RPLP0 and FN1 mRNA decay remained unaffected by the knockdown compared to the control siRNA transfected cells (Figure 3.11). The mRNA levels were determined over a time course of four hours. The mRNA half-life of LEF1 decreased from $t_{0.5}$= ~400 min in control cells to $t_{0.5}$ = ~200 min upon IGF2BP1 depletion.

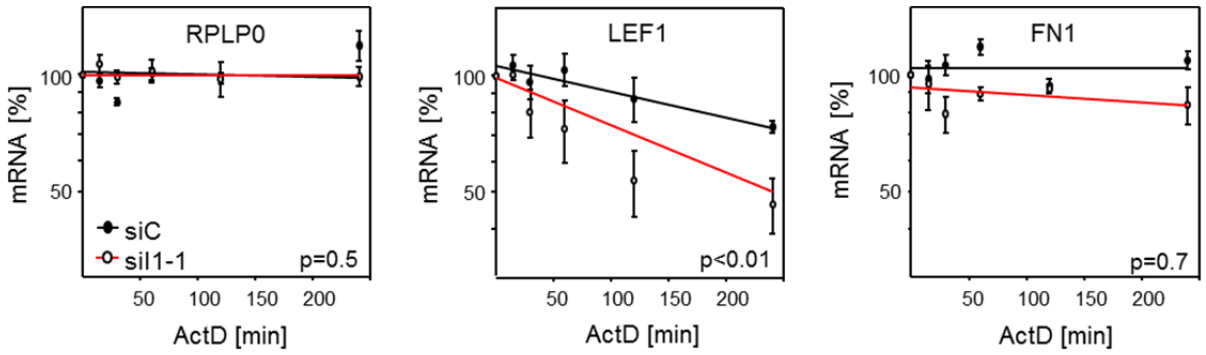


Figure 3.11: IGF2BP1 prevents turnover of LEF1 mRNAs. Decay analyses were performed by transfecting HEK293 cells with IGF2BP1-directed (sil1-1) or control (siC) siRNAs for 72 h. RNA synthesis was blocked using actinomycin D (ActD; 5µM) for indicated times. The abundance of RPLP0, LEF1 and FN1 mRNA levels was determined by quantitative RT-PCR relative to controls (siC). Input levels of transcripts were set to 100% and degradation of mRNAs is shown in semi-logarithmic scale. Standard deviation of three independent experiments is presented as error bars. Statistical significance was validated by Student's t-testing, depicted as p-values.

It still remained to be investigated which *cis*-element containing region of the LEF1 mRNA was essential for IGF2BP1 facilitated regulation. While it was proposed that IGF2BP1 stabilizes some of its target transcripts, e.g. MYC and PTEN via binding to regions of rare-codons within the coding sequence, for others like CD44 it was shown that degradation was prevented by binding to the 3' UTR (Lemm and Ross, 2002; Stohr et al., 2012; Vikesaa et al., 2006). It is suggested that tandem repetition of rare codons within the coding region of a transcript might interfer with translational efficiencies. At least for MYC it was shown that this rare codon enrichment led to enhanced endonucleolytic cleavage of the mRNA which is prevented by IGF2BP1 (IGF2BP1 in mouse = CRD-BP)(Lemm and Ross, 2002). Such an enrichment of rare codon repeats was not observed in the coding sequence of the LEF1 mRNA. However, recently published PAR-CLIP data suggested multiple binding site of IGF2BP1 in the 3'UTR of LEF1 (Hafner et al., 2010). Accordingly it was determined whether the siRNA-directed depletion of IGF2BP1 would influence luciferase reporter activity for those comprising the LEF1-3'UTR. Two alternative LEF1-3'UTR sequences were used for the analyses (Figure 3.12, left scheme). The published transcript variants 1, 2 and 4 (A: NM_016269, NM_001130713, NM_001166119) share the same 3'UTR whereas transcript variant 3 (B: NM_001130714) comprises a shorter 3'UTR due to alternative exon splicing. Nonetheless the sequence of this shorter 3'UTR can be found in all transcripts. Activity of the control reporter harboring the vector-encoded BGH-3'UTR resulted only in a modest decrease upon IGF2BP1 knockdown whereas the two LEF1-3'UTR-reporter activities were significantly reduced (Figure 3.12, right). These analyses were performed with two IGF2BP1-directed siRNAs strongly indicating that IGF2BP1 controls the stability of LEF1 mRNA via elements within its 3'UTR shared by all LEF1 isoforms.

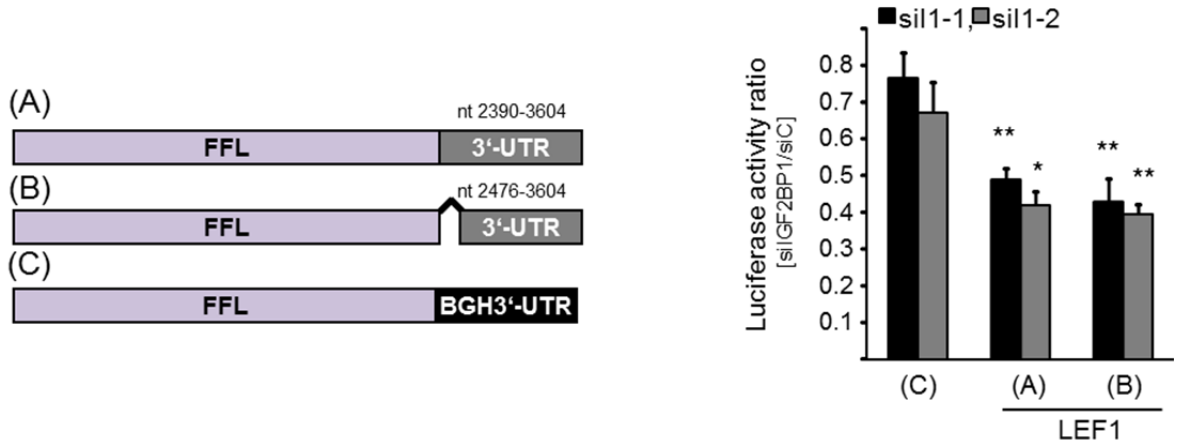


Figure 3.12: LEF1 is stabilized by IGF2BP1 in a 3'UTR dependent manner. HEK293 cells were transfected with two distinct siRNAs targeting IGF2BP1 or control siRNAs for 48 h before co-transfection of the indicated luciferase reporter for 24 h. A scheme of the used firefly luciferase reporter is depicted in the left panel. Luciferase reporter comprising two alternative LEF1 3'UTRs as well as a control reporter harboring the vector-encoded BGH 3'UTR were used for the analyses. A Renilla luciferase reporter served as an internal normalization control. Luciferase activities were determined by comparison of activities upon IGF2BP1 knockdown and the controls (siC) presented as activity ratio. Error bars indicate standard deviation of at least three independent analyses. Statistical significance was validated by Student's t-test: * $p < 0.05$; ** $p < 0.005$.

3.4 IGF2BP2 and IGF2BP3 do not control LEF1 mRNA turnover

The IGF2-mRNA binding proteins share a high homology of their domain structure which is essential for mediating their function. Therefore similar cellular functions could be assumed. Accordingly, it was determined wether the siRNA-mediated depletion of the two family members IGF2BP2 and IGF2BP3 control LEF1 expression in a similar manner. However the depletion of both proteins remained ineffective in regulating LEF1 mRNA steady state levels (Figure 3.13). This was furthermore supported by the findings that the activity of the shorter LEF1-3`UTR remained unchanged upon knockdown of IGF2BP2 and 3 (Figure 3.13). In summary, the data indicated that IGF2BP1 and not its family members IGF2BP2 and 3 was capable of controling the LEF1 mRNA turnover.

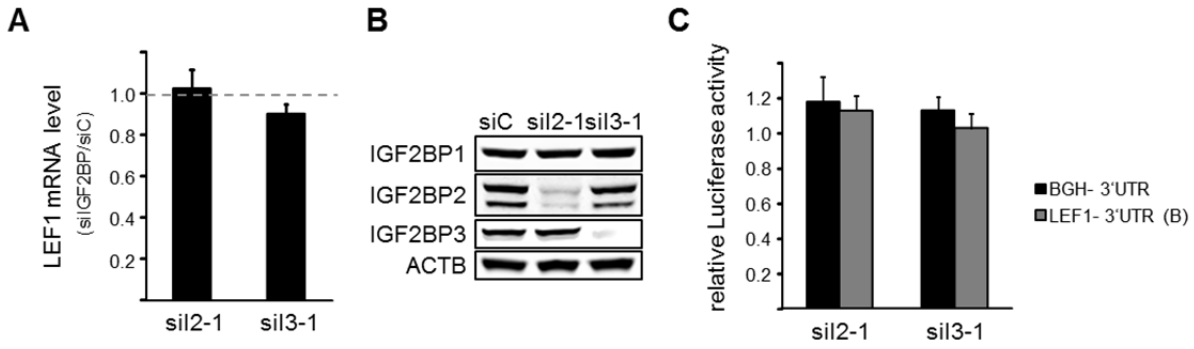


Figure 3.13: LEF1 mRNA levels remain unaffected upon IGF2BP2 and IGF2BP3 depletion. (A, B) HEK293 cells were transfected with IGF2BP2 (siI2-1) or 3 (siI3-1)-directed or control (siC) siRNAs for 72 h. **(A)** The abundance of LEF1 mRNA upon knockdown of IGF2BP2 or 3 was monitored by qRT-PCR relative to the controls (siC). **(B)** The knockdown efficiencies as well as the paralogue specificity of the siRNAs were evaluated by Western blotting using the indicated antibodies. Protein abundance of ACTB was used as loading control. **(C)** HEK293 cells were transfected with IGF2BP-directed (siI2-1 or siI3-1) or control siRNAs (siC) for 48 h followed by transfection of luciferase reporter plasmids for another 24 h. Luciferase activity analyses were performed as in figure 3.11. Mean values with standard deviation of at least three independent analyses are presented.

3.5 LEF1 expression is regulated by IGF2BP1 in U2OS and ES-2 cells

LEF1 was identified as a novel mRNA target of IGF2BP1 in U2OS cells using stressed conditions. It was demonstrated that IGF2BP1 prevented LEF1 mRNA degradation (Nadine Stöhr, PhD thesis 2008). The impact on cellular functions of such regulation remained elusive. Since we observed a down-regulation of FN1, a known target of LEF1-mediated transcriptional regulation, further investigations were performed. Accordingly, the knockdown of IGF2BP1 with two different siRNAs was analyzed regarding LEF1 expression in U2OS cells. In agreement with previous findings in HEK293 cells the siRNA-directed depletion of IGF2BP1 led to a significant decrease of LEF1 protein and mRNA (Figure 3.14A). U2OS cells express low level of endogenous IGF2BP1. A stable overexpression of ZBP1, the orthologous family member of IGF2BP1 from chicken, was used to evaluate a role in the regulation of LEF1 and FN1. This stable ZBP1 clone was previously described to express elevated levels of PTEN and reduced phosphorylation of HSP27 compared to the GFP clone (Stohr et al., 2012). In agreement with previous findings these cells revealed enhanced levels of LEF1 as well as FN1 proteins and mRNAs (Figure 3.14 B). Again CTNNB1 levels remained unchanged compared to the GFP-expressing cells.

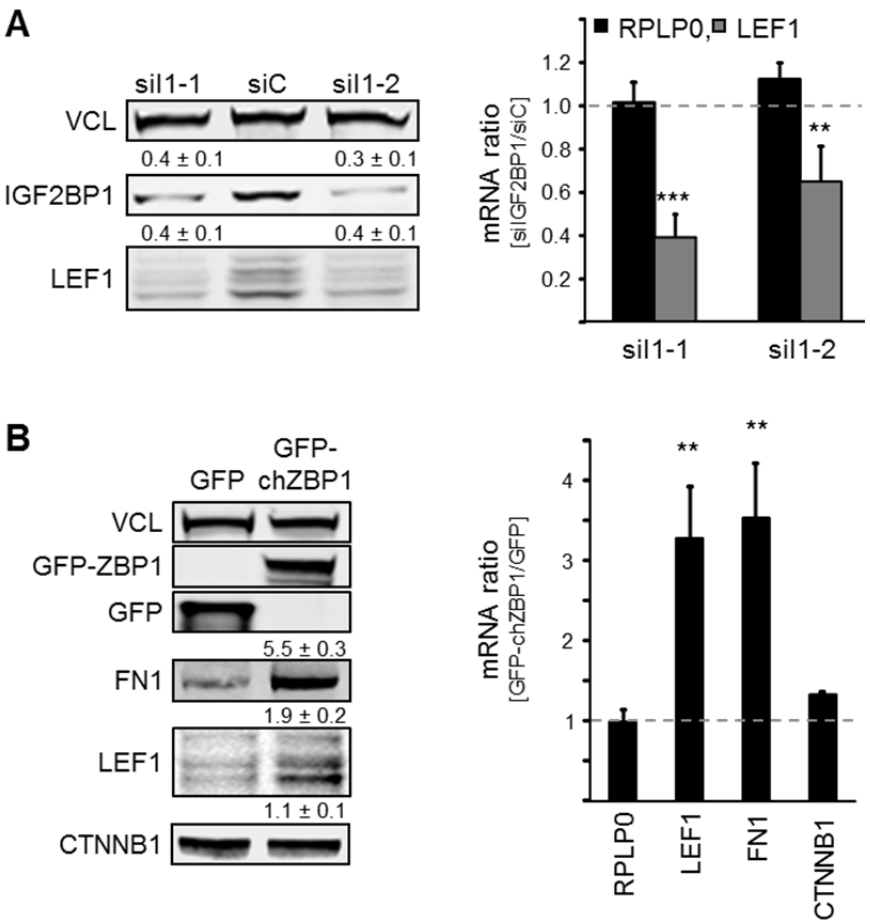


Figure 3.14: IGF2BP1 promotes LEF1 and FN1 expression in U2OS cells. (A) Loss of function analyses were performed by transfection of U2OS cells with two IGF2BP1-directed (siI1-1 or siI1-2) or control (siC) siRNAs for 72 h. Protein and RNA abundance of LEF1 upon knockdown was determined by Western blotting and qRT-PCR, respectively. **(B)** For gain of function analyses the chicken orthologue of IGF2BP1, GFP-chZBP1, was stably expressed as GFP-fusion protein in U2OS cells as previously described (Stohr et al., 2012). FN1 and LEF1 levels were determined on protein as well as mRNA levels by Western blotting and qRT-PCR relative to the GFP control. Error bars indicate standard deviation of at least three independent analyses that were used for quantification. Statistical significance was validated by Student's t-test: ** $p < 0.005$; *** $p < 0.0005$.

