Post-transcriptional networks control epithelial-to-mesenchymal transition in anaplastic thyroid carcinomas

and

miTRAP – an *in vitro* method to identify regulatory microRNAs

Dissertation

zur Erlangung des akademischen Grades
doctor rerum naturalium
(Dr. rer. nat.)

der

Naturwissenschaftlichen Fakultät I - Biowissenschaften -
der Martin-Luther-Universität Halle-Wittenberg,
vorgelegt

von Frau Juliane Braun
geboren am 16.07.1981 in Erfurt

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Halle, 10. November 2014

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1 ABSTRACT

Gene expression is severely deregulated in cancer, leading to uncontrolled cell growth, proliferation, and tissue dedifferentiation. In late-stage cancers, these processes culminate in the formation of metastasis, resulting in a poor patient prognosis. Carcinoma cell dissemination is essentially enhanced by the transition from an immotile epithelial cell phenotype to a motile mesenchymal-like phenotype. Such malignant form of epithelial-to-mesenchymal transition (EMT) is facilitated by the reprogramming of gene expression at the transcriptional as well as post-transcriptional level.

This doctoral study investigated the interplay of transcriptional and post-transcriptional regulators involved in malignant EMT of anaplastic thyroid carcinomas (ATCs). *E-cadherin* (*CDH1*) and the *miR-200-3p* family (f), genes strongly associated with the epithelial phenotype, were severely downmodulated in tumor tissues of patients suffering from ATCs. In contrast to ATCs, less aggressive subclasses of thyroid cancer preserved *CDH1* and *miR-200-3p* family expression. The onset of EMT in ATCs was accompanied by the increased abundance of the *CDH1*- and *miR-200-3p*-repressing transcription factor ZEB1. Malignant EMT as well as *ZEB1* expression are generally induced by cytokines of the transforming growth factor β (TGFB) family, which trigger SMAD-dependent transcription. Studies in ATC-derived cells revealed a negative feedback regulation between the TGFB signaling pathway and the *miR-200-3p* family. Consistent with this negative feedback loop, *TGFB receptor type I* (*TGFBR1*) and *SMAD2* expressions were elevated in ATCs. In addition to the tumor-suppressive *miR-200-3p* family, comparative microRNA (miRNA) expression analyses of ATC and non-transformed tissues identified the *miR-30-5p* family as significantly reduced in ATCs. Like *miR-200-3p f, miR-30-5p f* interfered with cancer cell invasion and mesenchymal marker expression.

The observed tumor-suppressive functions of *miR-200-3p f* and *miR-30-5p f* in ATC-derived cells suggested post-transcriptional regulators as powerful suppressors of malignant EMT in ATCs. However, in contrast to the reduced expression of tumor-suppressive miRNAs, the *IGF2* mRNA-binding protein 1 (IGF2BP1) was *de novo* synthesized in ATC. *IGF2BP1* expression is essentially lost in adult tissues; however, during development IGF2BP1 promotes cell migration and proliferation. Several studies identified *IGF2BP1* expression in neoplasia and suggested a role in tumor progression. In ATC tissues, re-expression of *IGF2BP1* correlated with the loss of epithelial marker

expression and the increased abundance of ZEB1. Consistent with this observation, loss-of-function studies revealed that IGF2BP1 sustained *ZEB1* expression, as well as the migratory, invasive and proliferative potential of ATC-derived cells. Interestingly, the reduced abundance of IGF2BP1 protein and *IGF2BP1* mRNA upon *ZEB1* depletion indicated that ZEB1 in turn sustains *IGF2BP1* expression. Accordingly, a positive feedback regulation between IGF2BP1 and ZEB1 may synergize in promoting ATC tumorigenesis. Taken together, this doctoral study identified post-transcriptional regulators as potent biomarkers of ATCs and putative targets for therapeutic protocols.

In addition to ATC-focused studies, the miTRAP (miRNA trapping by RNA *in vitro* affinity purification) approach was developed. MiTRAP allows miRNA co-purification from cellular extracts with immobilized *in vitro* transcribed MS2-tagged bait RNAs. With this protocol, reported miRNA binding to the *MYC* and *ZEB2* 3'UTRs was confirmed. Combined with next generation sequencing of miRNAs, miTRAP identified novel *MYC*-regulatory miRNAs. Loss-of-function studies confirmed their potency in controlling the expression of *MYC in vivo*. Hence, these findings proved the suitability of *in vitro* RNA affinity purifications to reliably and comprehensively identify regulatory miRNAs for a bait RNA of choice in a given cell context of interest.