IGF2BP1 was demonstrated to promote migration and invasiveness in ovarian-carcinoma-derived ES-2 cells (Kobel et al., 2007; Stohr et al., 2012). To correlate a pro-mesenchymal function of IGF2BP1 with the regulation of target gene expression additional analyses in this cell type were performed. In agreement with findings from HEK293 and U2OS cells the transcription factor LEF1 was also significantly down-regulated on protein and mRNA level in response to IGF2BP1 depletion in ES-2 cells (Figure 3.15A, B). In contrast, CTNNB1 protein levels again remained unchanged (Figure 3.15A). In conclusion, these findings supported the role of IGF2BP1 in the control of LEF1 expression in different cell types.

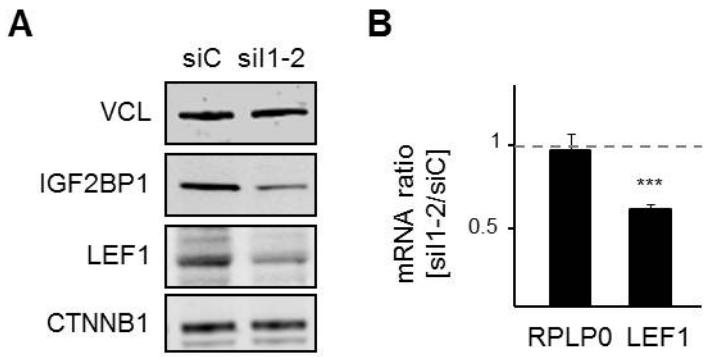


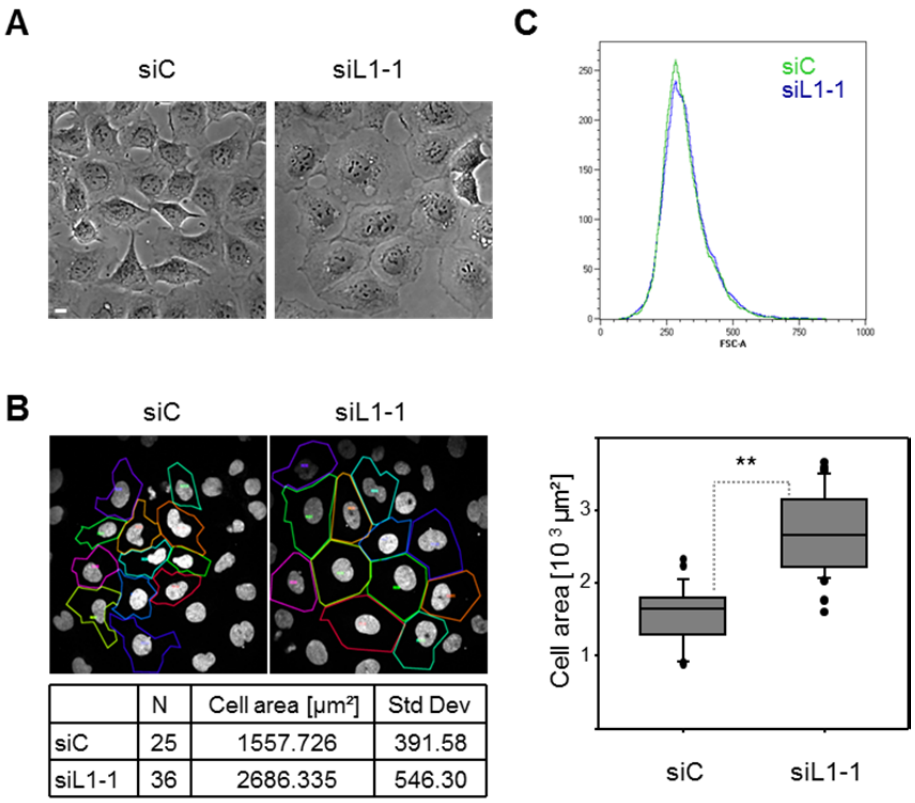
Figure 3.15: IGF2BP1 depletion leads to decreased expression of LEF1 in ES-2 cells. (A, B) ES-2 cells were transfected with IGF2BP1-directed (siI1-2) or control (siC) siRNAs for 72 h. **(A)** Protein abundance of LEF1 and CTNNB1 was determined by Western blotting using the indicating antibodies. **(B)** mRNA levels of LEF1 and RPLP0 were analyzed by qRT-PCR and depicted relatively to the controls (siC). Mean values with standard deviation of at least three independent analyses are presented. Statistical significance by Student's t-test: *** $p < 0.0005$.

3.6 LEF1 promotes a mesenchymal cell phenotype

The data presented so far suggested that IGF2BP1 sustains the expression of mesenchymal markers which become up-regulated during EMT. In contrast the expression of the epithelial marker CDH1 was enhanced upon IGF2BP1 depletion. Furthermore the mRNA of the transcription factor LEF1 was directly stabilized by IGF2BP1 in 3' UTR-dependent manner. Accordingly, it was investigated whether the expression of LEF1 affected mesenchymal cell properties in HEK293 cells. In previous studies LEF1 has been described to regulate transcription of many genes like CDH1, FN1 and SNAI2 which were shown to be essentially involved in EMT. These regulatory processes have been described to rely on signaling pathways like TGFβ and WNT/CTNNB1.

RNAi studies revealed that the siRNA-directed depletion of LEF1 induced similar morphological changes as observed for IGF2BP1 knockdown in HEK293 cells. The overall morphology was monitored by light microscopy (Figure 3.16A). Cells looked flattened with increased surface attached to the cell culture dishes. This increase in cell size was confirmed by analyzing the cell area of the cells by LSM microscopy and was in similar range as observed upon IGF2BP1 depletion (Figure 3.16B). Cell volume remained unchanged upon LEF1 knockdown indicating a cell

spreading rather than growth effect based on increased cell mass as observed upon IGF2BP1 depletion (Figure 3.16C).



	N	Cell area [µm²]	Std Dev
siC	25	1557.726	391.58
siL1-1	36	2686.335	546.30

Figure 3.16: LEF1 sustains a mesenchymal cell character of HEK293 cells. (A-C) HEK293 cells were transfected with LEF1-directed (siL1-1) or control (siC) siRNAs for 72 h. **(A)** The morphology of the cells was monitored by light microscopy. Bar indicates 10 µm. **(B)** The size of adherent cells was analyzed upon F-actin labeling by phalloidin as well as immunostaining of CTNNB1. Adherent cells were traced by manual labeling using CTNNB1-defined borders. The number of analyzed cells (N), mean cell area (μm^2) and standard deviation of cell size (μm^2) are presented in the table. Image acquisition was performed by LSM-microscopy. Additionally, cell area values are presented as box plots as described in 3.2. **(C)** Cells were transfected with the indicated siRNA and harvested by tryptic digestion after 72 h. The volume of cells was determined by flow cytometry using forward scattering by the MACSQuant (Miltenyi) software. Statistical significance was validated by Student's t-test: ** $p < 0.005$.

Subsequently, it was analyzed whether a re-localization or changes in the expression pattern of distinct markers was observed in consequence of depleting LEF1. Immunofluorescence studies demonstrated an enrichment of CTNNB1 as well as CDH1 at cell borders correlating to increased formation of cell-cell junctions in response to LEF1 knockdown (Figure 3.17A, B). However the F-actin cytoskeleton remained unchanged (Figure 3.17A, B) suggesting that increased cortical F-actin as observed upon IGF2BP1 knockdown was due to further regulatory functions of IGF2BP1 like interfering with ACTB or MAPK4 translation.

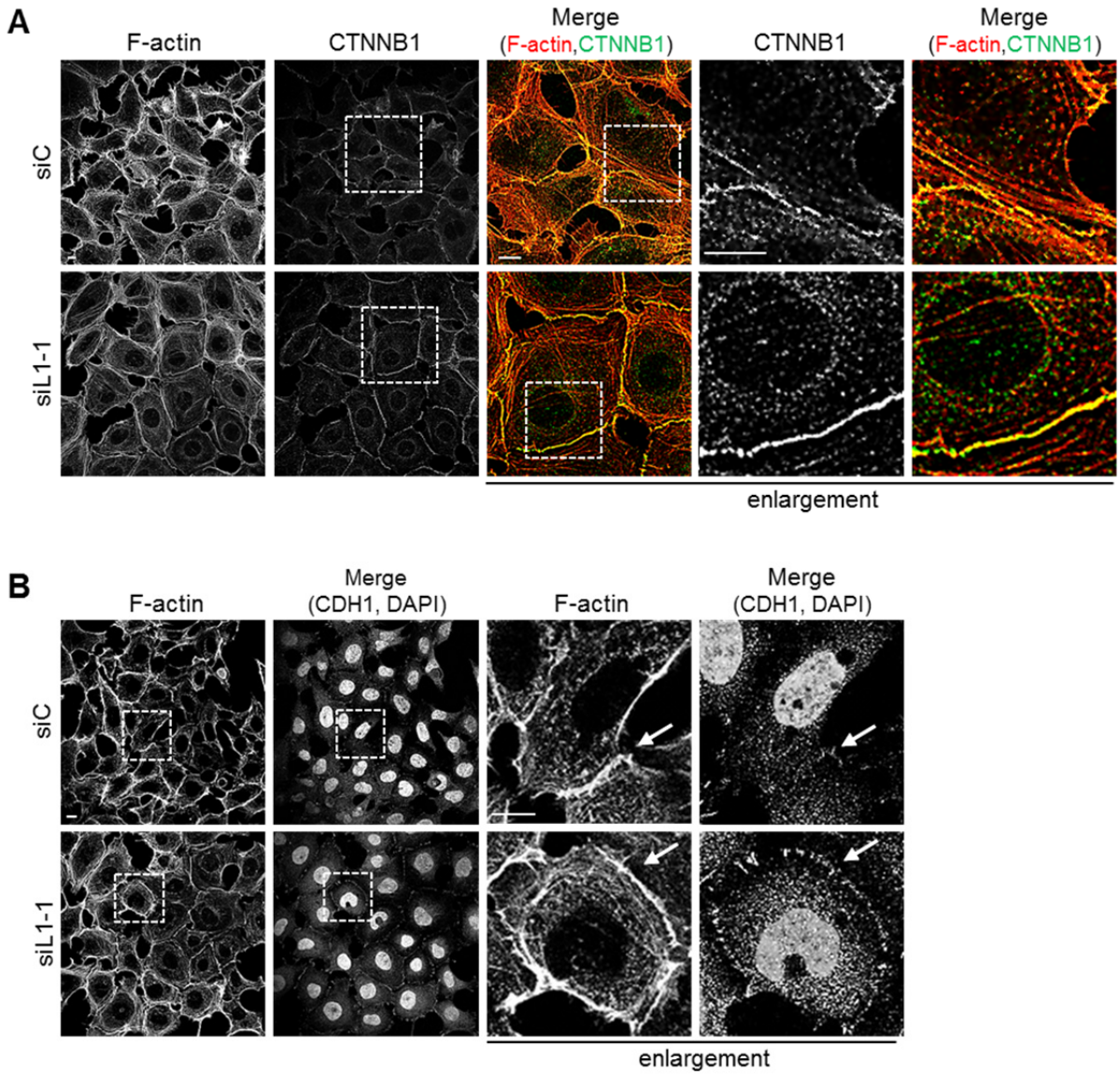


Figure 3.17: LEF1 depletion induces MET-like changes of cell morphology. (A, B) HEK293 cells were transfected with siRNAs targeting LEF1 (siL1-1) or a control (siC) for 72 h. The F-actin cytoskeleton and cell-cell contact formation was analyzed by phalloidin labeling and immunostaining for CTNNB1 (A) or CDH1 (B). Where indicated nuclei were stained by DAPI. Enlargements of the boxed regions are shown in the right panels. Image acquisition was performed by LSM-microscopy. Arrows indicate cell-cell border. Bars indicate 10 µm.

The abundance of marker proteins for a mesenchymal versus an epithelial state was further analyzed using a loss of function approach in HEK293 cells. This revealed a modest up-regulation of CDH1 on mRNA and protein level (Figure3.18A, B). In agreement with a pro-mesenchymal function the depletion of LEF1 led to a significant decrease of the mesenchymal marker FN1 on protein and mRNA level (Figure 3.18A, C).

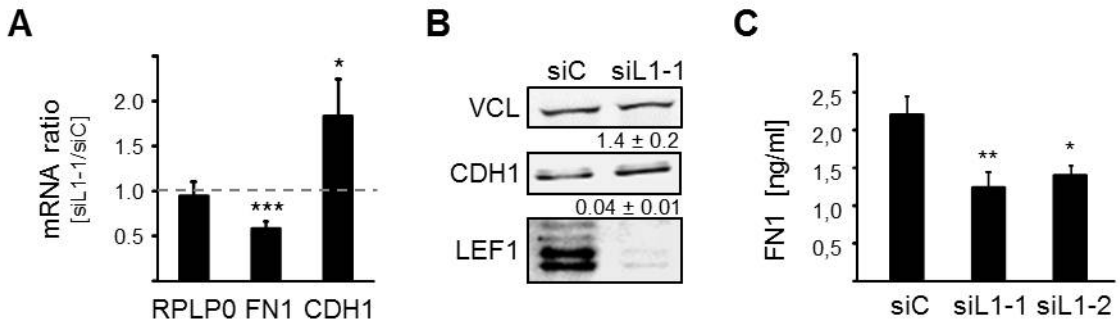


Figure 3.18: LEF1 depletion modulates CDH1 and FN1 expression. (A, B) HEK293 cells were transfected with the indicated LEF1-directed (siL1-1 or siL1-2) or control (siC) siRNAs for 72 h. **(A)** mRNA abundance of FN1 and CDH1 upon LEF1 knockdown was assessed by qRT-PCR relative to controls. **(B)** Western blotting was used to determine protein levels of CDH1 upon LEF1 depletion relative to controls (siC). **(C)** FN1 protein levels were analyzed by ELISA using two different LEF1-directed siRNAs (siL1-1, siL1-2). FN1 protein concentration was normalized to cell number determined by flow cytometry. Mean values with standard deviation of at least three independent analyses are presented. Statistical significance by Student's t-test: * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$.

The LEF1-dependent regulation of FN1 was confirmed in U2OS cells using two distinct LEF1-directed siRNAs. Here again a significant decrease of both FN1 protein and RNA levels (Figure 3.19A) was observed in response to LEF1 depletion. The stable overexpression of the longest LEF1 variant (corresponds to NM_016269) on the other hand induced an up-regulation of FN1 in U2OS (Figure 3.19B). This indicated that LEF1 regulated the expression of FN1 in HEK293 and U2OS cells.

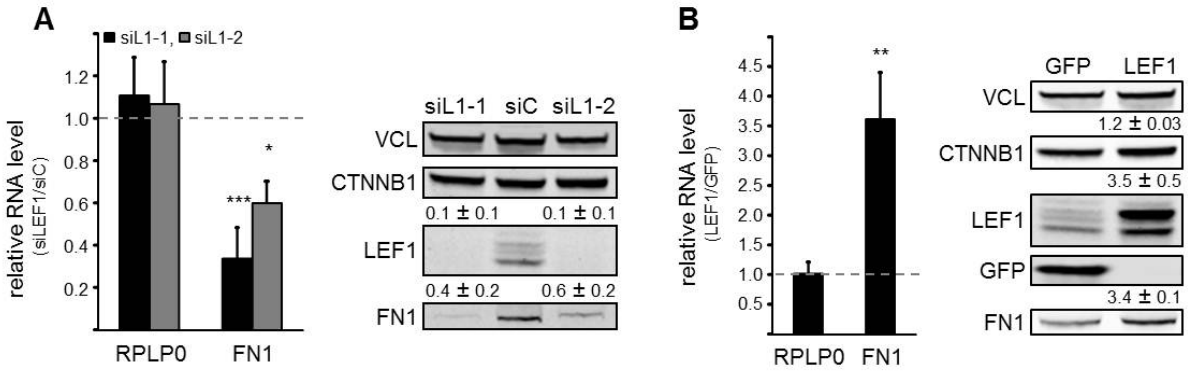


Figure 3.19: LEF1 controls FN1 expression in U2OS cells. (A) For loss of function analyses U2OS cells were transfected with two LEF1-directed (siL1-1 or siL1-2) or control (siC) siRNAs for 72 h. RNA abundance of FN1 upon LEF1 knockdown was assessed by qRT-PCR relative to controls (siC) Western blot analyses were used to determine FN1 protein levels upon LEF1 depletion relative to controls (siC) normalized to VCL. CTNNB1 levels remained unaffected. **(B)** Gain of function analyses were performed by stable transfection of LEF1 using lentiviral transduction. Changes in FN1 mRNA and protein abundance were determined as in (A). Statistical significance was validated by Student's t-test: * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$.

In summary, the data suggested that the knockdown of LEF1 induced the recruitment of CDH1 to cell –cell contacts with a moderate overall increase of the protein correlating to an enhancement of epithelial-like cell features. In agreement LEF1 was demonstrated to promote FN1 expression, a mesenchymal marker protein. This furthermore implied that IGF2BP1 could regulate epithelial-mesenchymal-like trans-differentiation by the control of LEF1 mRNA stability. It remained to be investigated how LEF1 mediates the regulation of FN1 and CDH1.

3.7 LEF1 promotes fibronectin transcription

Previous studies indicated a role of LEF1 in the transcriptional regulation of FN1 in Xenopus (Gradl et al., 1999). Others suggested a role of CTNNB1-dependent transcriptional activation of FN1 for LEF1 isoform 2, which is lacking exon VI (NM_001130713), in pancreatic carcinoma derived cells (Jesse et al., 2010). Yet, the transcriptional regulation of the human FN1 gene by LEF1 remained elusive. To address this question, Flag-tagged LEF1 was transiently overexpressed in HEK293 cells. The enforced expression of LEF1 led to a significant up-regulation of approximately two-fold of FN1 mRNA and protein compared to the GFP/Flag-controls (Figure 3.20B, C). In contrast to previous reports the overexpression of the LEF1 isoform 2 (LEF1-ΔExon VI) did not sufficiently enhance FN1 mRNA abundance (Figure 3.20C).

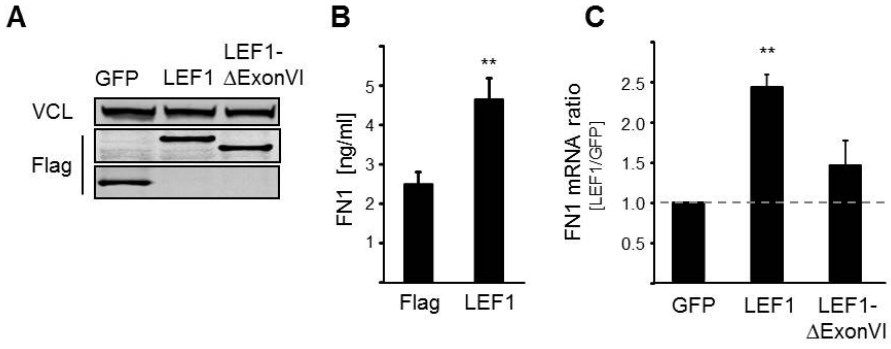


Figure 3.20: LEF1 overexpression enhances FN1 expression in HEK293 cells. (A-C) Gain of function analyses were executed by transient expression of Flag-LEF1 (LEF1 or LEF1ΔExonVI) and Flag-GFP or Flag-pcDNA3.1 as controls for 48 h. **(A)** Successful overexpression was monitored by Western blotting using monoclonal anti-Flag antibody. VCL served as a loading control. **(B)** Protein abundance of FN1 was determined by ELISA as described before. **(C)** FN1 mRNA levels upon LEF1 or LEF1ΔExonVI overexpression were analyzed by qRT-PCR relative to the control (GFP). Statistical significance of three independent analyses was validated by Student's t-test: ** $p < 0.005$.

The putative minimal promoter of the human FN1 gene was *in silico* predicted using the Promoter scan database Proscan (http://www-bimas.cit.nih.gov/molbio/proscan) upstream of the starting ATG of FN1 (Chr.2q34). A minimal promoter of approximately 1 kb was suggested which was used for luciferase reporter analyses. A second database called PROMO identified 5 putative binding sites for LEF1 within this FN1 promoter sequence (Farre et al., 2003; Messeguer et al., 2002). The sequence of the minimal promoter with the highlighted LEF1 binding sites is presented in Figure 3.21A. Luciferase promoter reporter studies were performed using the predicted minimal FN1 promoter (FN1-839) cloned into a promoterless firefly luciferase vector (pGL4.21) (Figure 3.21A, C). The promoter activity was analyzed in response to IGF2BP1- and LEF1 depletion. These loss-of-function studies revealed that the luciferase activity of the promoter reporter (FN1-839) was decreased upon IGF2BP1 and LEF1 knockdown (Figure 3.21B). FN1 promoter activity was further analyzed in response to LEF1 or RFP overexpression. Subsequent shortening of the FN1-839 reporter was used to identify the LEF1 responsive site of the promoter reporter. The fragments were created in a way that each shorter reporter was lacking one LEF1 binding site compared to the previous longer one. A Renilla luciferase served as an internal control and was used for normalization. Luciferase promoter activity, when co-expressing Flag-RFP, was significantly higher in cells transfected with the reporter FN1-689, FN1-739, FN1-789 and FN1-839 reporter compared to the shorter FN1-559, FN1-282 and FN1+1 reporter (Figure 3.21C). The data suggested that endogenous levels of LEF1 are sufficient to activate the FN1 promoter when comprising LEF1 binding site 4 as demonstrated by knockdown studies of the longest FN1 promoter reporter (Figure 3.21B). The overexpression of Flag-LEF1 led to a further significant increase of luciferase activity of the reporters FN1-689 to FN1-839 (Figure 3.21C). Luciferase activity was essentially abolished for the FN-559 reporter indicating that LEF1 binding site 4 was necessary for proper promoter activation. In conclusion, the obtained data showed that LEF1 is capable of activating the human FN1 reporter *in vitro*.

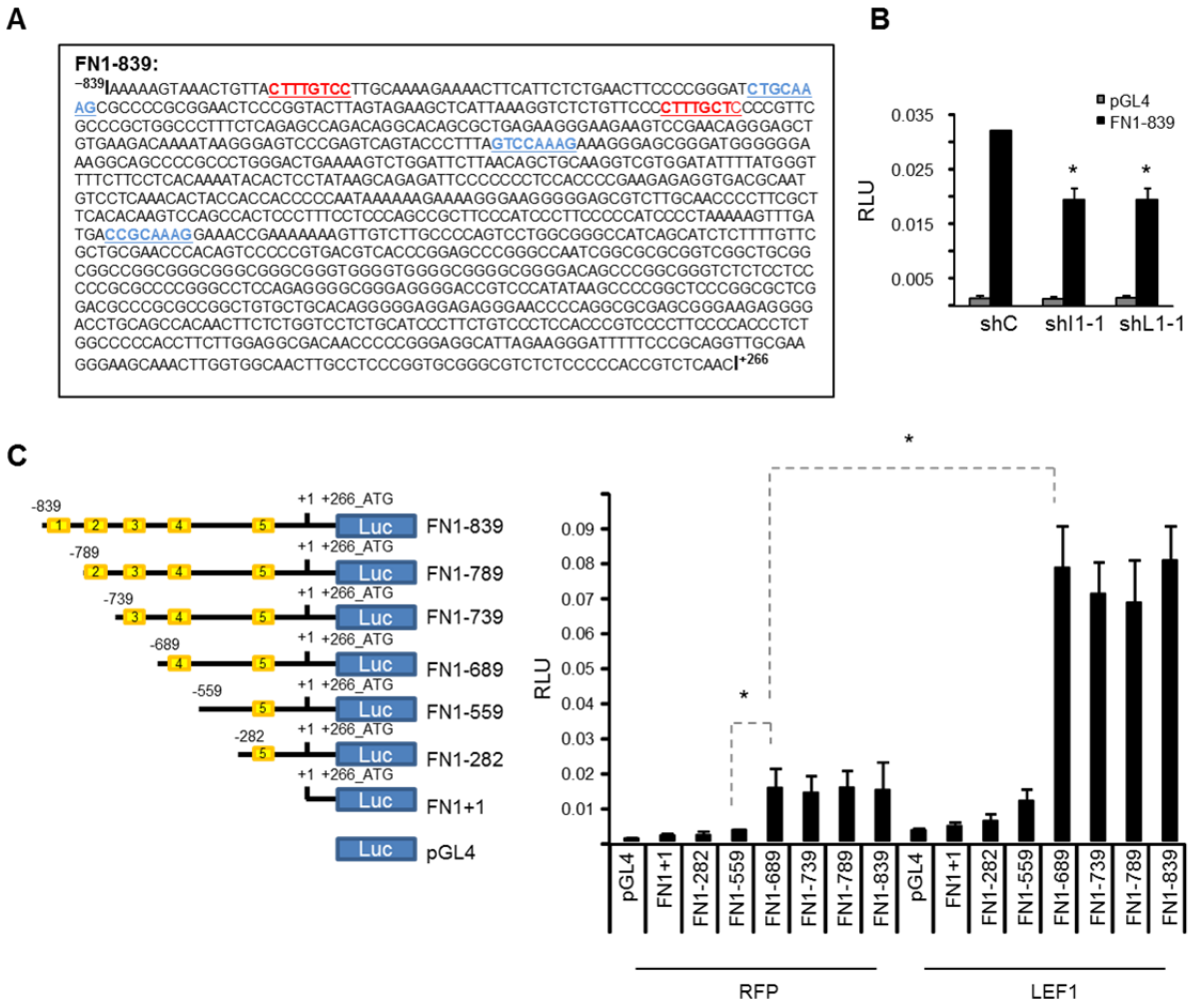


Figure 3.21: LEF1 regulates FN1 promoter activity. (A) The sequence of the putative minimal promoter of FN1 predicted by Proscan database is shown in the box. The putative LEF1 binding sites predicted by PROMO database are highlighted in blue (sense) and red (antisense). It spans approximately 1kb upstream of the starting ATG of the FN1 open reading frame (FN1-839). **(B)** HEK293 cells were transfected with control-, IGF2BP1- or LEF1-directed sh-plasmids and pGL4.21 or FN1-839 firefly luciferase plasmids for 48 h. **(C)** Gain of function analyses were performed by transfection of Flag-LEF1 or Flag-RFP and the indicated luciferase reporter for 30 h. The scheme indicates the constructs used for promoter studies including putative LEF1 binding sites (yellow boxes). The empty pGL4.21 vector served as control firefly reporter for background activity. The firefly activities were normalized to co-transfected Renilla activities and presented as relative luciferase units (RLU). Mean values with standard deviation of three independent analyses are shown. Statistical significance was validated by Student's t-test: * $p < 0.05$.

In order to map the LEF1 binding site responsible for FN1 promoter activity a region of 8 nucleotides containing the LEF1 consensus motif was deleted from the FN-839 reporter. However the deletion of this LEF1 binding sites in the full length minimal promoter (FN1-LEFΔ4) was still activated by the transient overexpression of LEF1 (Figure 3.22). It is very likely that the existing LEF1 binding sites (1-3) in this

reporter can substitute for the missing binding site 4. The sequences of the putative binding sites are highly similar as seen in Figure 3.21A.

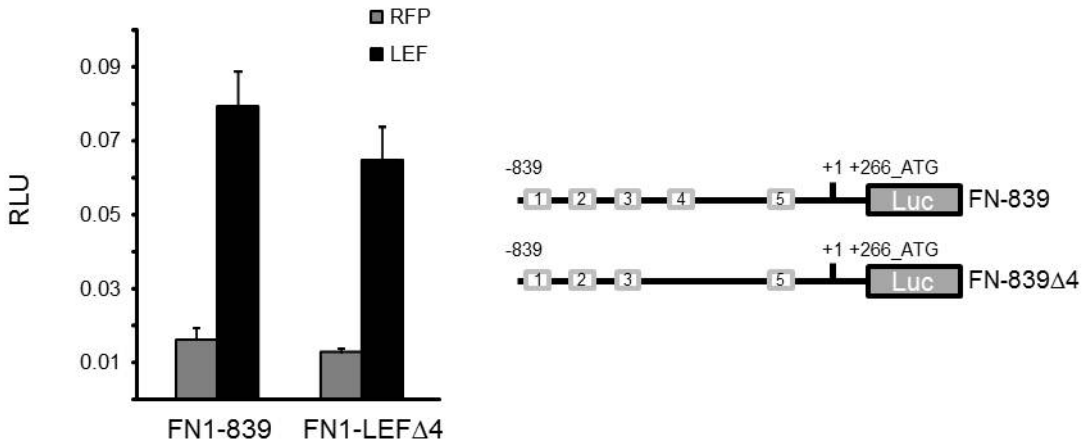


Figure 3.22: Deletion of LEF1 binding site 4 did not abolish promoter activation. A scheme of the used constructs is shown on the right. Promoter studies were performed as in 3.21. Luciferase activities are depicted as relative luciferase units (RLU). The promoter fragment covering the full minimal promoter (FN1-839) was used as positive control. Error bars indicate standard deviation of three independent measurements.