2 INTRODUCTION

Multicellular organisms consist of phenotypically distinct cell types. Although most cells comprise the same set of genetic information, cellular heterogeneity essentially relies on cell-specific gene expression signatures. Whereas some genes are expressed in every cell, others are expressed in a cell type-specific manner. To regulate these processes, the way from a gene to its product is controlled at every stage including transcription of the DNA, processing of the transcript, transport of the transcript to the cytoplasm, localization of the transcript within the cell and stability of the transcript. In the case of protein-coding genes also translation efficiency and protein stability are subjected to regulation. In addition to transcriptional regulators, which affect all of the following steps, a plethora of post-transcriptional regulators, e.g. microRNAs and RNA-binding proteins, are necessary to control gene expression, and thereby cell type-specific homeostasis and function, as well as cellular reorganization in developmental processes. The scientific interest to study post-transcriptional mechanisms increased strongly with the identification of aberrant post-transcriptional gene regulation associated to developmental defects and diseases like cancer.

Anaplastic thyroid carcinomas

Anaplastic thyroid carcinomas (ATCs) originate from follicular (thyroid epithelial) cells of the thyroid. The thyroid gland is composed of follicles filled with thyroid hormones containing colloid. Follicles are lined by follicular cells that produce thyroid hormones (T3, T4) and to a small proportion by parafollicular cells (C cells) that produce calcitonin. Thyroid cancer can originate from follicular or parafollicular cells. In contrast to the rare C cell-originating medullary thyroid carcinomas (MTCs), thyroid cancers of follicular origin are histopathologically classified into four major subclasses: well-differentiated follicular or papillary thyroid carcinomas (FTCs or PTCs), poorly differentiated thyroid carcinomas (PDTCs), and undifferentiated anaplastic thyroid carcinomas. PTCs are the most frequent thyroid carcinomas (approximately 90%). This subtype is composed of epithelial cells with visible changes in nuclear morphology and appearance. FTCs with a prevalence of less than 10% are also composed of differentiated epithelial cells, but lack nuclear features of PTCs. Although some of these well-differentiated carcinomas behave aggressively, the vast majority of PTCs and FTCs can be effectively cured. ATC tumors are composed of undifferentiated cells with a high mitosis rate, spindle-like cell

morphologies as well as osteoclast-like giant cells. The patients suffer from a rapidly growing neck mass and metastatic spread to other organs. Accordingly, all ATCs are classified as stage IV diseases, and the patient's median survival time is only six months. PDTCs represent an 'intermediate' entity between ATCs and well-differentiated thyroid carcinomas. They appear partially dedifferentiated compared to FTCs or PTCs, and behave more aggressively than these. (Thyroid tumor characteristics are reviewed in [Kondo *et al.*, 2006; Cornett *et al.*, 2007; O'Neill *et al.*, 2010; Schmid, 2010].)

Although ATCs account for a small fraction of thyroid cancers (2-7%), over half of thyroid cancer associated deaths are related to the anaplastic variant. The essential part of ATC treatment displays surgery, if manageable full resection, often involving vital organs like trachea or larynx, followed by chemotherapy and radiation. Risk factors for ATCs are age and female sex. The mean age of ATC diagnosis is 65 years and 60 - 70% of tumors occur in women. It was also suggested that ATCs arise from pre-existing FTCs and PTCs by post-malignant dedifferentiation. This suggestion is supported by the histological observation of concurrent well-differentiated forms of thyroid cancer, as well as the identification of FTC- and PTC-specific mutations in ATCs (Figure 1). Historical events exhibit radiation exposure as the major risk for PTCs, since atomic bomb survivors and Chernobyl victims frequently developed these tumors. The major risk for FTCs represents dietary iodine deficiency resulting in thyroid growth as a compensatory mechanism. Other risk factors for thyroid carcinomas are active oxygen-species like H_2O_2, which is necessary for thyroid hormone synthesis.