To evaluate whether LEF1 also binds to the human FN1 promoter *in vivo* chromatin immunoprecipitation (ChIP) experiments were performed. ChIP analyses revealed that LEF1 was *in vivo* associated with multiple sequences located in the FN1 promoter region (Figure 3.23). Primers were used for PCR that flank the putative LEF1 binding sites 1-4. An enrichment of the respective DNA sequences of about 10-fold was observed for LEF1 and also for the positive control Histon H3. An intergenic sequence was only enriched upon Histon H3 immunoprecipitation but not with LEF1 indicating the specific binding of LEF1 to regions within the FN1 promoter.

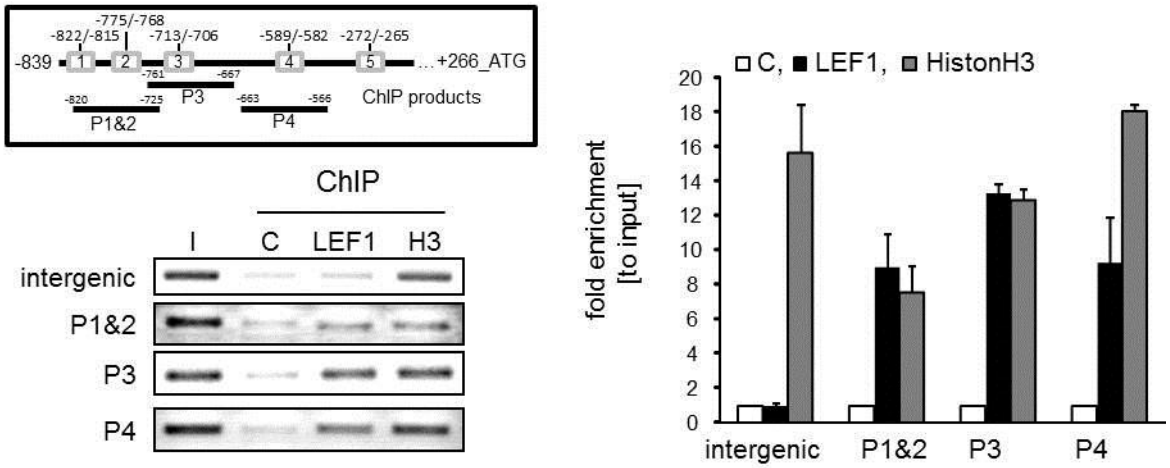


Figure 3.23: LEF1 binds to the FN1 promoter *in vivo*. Scheme indicates the positions of the analyzed PCR products. Binding of endogenous LEF1 protein to the human FN1 promoter in HEK293 cells was assessed by chromatin immunoprecipitation (ChIP). Cross-linked lysate was subjected to immunoprecipitation (IP) using anti-LEF1, anti-Histon H3 or mouse IgG antibodies. The Histon (H3) IP was used to confirm general association with chromatin (positive control). IgG-agarose was used to monitor unspecific binding (negative control). The association of LEF1 with binding sites in the FN1 promoter (P1&2, P3 or P4) or an intergenic control was analyzed by semi-quantitative as well as quantitative PCR. The enrichment of the indicated DNA fragments was determined relative to the input fraction (2% of lysate) normalized to the IgG control by the ΔC_t-method. Error bars indicate standard deviation of three independent ChIPs.

The LEF1-mediated regulation of FN1 expression was finally tested by RNA-recovery analyses. The shRNA-directed depletion of IGF2BP1 reduced the FN1 RNA levels to about 70% residual RNA (lane 3) compared to controls (shC+Flag, lane 1). FN1 mRNA levels upon IGF2BP1 knockdown (lane 3) were essentially recovered by co-expression of LEF1 (lane 4) to levels comparable with co-transfection of a shC-plasmid (lane 2) (Figure 3.24).

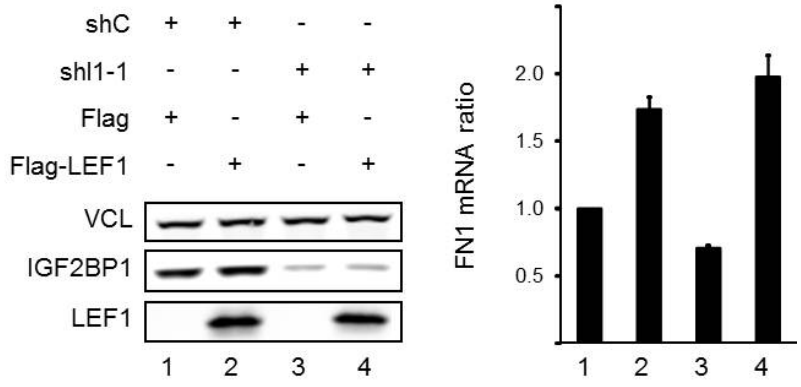


Figure 3.24: LEF1 recovers FN1 mRNA upon IGF2BP1 depletion. HEK293 cells were transfected with control (shC) or IGF2BP1-directed (shI1-1) shRNAs and Flag-pcDNA3.1 or Flag-LEF1 plasmids for 72 h indicated by numbers (1-4). The knockdown of IGF2P1 and the overexpression of LEF1 were monitored by Western blotting using the indicated antibodies. FN1 mRNA expression was analyzed by qRT-PCR. FN1 mRNA levels were determined relative to the control (shC + Flag) normalized to ACTB abundance using the $\Delta\Delta C_t$-method.

In conclusion the data indicated that LEF1 binds to and activates the human FN1 promoter. In addition, the findings supported that the IGF2BP1-directed regulation of FN1 was mediated via LEF1 since it essentially recovered FN1 RNA levels after reduction upon IGF2BP1 knockdown.

3.8 LEF1 overexpression is insufficient to repress CDH1 in HEK293 cells

The siRNA-directed depletion of LEF1 led to a moderate but significant increase of CDH1 levels. This indicated that LEF1 mediated a pro-mesenchymal phenotype by repressing this epithelial marker in addition to positively regulating FN1. Accordingly it was investigated whether LEF1 depletion could activate CDH1 promoter activity. However, the knockdown of LEF1 did not alter significantly the activity of the previously described human minimal promoter (Jesse et al., 2010) compared to a control knockdown (Figure 3.25A). In agreement, the transient overexpression of LEF1 led to a rather moderate increase of CDH1 mRNA abundance (Figure 3.25B). This led to the conclusion that the repressive function of LEF1 on CDH1 mRNA expression was insufficient in these cells and presumably involved additional mechanism.

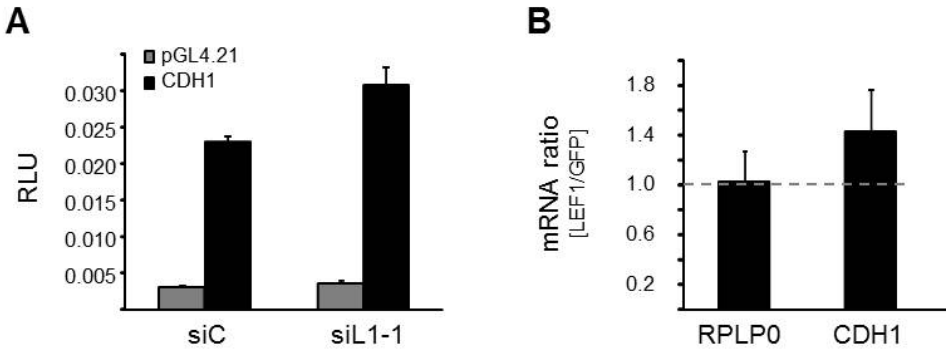


Figure 3.25: CDH1 repression is not mediated via LEF1. (A) CDH1 promoter (sequence published in (Jesse et al., 2010)) activity was monitored upon shRNA-mediated depletion of LEF1 compared to the control knockdown in HEK293 cells for 48 h. The empty pGL4.21 vector served as a control firefly reporter. Firefly activity was normalized to co-transfected Renilla activity (RLU). **(B)** Gain of function was analyzed in response to Flag-GFP or Flag-LEF1 overexpression for 48 h. RNA levels upon LEF1 overexpression were determined relative to the control (GFP). Mean values with standard deviation of at least three independent analyses are presented.

3.9 SNAI2 expression is modulated via an IGF2BP1-LEF1 axis

The results indicated that IGF2BP1 served a role in promoting mesenchymal cell characteristics (cell morphology and marker expression) via the enhanced expression of LEF1. Previous studies suggested a role of LEF1 in the regulation of the EMT-transcriptional regulators ZEB2 and Slug (SNAI2) in breast-cancer-derived MDA-MB-231 cells (Huang et al., 2012; Lambertini et al., 2010). The latter was shown to repress CDH1 expression in HEK293 cells by overexpression studies (Conacci-Sorrell et al., 2003). This raised the question whether IGF2BP1 facilitates its function through the LEF1-dependent induction of other pro-mesenchmymal factors like SNAI2. Accordingly, it was analyzed whether IGF2BP1 effected SNAI2 expression. The siRNA-directed depletion of IGF2BP1 led to a significant decrease of SNAI2 protein and RNA levels (Figure 3.26A). However, decay analyses upon IGF2BP1 knockdown revealed no changes of SNAI2 mRNA turnover compared to the control (siC) suggesting an indirect regulation of SNAI2 by IGF2BP1 potentially via promoting LEF1 (Figure 3.26B). This hypothesis was supported by findings that SNAI2 mRNA was not associated with IGF2BP1 in RIP studies (Figure 3.26C).

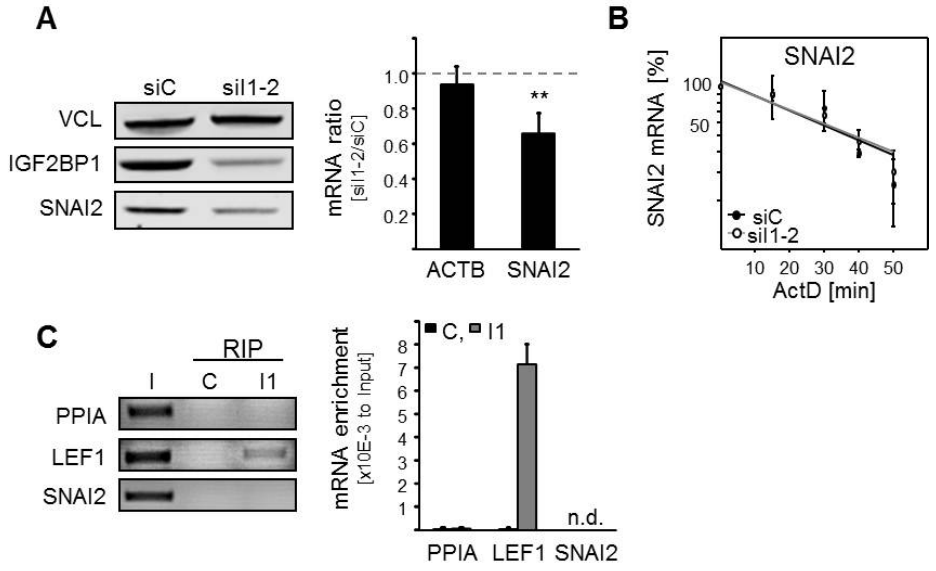


Figure 3.26: IGF2BP1 regulates SNAI2 indirectly. (A, B) HEK293 cells were transfected with IGF2BP1-directed (siI1-2) or control (siC) siRNAs for 72 h. **(A)** SNAI2 protein and mRNA levels were analyzed in response to IGF2BP1 knockdown by Western blotting and qRT-PCR, respectively. **(B)** mRNA turnover was determined by decay analyses. Cells were treated with actinomycin D (ActD) to block mRNA synthesis for the indicated time. **(C)** The association of IGF2BP1 with the indicated mRNAs was analyzed by RIP as described in Figure 3.9. Semi-quantitative (agarose gel) and quantitative real-time PCR (bar diagram) results are shown. The association of LEF1 mRNA was used as positive, PPIA as negative controls. Error bars indicate standard deviation of three independent analyses.

To evaluate the impact of LEF1 on SNAI2 expression loss of function analyses for LEF1 were performed. In these studies the depletion of LEF1 significantly decreased SNAI2 levels on protein and mRNA level (Figure 3.27A). However the ectopic expression of LEF1 (gain of function) did not alter SNAI2 mRNA levels (Figure 3.27B). Additional co-factors that are essential for a full regulation of SNAI2 by LEF1 may have been lacking in these analyses.

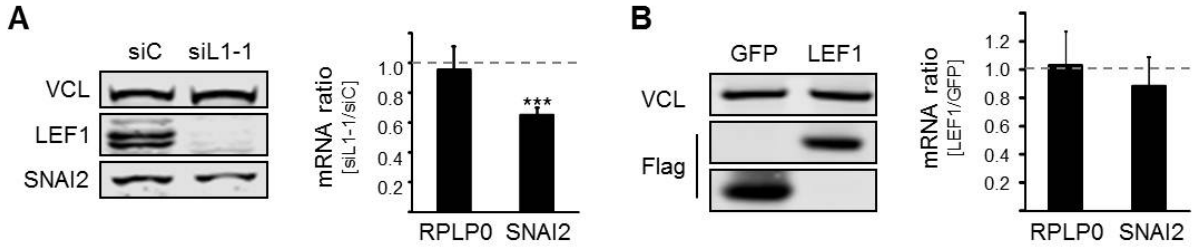


Figure 3.27: LEF1 depletion reduces SNAI2 levels. (A) HEK293 cells were transfected with LEF1-directed (siL1-1) or control (siC) siRNAs for 72 h. SNAI2 protein abundance upon LEF1 depletion was monitored by Western blotting. VCL served as loading control. SNAI2 RNA levels were determined relative to the controls (siC). **(B)** Gain of function studies were performed by transfection of HEK293 cells with Flag-LEF1 or Flag-GFP for 48 h. Overexpression of the Flag-tagged proteins was monitored by Western blotting. SNAI2 mRNA levels were determined in response to the LEF1 overexpression by qRT-PCR relative to the control (Flag-GFP) by cross-normalization to PPIA. RPLP0 was used as a control. Error bars indicate standard deviation of at least three independent analyses. Statistical significance was validated by Student's t-test: * $p < 0.05$; *** $p < 0.0005$.

It remained to be investigated whether IGF2BP1 facilitated an up-regulation of SNAI2 via LEF1. Recent studies showed that LEF1 binds to the SNAI2 promoter in osteosarcoma-derived cell lines (Lambertini et al., 2010) (Figure 3.28A). Thus SNAI2 promoter activity was studied in response to RFP or LEF1 overexpression in HEK293 cells. In addition, the promoter of SNAI1, a close family member of SNAI2, was analyzed in terms of LEF1 responsiveness (Figure 3.28A). Luciferase activity was very low for the promoterless pGL4.21 and the SNAI1 promoter by co-expression of Flag-RFP (Figure 3.28B). The SNAI2 luciferase reporter showed significantly higher activity in response to RFP expression compared to the SNAI1 promoter and the empty vector (Figure 3.28B). Transient overexpression of LEF1 increased activity of all luciferase reporters. However activation of pGL.4.21 and SNAI1 promoter reporter only reached activity levels of endogenous SNAI2 promoter therefore remained low. In contrast the SNAI2 reporter activity reached an about five-fold higher activity upon

LEF1 overexpression compared to RFP (Figure 3.28B). The SNAI2 promoter activation was much lower than correlating effects observed for FN1 promoter activation upon LEF1 overexpression indicating that LEF1 alone was only a weak inducer of the SNAI2 transcription.

In order to support the findings that SNAI2 expression was regulated at the transcriptional level via an IGF2BP1-LEF1 axis it was also investigated whether knockdown of either IGF2BP1 or LEF1 influenced SNAI2 promoter activity (Figure 3.28C). Since the two family members SNAI1 and SNAI2 are highly related the SNAI1 promoter was again tested in response to IGF2BP1- or LEF1 depletion. The shRNA-mediated depletion of IGF2BP1 or LEF1 significantly reduced promoter activity of SNAI2 but not of SNAI1 or the empty pGL4.21 (Figure 3.28C). This strongly suggested that SNAI2 down-regulation by IGF2BP1 and LEF1 was mediated via its promoter region. However *in vivo* binding of LEF1 to DNA sequences of the SNAI2 promoter was not observed in HEK293 cells (Figure 3.28D) although binding of LEF1 to the SNAI2 promoter was previously described in osteoscarcoma-derived cell lines (Lambertini et al., 2010). In addition, LEF1 binding to the CDH1 promoter was also not observed (Figure 3.26D). Note that, only data from ChIP experiments were evaluated that showed binding of LEF1 to the FN1 promoter.

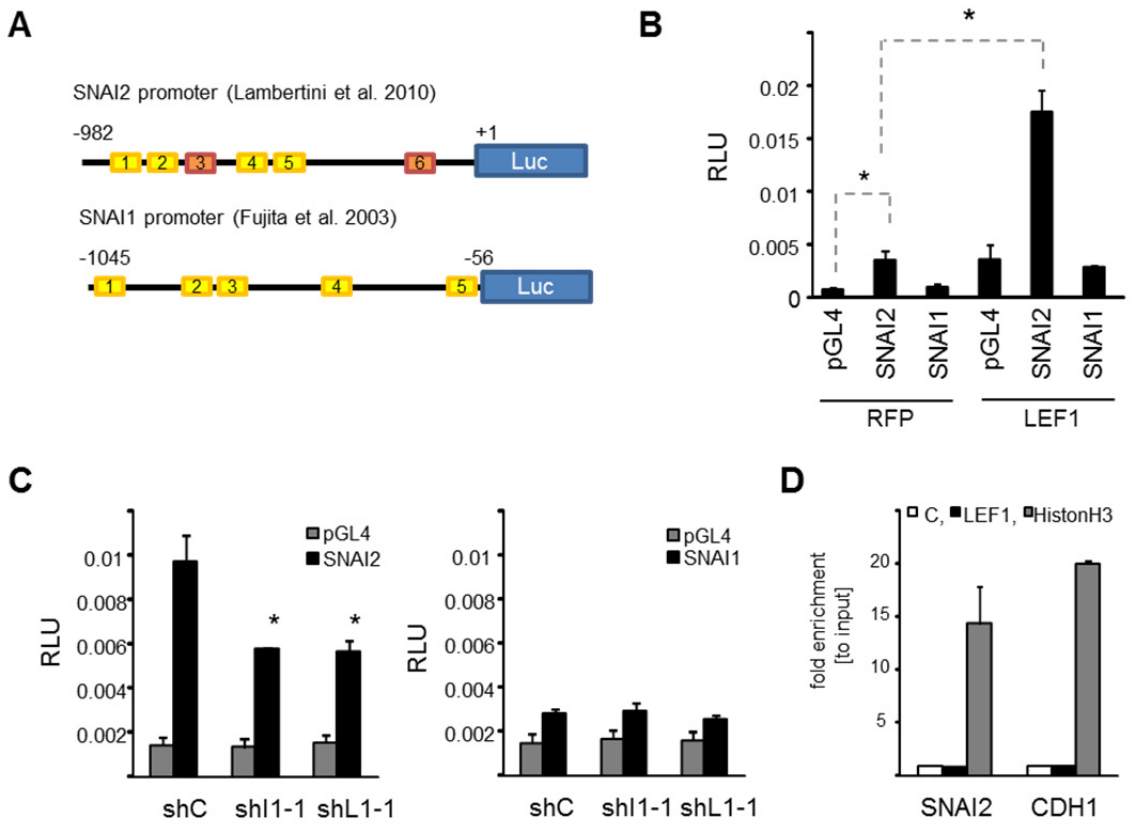


Figure 3.28: SNAI2 but not SNAI1 promoter is regulated by LEF1. (A) Scheme of the used SNAI1 and SNAI2 luciferase promoter constructs. Yellow boxes indicate putative LEF1 binding site determined by PROMO database, red boxes suggested sites by other groups. **(B)** HEK293 cells were transfected with firefly luciferase reporter plasmids (pGL4.21, SNAI2 or SNAI1) and Flag-RFP or Flag-LEF1 for 30 h. **(C)** HEK293 cells were transfected with sh-plasmids targeting IGF2BP1, LEF or controls. Firefly luciferase reporter and Renilla were co-transfected for 48 h. Relative luciferase activities were determined by normalization to co-transfected Renilla luciferase. **(D)** Binding of endogenous LEF1 protein to the human SNAI2 and CDH1 promoter in HEK293 cells was assessed by chromatin immunoprecipitation (ChIP) as described in 3.20. Enrichment of precipitated chromatin was determined by quantitative RT-PCR using self-designed primers for CDH1 and previously published primers for SNAI2 promoter region (Lambertini et al., 2010). Error bars indicate standard deviation of three independent analyses. Statistical significance was validated by Student's t-test: * $p < 0.05$.

In summary, the EMT-driver SNAI2 was shown to be regulated by IGF2BP1 and LEF1. This regulatory mechanism presumably involves an indirect control of the SNAI2 transcription via its promoter region. Although it remained elusive which factors directly modulate SNAI2 expression it was demonstrated that LEF1 is capable of controlling SNAI2 promoter activity in HEK293 cells. These findings strongly supported a pro-mesenchymal function of IGF2BP1 via an IGF2BP1-LEF1 regulatory axis.

3.10 IGF2BP1 - a pro-mesenchymal marker in tumor-derived cell lines

Based on the data it became apparent that IGF2BP1 promotes the expression of mesenchymal genes like FN1 and SNAI2 through LEF1. On the other hand IGF2BP1 interfered with the expression of the epithelial marker CDH1 by a so far unsolved mechanism. It was expected that the expression of IGF2BP1 and LEF1 correlated to a pro-mesenchymal phenotype in different cellular systems. Thus cell lines derived from ovarian-, colorectal- and breast carcinomas as well as melanoma were analyzed for IGF2BP1, LEF1 and epithelial or mesenchymal marker expression. The epithelial markers CDH1 and Keratin 8 (KRT8) were highly expressed in OVCAR, HT29, SW480 and MCF7 (Figure 2.29). The mesenchymal markers Vimentin (VIM), N-cadherin (CDH2), SNAI2, ZEB1 and FN1 were mainly expressed in ES-2, MDA-MB 231, HBL-100, HT-144, 1F6 and MV3 although to different extends. Due to the diverse nature of the cell types a 100-percent overlap of markers was not observed. For example, CDH2 was not expressed in the breast-cancer derived MDA-MB231 cells but other mesenchymal markers like VIM and SNAI2 were. Surprisingly, SNAI1 which is described in the literature as pro-mesenchymal marker did not correlate well in this respect. Note that FN1 levels determined by Western blotting of cell lysates could be misleading, since such analyses underestimate soluble FN1 levels. Moreover the evaluation of the protein expression pattern indicated that OVCAR, HT29 and MCF7 cell lines can be characterized as epithelial (Figure 2.29). In contrast ES-2, MDA-MB 231, HBL-100, HT144, 1F6 and MV3 displayed high expression of mesenchymal markers and can therefore be categorized as mesenchymal cell lines. The colorectal carcinoma-derived cell line SW480 showed features of both epithelial and mesenchymal cell properties as frequently observed for colorectal carcinoma-derived cells. Regarding expression of IGF2BP1 the protein levels of the RBP correlated to high expression levels with the mesenchymal markers VIM and SNAI2 and to lesser extend also to LEF1 in the analyzed tumor derived cells. IGF2BP1 was barely expressed in cells with high levels of CDH1 or KRT8. The pro-mesenchymal expression of IGF2BP1 and LEF1 became also apparent with regard to cells derived from the same/ or similar tumor type. ES-2

cells expressed higher levels of IGF2BP1 and LEF1 than OVCARs. SW480 also revealed higher levels of IGF2BP1 and LEF1 than HT-29 cells. Exceptions can be found in breast cancer derived cells. MDA-MB 231 cells for example express mainly mesenchymal markers like VIM, SNAI2 and LEF1 but don't express IGF2BP1. Melanoma, a typical mesenchymal tumor type, was represented by three tumor and metastasis-derived cell lines. Two of which, namely HT-144 and 1F6, showed the highest expression levels of LEF1 which correlate to high amounts of FN1 and SNAI2 levels.

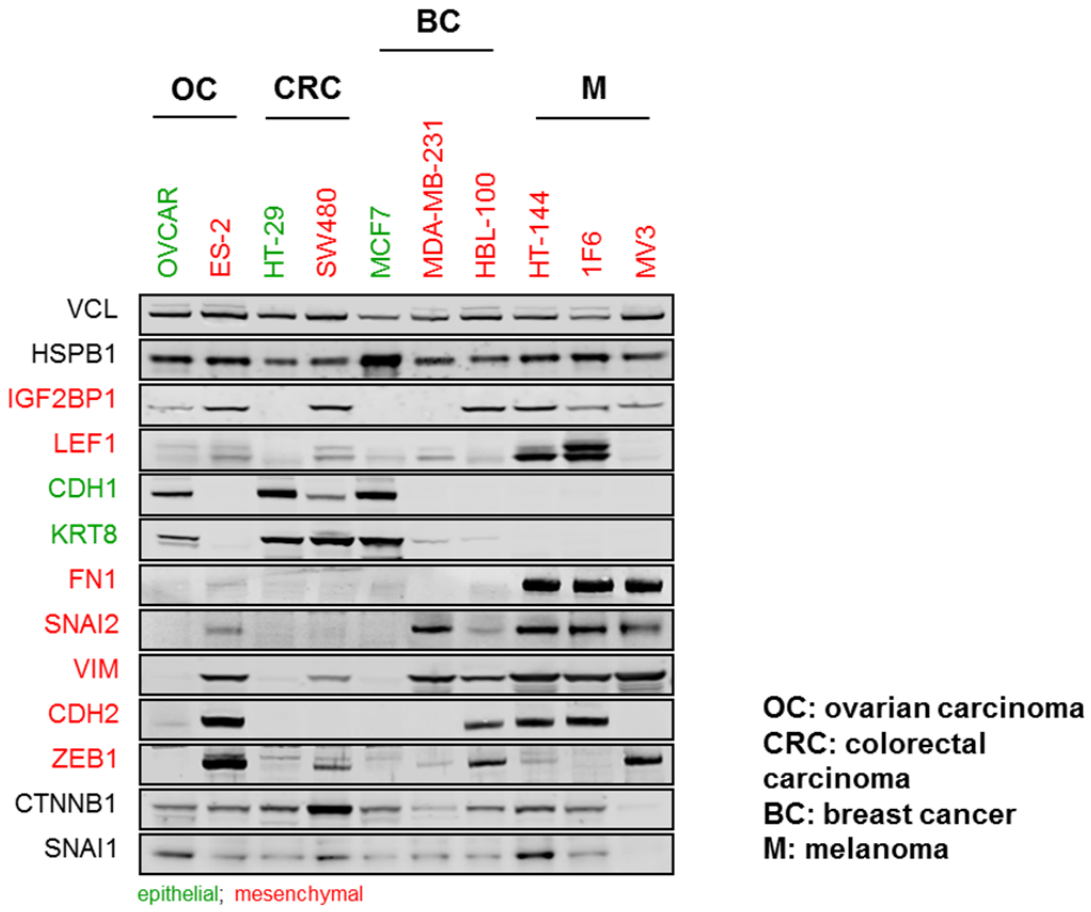


Figure 3.29: Marker expression in tumor derived cell lines. Protein abundance of different marker proteins for epithelial or a mesenchymal characteristics was analyzed by Western blotting using the indicated antibodies and the indicated cell lines. VCL and HSPB1 served as loading controls.

In conclusion, analyses of protein marker expression indicated that IGF2BP1 can be mainly found in mesenchymal-like tumor- and metastasis-derived cell lines. This correlates with the proposed function of IGF2BP1 in promoting mesenchymal cell properties.