For neoplastic transformation activating mutations or rearrangements in genes that encode MAPK (mitogen-activated protein kinase) pathway effectors like RAS (rat sarcoma viral oncogene homologue) or BRAF (v-raf murine sarcoma viral oncogene homologue B) seem to be required. Such oncogenic activation of the MAPK signaling pathway was suggested to promote proliferation and genomic instability, which possibly causes additional somatic mutations. Moreover, activating mutations in *PIK3CA* (*phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha*) occur in ATCs and FTCs, and potentially enhance phosphatidylinositol-3 kinase (PI3K)/Akt signaling. Increased expressions of growth factor receptors have been identified in all subclasses of thyroid carcinomas. Activated by growth factors, these receptors were suggested to trigger in addition to the MAPK pathway also the PI3K/Akt pathway. Tumor growth activity of ATCs and PTCs was also suggested to be governed by the increased expression of *cyclins* and the downregulation of *cyclin-dependent kinase inhibitor* (*CDKI*) genes. The predominant association of *TP53* (*tumor protein p53*) mutations with ATCs

and PDTCs suggested TP53 as a crucial gatekeeper in the progression from manageable to lethal thyroid cancers. Loss-of-function mutations of *TP53* were suggested to induce genomic instability, cell cycle progression and epithelial dedifferentiation. Additionally, ATC and PDTC-specific mutations in *CTNNB1 (β-catenin*) were proposed as precautions for aberrant Wnt signaling, which also promotes proliferation and epithelial dedifferentiation. Accordingly, ATCs were proposed to exhibit a genetic status of maximal activated signal transduction, which promotes cell growth, proliferation, survival and dissemination. (Classification, histological and pathogenic characteristics, risk factors, and treatment of thyroid carcinomas are reviewed in [Are and Shaha, 2006; Kondo *et al.*, 2006; Cornett *et al.*, 2007; O'Neill *et al.*, 2010; Schmid, 2010; Regalbuto *et al.*, 2012; Denaro *et al.*, 2013; Pallante *et al.*, 2014], genetic alterations of thyroid carcinomas are reviewed in [Kondo *et al.*, 2006; Sobrinho-Simoes *et al.*, 2008; O'Neill *et al.*, 2010; Schmid, 2010; Xing, 2010; Pallante *et al.*, 2014].)

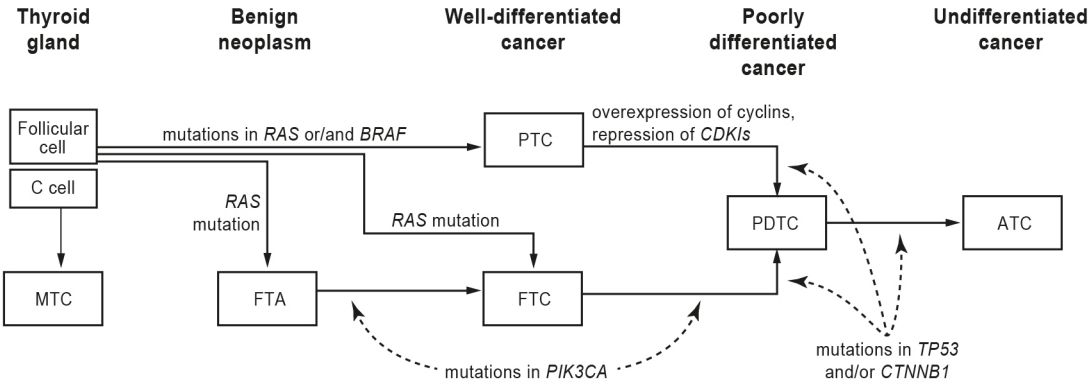

Figure 1 | Multistep carcinogenesis model of thyroid cancer and the occurrence of alterations in gene expression.
The thyroid gland contains follicular and C cells. Medullary thyroid carcinomas (MTC) derive from C cells. The vast majority of benign thyroid tumors are follicular thyroid adenomas (FTA) that often carry *RAS* point mutations. Carcinomas of follicular origin are subdivided in well-differentiated follicular thyroid carcinomas (FTC) and papillary thyroid carcinomas (PTC), poorly differentiated thyroid carcinomas (PDTC) and undifferentiated anaplastic thyroid carcinomas (ATC). *RAS* mutations have been associated with FTCs, PTCs, PDTCs and ATCs. In addition to *RAS*, genetic defects that activate *BRAF* often represent PTC-initiating events. Both *RAS* and *BRAF* mutations have been identified in PDTCs and ATCs. Additionally, the overexpression of genes encoding cyclins and the repression of genes encoding CDKIs has been identified with increasing incidence from PTCs, over PDTCs to ATCs. Like *BRAF* mutations in PTCs, mutations activating *PIK3CA* were identified in FTCs, and with high incidence in ATCs. ATC- or PDTC-specific genetic events represent the mutations of *TP53* and *CTNNB1*, which potentially lead to an undifferentiated state. Note, figure displays only those alterations of FTAs, FTCs, PTCs, and PDTCs yet identified in ATCs. (Scheme modified from [Kondo et al., 2006; Pallante et al., 2014].)