3.11 IGF2BP1 promotes migration via LEF1 and SNAI2 in HT-144 cells

In support of its function as a pro-mesenchymal regulator Dr. Marcell Lederer and Dr. Nadine Stöhr generated additional data sets in HT-144 cells, a melanoma-derived cell line of a mesenchymal phenotype. In agreement with data obtained from HEK293 cells the transient depletion of IGF2BP1 and LEF1 in HT-144 cells correlated to reduced levels of FN1 and SNAI2 (Figure 3.30A). Changes in cell morphology were also observed upon knockdown of IGF2BP1, LEF1 as well as SNAI2 indicating a role of these factors in tumor cell dissemination (Figure 3.30B). Furthermore the depletion of IGF2BP1 reduced migration of HT-144 cells using wound closure analyses, as previously described for U2OS and ES-2 cells (Stohr et al., 2012) (Figure 3.30D, E). The impaired cell migration was essentially recovered by re-expression of LEF1 or SNAI2 (Figure 3.30D, E). Reduced migration was also observed upon shRNA-mediated depletion of LEF1 and SNAI2 (Figure 3.30D, E). These findings strongly supported that both factors were essential for the pro-migratory capacity of IGF2BP1 by promoting mesenchymal gene expression. Surprisingly, Western blot analyses showed that LEF1 re-introduction down-regulated CDH1 whereas SNAI2 re-expression recovered FN1 levels (Figure 3.30C). In addition, long-term knockdowns by IGF2BP1 and LEF1 by lentiviral transduction even enhanced pro-epithelial character of HT-144 cells revealing increased expression of CDH1 and down-regulation of another mesenchymal marker VIM which were not changed upon transient depletion of IGF2BP1 or LEF1 (Figure 3.30A).

In summary these results confirmed a pro-mesenchymal role of IGF2BP1 in tumor –derived HT-144 cells. The depletion of IGF2BP1 and its downstream effectors LEF1 and SNAI2 were correlated to promote tumor cell migration, a key feature of mesenchymal cells.

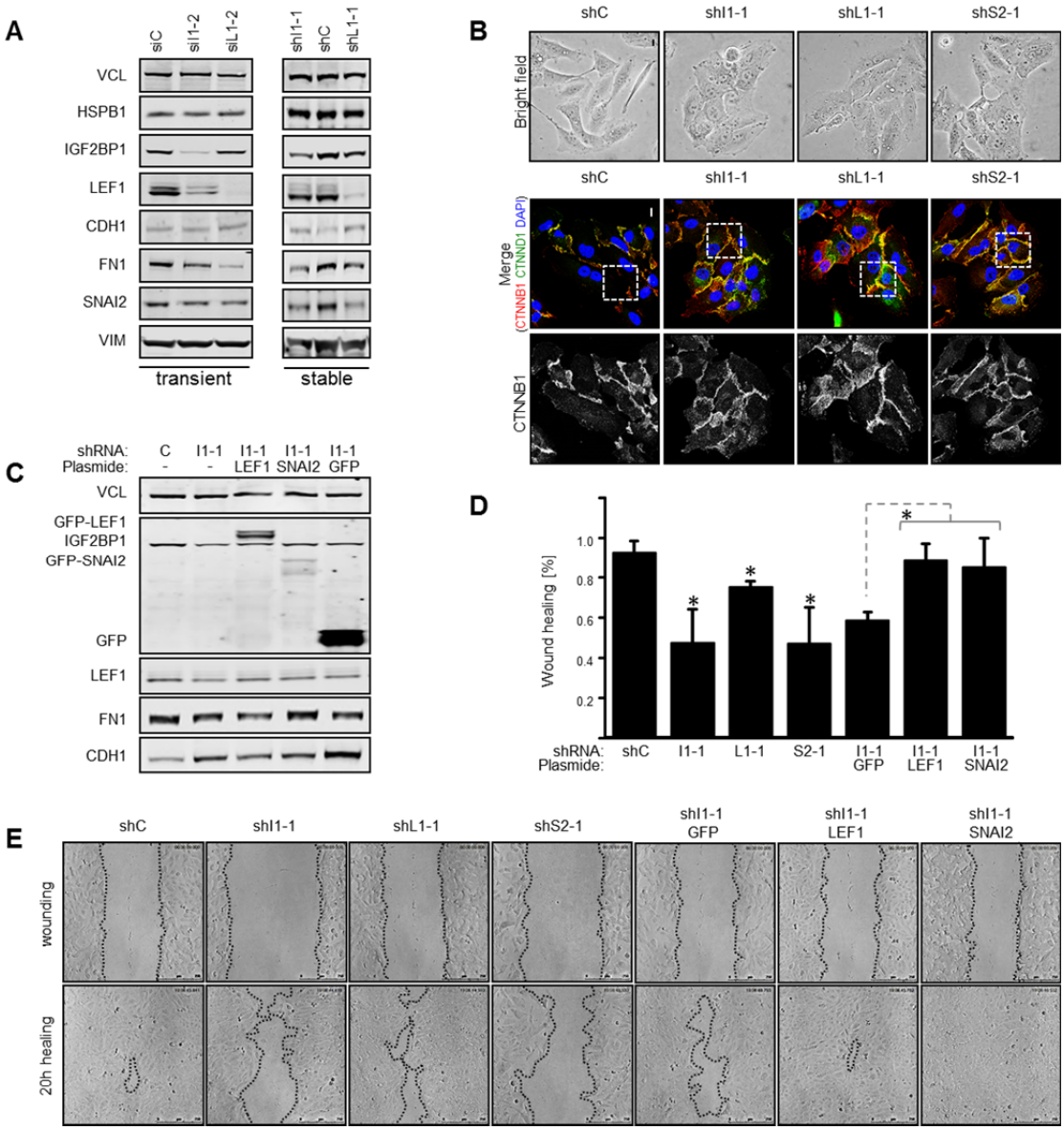


Figure 3.30: IGF2BP1 promotes migration and mesenchymal-like cell morphology via LEF1 and SNAI2. (A, B) Melanoma-derived HT-144 cells were transiently (siRNA) transfected or stably (shRNA) transduced with indicated siRNAs or lentiviral constructs. **(A)** The abundance of indicated proteins was analyzed by Western blotting. Transient depletion of IGF2BP1 as well as LEF1 was analyzed 72 h post transfection (A, left panel). To create stable knockdown cell populations HT-144 cells were stably transduced by lentiviral vectors encoding IGF2BP1 (shI1-1), LEF1 (shL1-1), SNAI2 (shS2-1) directed or control (shC) shRNAs. Three weeks after transduction, cells were cultured for 48h before analyzing protein abundance by Western blotting with indicated antibodies (A, right panel). Cell morphology upon stable knockdown of IGF2BP1, LEF1 and SNAI2 was monitored by bright field microscopy and immunostaining **(B)**. Cells were cultured on collagen coated coverslips for 48h before immunostaining of CTNNB1 and CTNND1 (p120 Catenin) to label cell-cell contacts. **(C-E)** HT-144 cells were stably transduced by lentiviral vectors encoding IGF2BP1 (shI1-1), LEF1 (shL1-1), SNAI2 (shS2-1) directed or control (shC) shRNAs. Where indicated IGF2BP1 knockdown populations were transduced with GFP, GFP-LEF1 or GFP-SNAI2 cDNA encoding lentiviral vectors three weeks after the infection with shRNA encoding vectors. **(C)** The abundance of indicated epithelial or mesenchymal markers was analyzed by Western blotting in indicated cell populations. **(D, E)** Cell migration was analyzed using wound closure analyses monitored by time lapse microscopy over 20h (D; Bars, 250µm). Cell migration was assessed by quantitative means relative to cells transduced with control shRNA (shC) using automated segmentation algorithms (E), as recently described (Glaß et al., 2012). Standard deviation was determined over three independent analyses. Statistical significance was validated by Student´s ttesting: * $p < 0.05$.

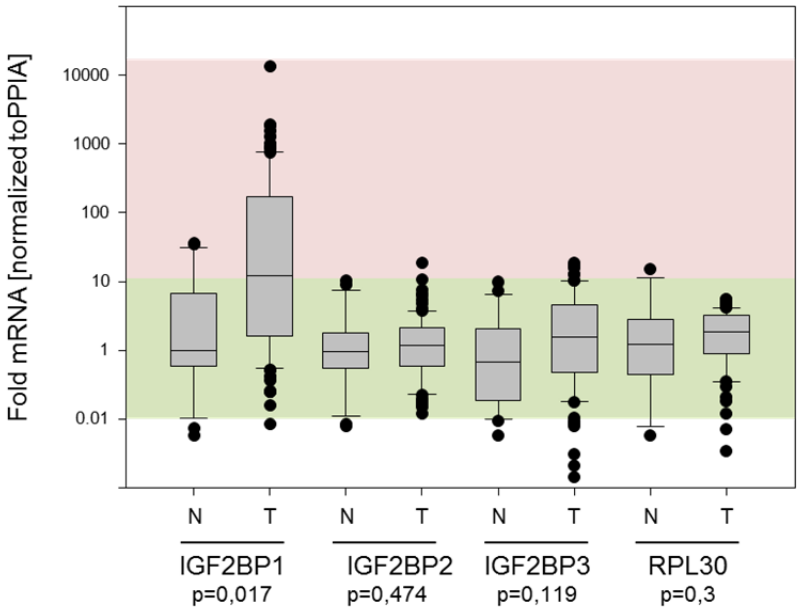
3.12 IGF2BP1 is selectively up-regulated in squamous cell carcinoma

So far the function of IGF2BP1 was studied in the context of non-malignant and tumor-derived cells. In addition it was of great interest to analyze the expression of IGF2BP1 in cancerous tissue. A cancer type that arises at the mucosal surface inside the head and neck has been barely investigated regarding the expression levels of the IGF2BPs at the mRNA level. This type of cancer, the squamous cell carcinoma of the head and neck (HNSCC) is the sixth most common cancer worldwide with 600000 new cases every year (Rothenberg and Ellisen, 2012). Only 40-50 % of patients with HNSCC survive 5 years (Leemans et al., 2011). Due to the heterogeneity of the tissue origin the consideration as one disease is complicated. The common origin of HNSCC is the squamous mucosa of the upper aerodigestive tract including structure like lip, tongue, nasopharynx, oropharynx, larynx and hypopharynx (Rothenberg and Ellisen, 2012). Risk factors for this disease are tobacco use and alcohol consumption but HNSCC has also been linked to infections of high-risk types of human papilloma virus (HPV) (Rothenberg and Ellisen, 2012). Several mutations of tumor suppressive as well as oncogenic factors have been correlated to tumor development of HNSCC. The most common mutation of the TP53 tumor suppressor occurs in 60-80 % of the cases (Balz et al., 2003; Poeta et al., 2007; van Houten et al., 2002). Furthermore loss of CDKN2A, TGFBR2/SMAD4 and factors of the NOTCH pathway through mutations are considered to be involved in tumor development and progression (Rothenberg and Ellisen, 2012). In contrast to loss of function mutations, the occurrence of somatic mutations and genetic changes indicates that the PI3K–PTEN–AKT pathway is frequently activated in HNSCC (Leemans et al., 2011).

Various groups have examined the IGF2BP expression in squamous cell carcinoma (SCC) and mainly reported IGF2BP3 to be up-regulated in malignant neoplasms (reviewed in (Bell et al., 2012; Findeis-Hosey and Xu, 2011). Moreover IGF2BP3 is used as a prognostic biomarker for some of these tumor types. In squamous cell carcinoma again IGF2BP3 has been correlated to metastasis formation of tongue squamous cell carcinoma and poorer overall survival in oral squamous cell carcinoma (Li et al., 2011a; Li et al., 2011b). Another group identified

KOC (IGF2BP3 homologue) to be highly up-regulated in lung squamous cell carcinoma based on microarray studies (Wang et al., 2000).

Accordingly, analyses of ~120 patient´s samples were performed to determine mRNA levels of IGF2BP1, 2 and 3 in normal and tumor-derived tissue samples. Tumor as well as normal tissue samples were provided by Dr. Matthias Kappler and analyzed in collaboration with the group of Prof.Dr. Vordermark (Section Molekulare Strahlenbiologie) from the university hospital of Halle. Based on the evaluation by the local pathologist samples were categorized into normal or tumor tissue samples. Up to now the status of a HPV infection or TP53-mutation of the patient's tumor samples is not available. Expression levels of IGF2BP1, 2 and 3 were assessed by quantitative real-time PCR. mRNA levels of the IGF2BPs were determined relative to PPIA using the ΔCt-method. Cyclophilin A (PPIA) was used as house-keeping gene for normalization to exclude variations in mRNA concentration and quality throughout the samples. Surprisingly, these analyses revealed a significant up-regulation of IGF2BP1 and not the family member IGF2BP3 compared to normal tissue samples (Figure 3.31). mRNA levels of IGF2BP2 and RPLP30 remained unaffected in tumor samples compared to normal tissue.



	N-values
IMP1-N	21
IMP1-T	93
IMP2-N	22
IMP2-T	93
IMP3-N	22
IMP3-T	93
RPLP30-N	16
RLP30-T	70

Figure 3.31: IGF2BP1 is selectively up-regulated in squamous cell carcinoma from head and neck. mRNA levels of tissue samples from patients suffering from head and neck squamous cell carcinoma (HNSCC) were analyzed for IGF2BP1-3 expression by qRT-PCR. RNA abundance was determined relative to PPIA levels by the ΔC_t-method. RPLP30 was used as control mRNA. N-values of normal (N) or tumor (T) tissue samples are summarized in the table. Mean values with error bars are presented as box plots. RNA levels are presented in semi-logarithmic scale. Statistical significance was validated by Student's t-test and indicated as p-values.

Moreover mRNA abundance of the IGF2BPs was determined in the three SCC-derived cell lines FaDu, SAS and Cal33. The quantitative analyses of IGF2BP mRNA levels in these cell lines nicely support the findings from tumor samples. The fold increase of IGF2BP1 compared to normal (N) was higher than to tumor (T) whereas this is not observed for the other IGF2BPs. The measurements indicated that an up-regulation of IGF2BP1 remained preserved in these tumor-derived cell lines (Figure 3.32).