Epithelial-to-mesenchymal transition in cancer

ATC patients suffer from a rapidly growing tumor mass and a high metastatic spread [O'Neill *et al.*, 2010]. Metastases were suggested to be responsible for more than 90% of cancer-associated mortality [Brabletz, 2012]. Accordingly, the investigation of these phenomena is of special interest. The prerequisite for carcinoma cell invasion and metastasis formation is the conversion of an immotile epithelial to migratory/invasive mesenchymal-like cell phenotype. Interconnected by cell-cell junctions (Figure 2A), epithelial cells line cavities and free surfaces of the body, and form barriers to inhibit the movement of solutes and cells. Mesenchymal cells, by contrast, are predominantly attached to the interstitial extracellular matrix (ECM), where they can move in three dimensions (Figure 2B). Accordingly, the biological process of an epithelial-to-mesenchymal transition (EMT) requires changes in cell morphology, cell architecture, adhesion and migration capacity. To achieve these changes, cellular gene expression is reprogrammed resulting in the downregulation of epithelial genes and the upregulation of mesenchymal genes. In contrast to cancer, coordinated EMTs, as well as the reverse mesenchymal-to-epithelial transitions (METs) are fundamental processes during embryogenesis to allow germ layer formation and organ development (type 1 EMT). In adult tissues, EMTs are also necessary for wound healing, tissue regeneration and fibrosis (type 2 EMT). These events generate fibroblasts or myofibroblasts to reconstruct tissues that were injured by trauma or inflammation (type 2 EMT). (Epithelial and mesenchymal cell characteristics, as well as EMT and MET are reviewed in [Alberts *et al.*, 2002; Kalluri and Weinberg, 2009; De Craene and Berx, 2013; Jaime A. Rivera-Pérez, 2013; Nisticò *et al.*, 2013].)

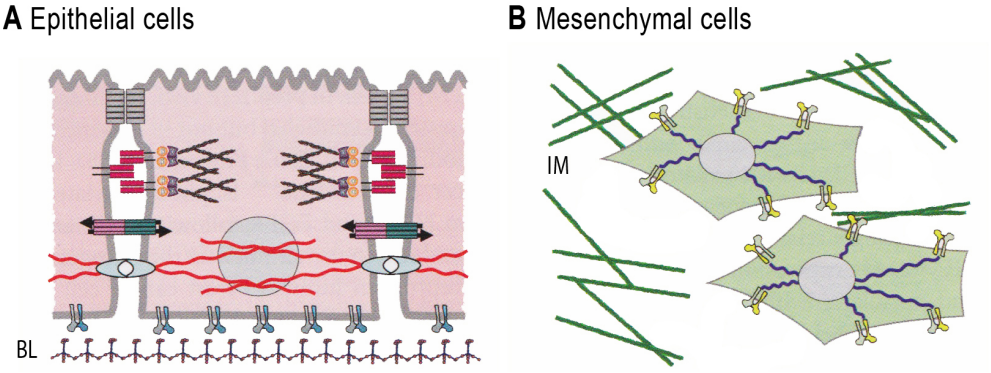

Figure 2 | Epithelial and mesenchymal cell characteristics.
(A) Epithelial cells are interconnected through tight junctions (grey), adherens junctions (red), which are connected to the actin cytoskeleton (black filaments), gap junctions (pink/blue), and desmosomes (cyan), which are connected to the cytokeratin-based intermediate filament cytoskeleton (red filaments). Epithelial cells also have specialized cell-ECM (extracellular matrix) interactions (hemidesmosomes) for adhesion to the laminin-rich basal lamina (BL), a specialized form of the ECM that underlies epithelial tissue. Note scheme represents epithelium consisting of one cell layer, however, dependent on the organ, epithelial tissue is composed of two or more epithelial cell layers. (B) Mesenchymal cells show a shift to a vimentin-based intermediate filament (dark blue) cytoskeleton and altered composition of cell-ECM interactions optimized for adhesion to the interstitial matrix (IM, green fibers). (Illustrations modified from [Nisticò *et al.*, 2013])