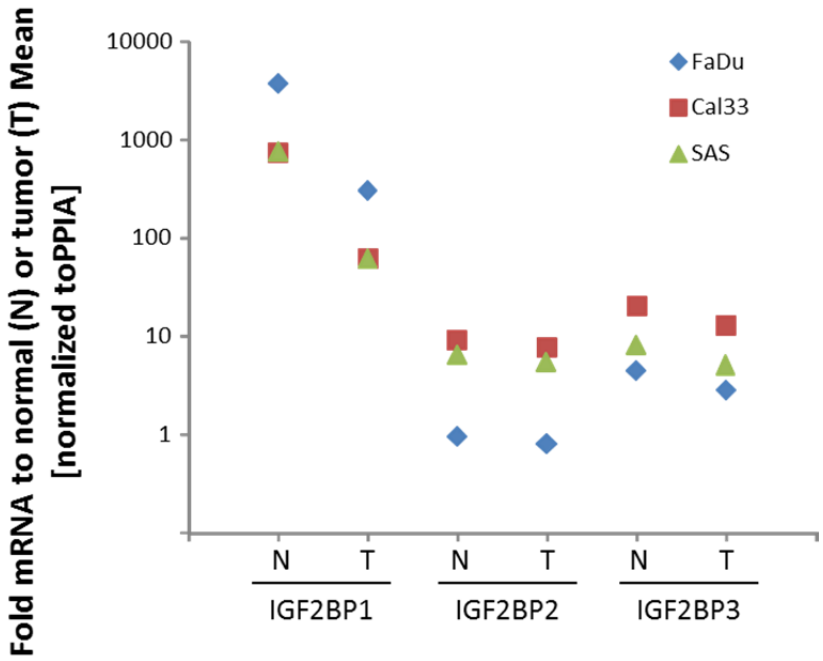


Figure 3.32: IGF2BP1 up-regulation is preserved in HNSCC-derived cell lines. The mRNA levels of the IGF2BPs in the SCC-derived cell lines FaDu, SAS and Cal33 were analyzed by qRT-PCR and normalized to PPIA levels using the ΔC_t-method. RNA abundance is presented as fold mRNA to normal (N) or tumor (T) Mean of HNSCC-tissue samples in semi-logarithmic scale.

In conclusion IGF2BP1 was found to be up-regulated in HNSCC tumor samples compared to non-cancerous tissue. Such elevated expression of IGF2BP1 was further preserved in SCC-derived cell lines in contrast to its family members IGF2BP2 and IGF2BP3. However the consequences of such an elevated IGF2BP1 expression remained elusive. Further studies need to be performed to evaluate the role of IGF2BP1 in regulating cellular processes like migration, invasiveness or tumor cell dissemination in squamous cell carcinoma. Nonetheless the data indicated that IGF2BP1 rather than the family members IGF2BP2 or 3 is a decisive factor in tumorigenesis in squamous cell carcinoma based on the patient's tissue samples at least on RNA level.

4 Discussion

4.1 IGF2BP1 a pro-mesenchymal oncogene in tumor-derived cell lines

IGF2BP1 controls the fate of distinct target transcripts by regulating their mRNA turnover, localization and/or translation. This study identifies a new mechanism by which the protein promotes pro-mesenchymal cell properties. The direct stabilization of the LEF1 mRNA by IGF2BP1 leads to elevated levels of this transcription factor which in turn enhances transcription of the extracellular matrix component FN1. Furthermore IGF2BP1 promotes, presumably via LEF1, indirectly the expression of SNAI2, a transcriptional driver of EMT programs. Decreased levels of IGF2BP1 by knockdown experiments lead to changes in morphology and reduced cell migration which correlate to a down-regulation of the mesenchymal proteins LEF1, SNAI2 and FN1. In contrast, an up-regulation of the epithelial cell-contact protein CDH1 upon IGF2BP1 knockdown supports its role in sustaining mesenchymal cell properties in embryonic- as well as tumor-derived cell lines.

IGF2BP1 interferes with the degradation of the lymphoid-enhancer binding factor 1 (LEF1) mRNA in a 3' UTR-dependent manner resulting in the enhanced expression of this TCF-family member. Yet the exact binding motifs of IGF2BP1 to LEF1 mRNAs remain to be determined. However, recent data obtained by the PAR-CLIP method indicated binding to various sites in the 3'UTR of the LEF1 mRNA (refer to doRiNA database: http://dorina.mdc-berlin.de/rbp_browser/dorina.html.). The work of Hafner and colleagues revealed a common recognition motif for all three IGF2BPs of only four nucleotides (CAUH (H = A, U, or C)) (Hafner et al., 2010). The shared recognition motif suggested a redundant role of all three IGF2BPs in RNA regulation. In contrast, findings from the present study suggest that only IGF2BP1 and not IGF2BP2 or 3 are capable of regulating LEF1 mRNA stability although all three were expressed in HEK293 cells. This implicates that the regulatory function of the IGF2BPs cannot be simply deduced from the identification of the four-nucleotide consensus motif. Recognition sequences creating proper binding sites by spacing and folding as suggested for the ACTB 3'UTR present a better model to enhance

specific regulation of various target mRNAs and might explain differences between the PAR-CLIP data and functional approaches (Chao et al., 2010; Patel et al., 2012).

In consequence of elevated LEF1 levels, the transcriptional control of target gene expression by LEF1 is modified. Fibronectin (FN1) and Slug (SNAI2) promoter activities increase in response to LEF1 expression. The identification of regulatory LEF1 binding sites in the human FN1 promoter indicates that LEF1 is a decisive factor in human FN1 transcriptional regulation. Binding of LEF1 to sequences in the FN1 promoter region strongly supports this view. Shortening of the longest promoter luciferase reporter (FN1-839) indicates that binding site 4 is sufficient to sustain transcriptional activation without binding sites 1-3. However the deletion of the LEF1 binding site 4 does not abolish promoter activation by LEF1. The data suggests that LEF1 binding sites may cooperate in mediating transcriptional regulation. As indicated in Figure 3.18 the consensus motif of these sites only varies in few nucleotides. The LEF1 consensus binding motif (A/T)(A/T)CAA(A/T)G for example can be found as GTCCAAAG in site 4 and as CTGCAAAG in site 2 with variations in position 1 and 3. Note that PROMO database selects putative binding sites via the consensus sequence GATCAAAG thereby creating motifs of 8 instead of 7 nucleotides. One nucleotide within the consensus motif was demonstrated to be indispensable for binding (TTCAAAG) (Love et al., 1995). In addition, LEF1 was shown to bind to other sequences than the consensus motif. WNT responsive elements (WRE) in the Cacna1g promoter were identified revealing no similarities to the consensus motif (Wisniewska et al., 2010). One can assume that surrounding sequences of the binding site 4 that differ from the consensus motif could possibly mediate transcriptional activation of the FN1 promoter by LEF1. However LEF1 binding sequences published for the Cacnag1g promoter are not present in the FN1 promoter sequence used for investigations here.

Previous studies revealed a role of LEF1 in the transcriptional regulation of the *Xenopus* FN1 (xFN1) promoter (Gradl et al., 1999). This was shown to rely on transcriptional co-activation by CTNNB1 (Gradl et al., 1999). Others suggested that the human LEF1 isoform lacking Exon VI (LEF1ΔExonVI) specifically activates the xFN1 promoter in pancreatic cancer cell lines (Jesse et al., 2010). This study also showed the repression of the CDH1 promoter by LEF1ΔExonVI (Jesse et al., 2010). However, findings presented here in this study suggest that the full length LEF1 was

capable of activating the human FN1 promoter correlating to increased levels of endogenous FN1 at least in HEK293 and U2OS cells. In contrast, the transient overexpression of LEF1ΔExonVI did not significantly increase FN1 mRNA levels in HEK293 cells. In contrast, full length LEF1 was incapable of recovering FN1 levels upon IGF2BP1 depletion in HT-144 cells indicating that the control of FN1 transcription is cell-type dependent. The siRNA mediated depletion of LEF1 efficiently reduced levels of all isoforms in the used cell lines. This resulted in a down-regulation of FN1 expression in all analyzed cell types regardless of the expressed LEF1 isoforms. Although knockdown experiments were performed independently of isoform specificity the data suggest that LEF1 controls FN1 expression. It remains to be investigated whether distinct LEF1 isoforms contribute to the regulation of FN1 in different cellular systems.

Furthermore IGF2BP1 is demonstrated to promote the expression of the transcription factor SNAI2 most likely via LEF1. In HEK293 cells a direct regulation by IGF2BP1 is excluded by the findings that the protein does not associate with the SNAI2 transcript and mRNA turnover remains unaffected by IGF2BP1 depletion. Recent publications suggested the LEF1-dependent control of SNAI2 expression in osteosarcoma-derived cell lines (Lambertini et al., 2010).Transiently overexpressed LEF1 induced activation of the previously published SNAI2 promoter reporter in HEK293 cells. However, LEF1 does not bind to the SNAI2 promoter regions suggested by Lambertini and colleagues indicating also an indirect regulation by this factor. Moreover the LEF1-dependent activation of the promoter reporter was less potent for SNAI2 compared to the activation of the FN1 reporter. In addition, the transient overexpression of LEF1 did not enhance SNAI2 mRNA levels indicating that additional co-factors or epigenetic modifications may be necessary to facilitate proper promoter activation.

LEF1 binds to DNA sequences via its HMG box and is incapable to regulate gene transcription without co-activators or repressors. The majority of published studies showed co-activation of LEF1-dependent transcription by CTNNB1 through functional nuclear complex formation (Behrens et al., 1996; Porfiri et al., 1997). However, it remains elusive how transcriptional regulation via LEF1 is facilitated in the present study. In agreement with previous finding endogenous transcriptional activity of CTNNB1 is dispensable in HEK293 cells as demonstrated by CTNNB1

driven promoter activation and localization studies. The protein is mainly located at cell-cell junctions instead of the nucleus to facilitate transcriptional regulation. Previous studies showed that although CTNNB1 and LEF1 were expressed in 293 cells no functional complex formation was observed (Porfiri et al., 1997). A mechanism by which transcriptional regulation via LEF1 involved additional co-factors is likely. CDH1 repression for example was shown to be mediated by complex formation of LEF1 with the SMAD2/SMAD4 co-regulators and independently of CTNNB1 during palatal fusion (Nawshad et al., 2007). Although no direct repression of the CDH1 promoter could be validated at least in HEK293 cells a co-regulation of LEF1- SMAD complexes at distant gene promoters represents an option for future studies. In addition, co-factors like IGF2R, AP-1, ALY and MITF were suggested to function as co-regulators of LEF1 (Hsu et al., 1998; Rivat et al., 2003; Warsito et al., 2012; Yasumoto et al., 2002). Thus further mechanistic details remain to be investigated. The role of CTNNB1 in the regulation of IGF2BP1 remains puzzling. It was reported that CTNNB1-TCF4 driven promoter regulation enforced the expression of IGF2BP1 in HEK293 cells (Gu et al., 2008; Noubissi et al., 2006). Furthermore it was demonstrated that IGF2BP1 controls CTNNB1 stability via a feedback mechanism in breast cancer-derived cell lines (Gu et al., 2008). The siRNA-mediated depletion of endogenous CTNNB1 does not result in changes of IGF2BP1, FN1 or CDH1 gene expression. In agreement, nuclear localization of the protein is not observed in the analyzed cell lines with the exception of HTC-116 cells which were used as control cells. Therefore it seems to highly rely on nuclear translocation of CTNNB1 which was enforced by the expression of a stabilized mutant in the cited study. This raises also the question whether CTNNB1-mediated transcription of IGF2BP1 is even essential. Although revealing high expression levels of IGF2BP1 no nuclear CTNNB1 is observed, e.g. in HEK293 and ES-2 cells. Other mechanisms must occur in order to promote IGF2BP1 transcription at least in these cell types. In agreement, previous studies reported that although nuclear CTNNB1 was observed in many colon cancers no correlation was found between IGF2BP1 and CTNNB1 expression in tumors (Mongroo et al., 2011). This indicated an up-regulation of IGF2BP1 independently of CTNNB1 *in vivo* (Mongroo et al., 2011). In addition, the chosen T-cell factor (TCF4) involved in the trans-activation of IGF2BP1 was different from LEF1. In the current study the involvement of TCF4 in the regulation of EMT-target genes is not evaluated. The differential expression of the T-cell factor family

members has been reported and has been correlated to distinct functional characteristics of LEF1 and TCF4 for example in melanoma-derived cell lines (Eichhoff et al., 2011). The cell context dependent TCF/LEF expression and function therefore presents a usable tool for the control of cell fate and would explain the controversial data obtained (Mao and Byers, 2011). This, however, is only speculative at this point and may be unraveled in future studies.

The current study revealed that IGF2BP1 promotes the expression of the pro-mesenchymal factors LEF1, FN1 and SNAI2 and interferes with CDH1 expression, an epithelial marker. Changes in cell morphology correlate to a more epithelial-like morphology upon IGF2BP1 depletion in HEK293 and ES-2. Cortical F-actin cytoskeleton is more pronounced in both cell lines upon IGF2BP1 knockdown supporting its role in controlling F-actin organization as previously described (Stohr et al., 2012). In both cell lines the changes of the F-actin cytoskeleton differed from those observed for U2OS cells. Even in ES-2 cells were a disruption of the F-actin fibers upon IGF2BP1 depletion was previously described the fibers appear rather thickened than shortened. Different time points of fixation may have led to different observations in addition to differences in the seeding density. It seemed likely that the shortened F-actin fibers represented an intermediate stage of remodeling which finally led to the cortical F-actin fibers. Differences in re-modeling of the F-actin architecture are also likely to depend on cellular levels of HSP27. HEK293 cells for example express lower levels of HSP27 (data not shown). The IGF2BP1-dependent phosphorylation of HSP27, as previously reported (Stohr et al., 2012), may play a minor role in these cells.

In agreement with its function in promoting mesenchymal characteristics, IGF2BP1 is found to be mainly expressed in mesenchymal-like tumor-derived cell lines. Cell migration is a key feature of mesenchymal cells. The role of IGF2BP1 in cell migration has been discussed controversially in the literature. In agreement with previous findings in U2OS and ES-2 cells (Stohr et al., 2012), IGF2BP1 enhances cell motility in HT-144 melanoma-derived cells. Reduced migration upon IGF2BP1 depletion is essentially restored by LEF1 and SNAI2. In contrast, studies in breast cancer-derived T47D cells revealed increased cell motility upon IGF2BP1 depletion (Gu et al., 2009). Later this was correlated to decreased formation of cell-cell adhesion via down-regulation of CDH1 upon IGF2BP1 depletion in this cell type (Gu

et al., 2012). However, findings presented here from non-malignant HEK293 as well as tumor-derived cell lines support a pro-mesenchymal role in a largely cell context independent manner. Severe morphological changes in response to IGF2BP1 depletion in HEK293 cells correlate to enhanced recruitment of CDH1 and CTNNB1 to cell-cell contacts. The increased expression of CDH1 and on the other hand a down-regulation of the extra cellular matrix protein FN1 upon knockdown emphasizes the function of IGF2BP1 as post-transcriptional driver of mesenchymal gene expression.

Unfortunately, this study was unable to elucidate the mechanism by which IGF2BP1-mediated repression of CDH1 is facilitated. It is very likely that IGF2BP1 regulates additional target mRNAs at the post-transcriptional level that mediate the repression of CDH1. The potent CDH1 repressors ZEB1/2, TWIST and E47 for example were not evaluated in this study. Yet unpublished data by Alexander Mensch showed that IGF2BP1 promotes the expression of ZEB1 in anaplastic thyroid carcinoma derived tumor cells indicating that indeed the regulation of additional EMT-relevant target mRNAs is involved.

IGF2BP1 and its family member IGF2BP3 were described to be up-regulated and *de novo* synthesized in a variety of tumor types (Bell et al., 2012). In addition, IGF2BP1 was identified as a pro-migratory factor at least *in vitro* in tumor-derived cell lines (Stohr and Huttelmaier, 2012). Its oncogenic potential was strongly supported by the transgenic expression of IGF2BP1 (CRD-BP) in murine mammary tissue which induced breast carcinomas as well as metastases (Tessier et al., 2004). The formation of metastases from a solid tumor relies on substantial changes in gene expression and the underlying mechanism of EMT. Although *in vivo* data are rare up to now, driving pro-mesenchymal cell properties by IGF2BP1 may contribute to tumor aggressiveness and metastasis formation with enhanced migratory and invasive capacities as, for example, described by Köbel and colleagues in ovarian carcinoma (Kobel et al., 2007). The mainly cell type independent post-transcriptional control of pro-mesenchymal proteins like LEF1 and SNAI2 indicates a more general role of IGF2BP1 in sustaining a mesenchymal character and promoting tumor cell migration. Enhanced expression levels of LEF1 in highly migrating cells from metastatic melanomas were reported as well as its up-regulation in lung adenocarcinoma metastasis supporting the metastatic potential of LEF1 (Murakami et al., 2001;

Nguyen et al., 2009). Thus elevated levels of LEF1 through stabilization by IGF2BP1 could affect tumor progression which, of course, needs substantial *in vivo* validation.

In summary, IGF2BP1 plays a pivotal role in tumor cell dissemination by synergistic cooperation of transcriptional and post-transcriptional control of mesenchymal gene expression (see Figure 4.1).

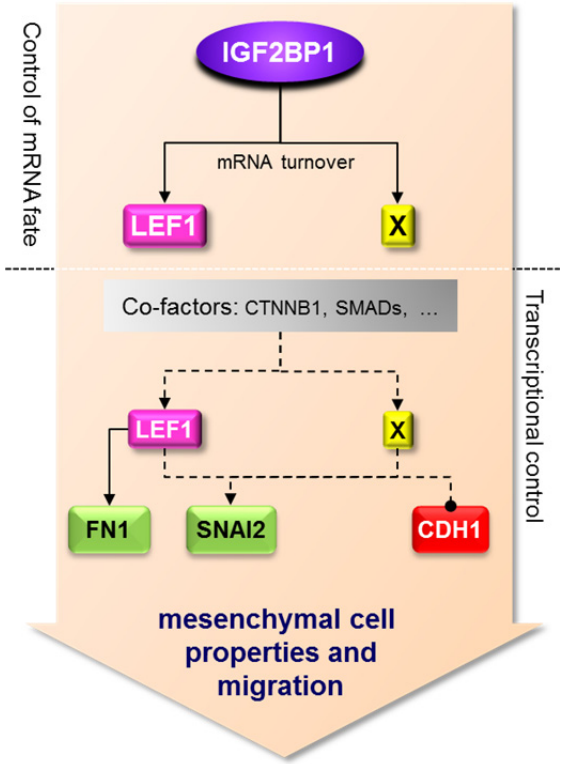


Figure 4.1: Scheme proposing that IGF2BP1 promotes mesenchymal cell properties at the post-transcriptional level by preventing LEF1 mRNA degradation and potentially other targets (indicated as x). Up-regulation of LEF1 enhances transcription of mesenchymal markers (indicated in green) like fibronectin (FN1) and SNAI2. Moreover, up-regulation of transcriptional repressors like SNAI2 interferes with the expression of epithelial markers (e.g.CDH1, indicated in red). Direct regulation: solid lines; indirect or yet to be validated regulation: dashed lines.

4.3 IGF2BP1 a prognostic marker in squamous cell carcinoma?

In addition to analyzing the function of IGF2BP1 in post-transcriptional control of gene regulation in tumor-derived cells this study evaluated expression levels of the IGF2BP family members in squamous cell carcinoma of the head and neck (HNSCC) from patient`s tissue samples. This type of cancer is relatively common and is characterized by high morbidity, high mortality and few therapeutic options besides surgery, cytotoxic chemotherapy and radiation (Rothenberg and Ellisen, 2012). Although new therapeutic approaches like the use of the epidermal growth factor receptor (EGFR)-specific antibody cetuximab combined with radiotherapy are

available to treat HNSCC, survival has not markedly improved in recent decades (Leemans et al., 2011). This is due to the fact that patients still frequently develop locoregional recurrences, distant metastases and second primary tumors (Leemans et al., 2011). The information available on the molecular carcinogenesis of HNSCC is still limited. Therefore the identification of new prognostic markers will help to understand cancer development and improve treatment of squamous cell carcinomas.

IGF2BP3 was suggested as prognostic biomarker in oral and tongue squamous cell carcinomas in two independent studies (Li et al., 2011a; Li et al., 2011b). High expression of IGF2BP3 was observed in tongue SCC with lymphoid metastases compared with non-metastatic tumors (Li et al., 2011a). IGF2BP3 up-regulation was also related to tumor invasion and metastases in oral squamous cell carcinoma (Li et al., 2011b). Recent studies correlated the expression of IGF2BP3, besides p53, with the risk of death in oral squamous cell carcinoma (Kim et al., 2012). However the studies regarding IGF2BP3 expression in SCC were performed using immunohistochemistry rather than mRNA analyses. This may be misleading since highly paralogue specific antibodies are barely available for IGF2BPs due to their high homology of their protein sequences (70-99% among all VICKZ protein members (Yisraeli, 2005)). The published expression studies in SCC did not show any negative results for IGF2BP1 or 2 expressions in these tumor types. Therefore one cannot exclude cross-reactivity e.g. by the used polyclonal goat anti-IGF2BP3 antibody with the other IGF2BP-family members. An up-regulation of IGF2BP1 for example could interfere with the obtained results.

In the present study tumor samples from ~120 patients suffering from HNSCC were analyzed for the expression of all IGF2BPs on the mRNA level. In contrast to previous findings a significant up-regulation of IGF2BP1 and not IGF2BP3 was observed in tumor samples compared to tissue samples classified as normal. IGF2BP2, IGF2BP3 as well as the control RPLP30 remained equally expressed in normal and tumor-derived tissue samples. Furthermore the enhanced expression of IGF2BP1 was preserved in SCC-derived cell lines when compared to the mean expression level of IGF2BP1 in normal tissue samples. However this study needs further evaluation to support the findings for an up-regulation of IGF2BP1 for example by immunohistochemistry. Analyses regarding biological relevance for example by

determining the survival rate of the patients in correlation with IGF2BP1 expression levels are also required to elucidate the role of IGF2BP1 in HNSCC. In addition, functional analyses in the SCC-derived cell lines are needed to correlate the de-regulation of this RBP with tumor-relevant cell properties like migration or invasiveness. These analyses, however, indicate that IGF2BP1, rather than IGF2BP3, may be used as biomarker for HNSCC and may present a valid candidate for new therapeutic approaches.

5 Summary

The oncofetal IGF2 mRNA-binding protein 1 (IGF2BP1) controls the migration and invasiveness of primary as well as tumor-derived cells *in vitro* by regulating the fate of target mRNAs at the post-transcriptional level. Whether the protein is also involved in epithelial-mesenchymal-transition (EMT), a fundamental process of tumor cell dissemination, remained elusive.

In this study, it was disclosed that IGF2BP1 sustains and/or promotes mesenchymal-like cell properties of non-tumor- and tumor-derived cells by enhancing the expression of the transcriptional regulator LEF1. IGF2BP1 directly associates with LEF1 mRNAs and prevents their degradation in a 3`UTR dependent manner. The elevated expression of LEF1 in turn up-regulates the transcription of the mesenchymal marker fibronectin (FN1) by associating with its promoter region. Moreover, IGF2BP1 enhances the expression of the EMT-driving factor SNAI2, presumably via LEF1-dependent indirect promoter activation. Accordingly, IGF2BP1 depletion causes MET-like morphological changes. Cell-cell contact formation is enhanced and extracellular matrix composition becomes modified in various cell types. In addition, IGF2BP1-driven cell motility was demonstrated to depend on the expression of LEF1 and SNAI2. These findings identify a novel function of IGF2BP1 as a pro-mesenchymal post-transcriptional determinant. Through the elevated expression of EMT-driving transcriptional regulators and mesenchymal markers IGF2BP1 is suggested to play a role in tumor cell dissemination.

In addition, expression studies of the IGF2BPs in head and neck squamous cell carcinoma (HNSCC) revealed an up-regulation of IGF2BP1 in this type of cancer which remained preserved in SCC-derived cell lines. These findings emphasize the oncogenic role of IGF2BP1 and support its function in tumor development and progression.

6 References

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7 Abbreviations

µ	micro
α-SMA	alpha smooth muscle actin
Ap-1	activator protein 1
APC	adenomatous polyposis coli
BMP	bone morphogenetic protein
bp	base pair
BSA	bovine serum albumin
BTRC	beta-transducin repeat containing E3 ubiquitin protein ligase
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CK1	casein kinase 1
cm	centimeter
DNA	deoxynucleic acid
dNTPs	deoxyribonucleotide triphosphates
DVL	dishevelled
e.g.	exempli gratia
ECM	extra cellular matrix
EGF	epidermal growth factor
eIF4E	eukaryotic translation initiation factor 4E
ELISA	Enzyme Linked Immunosorbent Assay
EMT	epithelial-mesenchymal-transition
et al.	*et altera*
FBS	fetal bovine serum
FER	feline encephalitis virus-related kinase
FF	Fopflash
FFL	firefly luciferase
FGF	fibroblast growth factor
FSP1	fibroblast-specific protein 1
g	gram
GFP	green fluorescent protein
GSK3B	glycogen synthase kinase beta 3
h	hour
HBS	HEPES buffered saline

HCV	hepatitis C virus
HGF	hepatocyte growth factor
HMG	high-mobility group
HMGA2	high mobility group AT-hook 2
HNSCC	head and neck squamous cell carcinoma
IGF2	insulin-like growth factor 2
IGF2BP1-3	insulin-like growth factor 2-mRNA binding protein1-3
IGF2R1	insulin-like growth factor 2 receptor 1
IP	Immunoprecipitation
IRES	Internal ribosome entry site
l	liter
LEF1	lymphoid enhancer-binding factor 1
LRP5/6	low density lipoprotein receptor-related protein 5/6
m	milli
M	molar
MAPK	mitogen-activated protein kinase
MDCK	Madin Darby canine kidney
MDR1	multidrug resistance protein 1
MEK	mitogen-activated protein kinase kinase
MET	mesenchymal-epithelial-transition
min	minutes
MITF	microphthalmia-associated transcription factor
MMP	matrix metalloprotease or matrix-metallopeptidase
mRNA	messenger ribonucleic acid
mTOR	mechanistic target of rapamycin/ mammalian target of rapamycin
n	nano
NFκB	nuclear factor kappa B
nm	nanometer
p	pico
PAR-CLIP	Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
pH	*Potentium Hydrogenii*
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
qRT-PCR	quantitative real-time PCR
RBP	RNA binding protein

RFP	red fluorescent protein
RIP	RNA-immunoprecipitation
RNA	ribonucleic acid
RNP	ribonucleoprotein
rpm	rounds per minute
RT	reverse transcriptase
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription PCR
SCC	squamous cell carcinoma
SDS	sodium dodecyl sulfate
shRNA	small hairpin RNA
siRNA	small interfering RNA
SMAD	mothers against decapentaplegic homolog
T2D	type 2 diabetes
TCF	T-cell factor
TF	Topflash
TGF-α	transforming growth factor alpha
TGF-β	transforming growth factor beta
TGF-βR	transforming growth factor beta receptor
TP53	p53, tumor protein p53
UTR	untranslated region
v/v	volume per volume
VCL	Vinculin
Vg1RBP	Vg1-mRNA binding protein
w/v	weight per volume
WB	washing buffer
WRE	WNT responsive element
ZBP1	zipcode binding protein 1

Acknowledgements

My doctoral studies have been a great experience. I owe sincere and earnest thankfulness to the people that contributed to its successful completion.

I am truly grateful to Prof. Dr. Stefan Hüttelmaier for providing an extremely interesting research topic and giving me the opportunity to work in an excellent research environment. Through his guidance I was able to take on challenges and grow with their tasks.

I would like to thank all the members of the Hüttelmaier lab for their support and discussions but also for their friendships. I´m especially thankful to Dr. Nadine Stöhr for her advice, patience and motivation - in the lab as well as outside. In addition I would like to express my gratitude to Marcel Köhn and Dr. Marcell Lederer for suggestions and scientific support. Dr. Marcell Lederer and Dr. Nadine Stöhr greatly contributed to the success of this project. I´d like to thank Marlen Mrotzek as equal contributor for her work in analyzing HNSCC tumor samples as well as Dr. Matthias Kappler for providing the samples.

I am grateful to Prof. Dr. Sven-Erik Behrens, Prof. Dr. Stefan Hüttelmaier and Assistant-Professor Dr. Daniel Zenklusen for reviewing my thesis.

I want to thank Prof. Dr. Sylvain Meloche for giving me the opportunity to work in his lab in Montreal for three months. It was a great experience and helped me to improve my scientific expertise.

I am sincerely thankful to my friends for encouraging and supporting me.

Most importantly, none of this would have been possible without the love and strength of my family. They helped and supported me at every stage of my personal and academic life.

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DECLARATION

I, Anne Zirkel, hereby declare that this dissertation entitled, “IGF2 mRNA-binding protein 1 promotes mesenchymal cell properties by enhancing the expression of LEF1” is my own work, and that all the sources I have used or quoted have been indicated or acknowledged by means of completed references.

Publications

Zirkel A, Lederer M, Stöhr N, Pazaitis N, Hüttelmaier S. IGF2BP1 promotes mesenchymal cell properties and migration of tumor-derived cells by enhancing the expression of LEF1 and SNAI2 (SLUG), Nucleic Acids Research, 2013 - accepted

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