An important hallmark of malignant EMT (type 3 EMT) is the loss of E-cadherin (CDH1)-based adherens junctions (see Figure 2). Such adherens junctions (AJs) link neighboring epithelial cells together, mechanically stabilize the tissue and control the cell shape through the association with the actin cytoskeleton (reviewed in [Lecuit and Lenne, 2007; Guillot and Lecuit, 2013]). CDH1 is a trans-membrane protein consisting of five 'extracellular cadherin' domains (ECs), EC1–5, which form calcium-dependent interactions with AJs of the same kind on adjacent epithelial cells (reviewed in [Perez-Moreno and Fuchs, 2006]). Through these interactions cadherins function as surface markers. Experiments in cell culture revealed that epithelial cells expressing the same levels of CDH1 aggregate into tissue-like structures, whereas cells expressing different CDH1 levels are sorted out [Friedlander *et al.*, 1989; Chanson *et al.*, 2011]. Beyond the cell surface, the cytoplasmic tail of CDH1 binds CTNNB1, which connects CDH1 via CTNNA1 (α-catenin) to the actin cytoskeleton [Perez-Moreno and Fuchs, 2006].

CDH1 expression was demonstrated to be directly repressed by various pro-mesenchymal transcription factors (TFs) such as the zinc-finger proteins SNAI1, SNAI2 (*Drosophila melanogaster* homologues Snail 1 and 2) and KLF8 (Kruppel-like factor 8), the zinc-finger E-box-binding homeobox proteins ZEB1 and ZEB2, and the basic helix-loop-helix (bHLH) factors TWIST1, TWIST2 (twist family bHLH transcription factor 1, 2) and E47 (transcription factor 3). These factors were suggested to be the master EMT-inducing transcription factors. Their high potency to induce EMT relies in addition to *CDH1* repression on the repression of other epithelial genes that encode epithelial intermediate filaments (cytokeratins), components of tight junctions (claudins, occludin) as well as desmosomes (plakophilins, desmoplakin). Moreover, they activate the expression mesenchymal genes encoding for vimentin (mesenchymal intermediate filament), proteins of the ECM (fibronectin, vitronectin, collagen III, V), and matrix metalloproteases (MMPs). These pleiotropic changes in gene expression were suggested to be facilitated by the interaction with epigenetic modifiers like histone deacetylases.

All master EMT-inducing TFs bind to E-box elements formed by the consensus sequence CANNTG (N = C/G). Although targeting highly similar DNA elements, each factor exhibits a different target specificity, generated most likely by specific interactions with transcriptional co-activators or co-repressors. This hypothesis is supported by two findings: 1) Each TF modulates gene expression in a cell context-dependent manner. 2) Protein-protein interaction domains of some EMT-driving TFs, for instance the homeodomain of ZEB1 and ZEB2 share, in contrast to other domains, little sequence

similarity. (EMT-inducing TFs are reviewed in [Peinado *et al.*, 2007; Xu *et al.*, 2009; Gheldof *et al.*, 2012; Scheel and Weinberg, 2012; De Craene and Berx, 2013].)

TGFB signaling directly activates the expression of EMT-inducing transcription factors as demonstrated in various biological systems e.g. lung carcinoma-derived A549 cells [Kasai *et al.*, 2005] and kidney-derived MDCK cells [Gregory *et al.*, 2008a]. TGFB binds to a heterotetrameric complex of type I and type II serine/threonine kinase receptors (TGFBR1/2). Upon ligand binding, both receptors come in close proximity and the constitutive active TGFBR2 phosphorylates TGFBR1, which subsequently phosphorylates SMAD2 and SMAD3 (similar to the gene products of the *Drosophila melanogaster* gene 'mothers against decapentaplegic' (Mad)). Together with SMAD4, the SMAD2/3/4 complex translocates into the nucleus, where it interacts with DNA-binding transcription factors (Figure 3). (TGFB signaling is reviewed in [Xu *et al.*, 2009; Ikushima and Miyazono, 2010].) In addition to such canonical TGFB signaling, activated TGFBR complexes can transduce TGFB signals via the MAPK or Wnt signaling pathway (non-canonical TGFB signaling) (Figure 3). Both pathways have been shown to activate the expression of various EMT-inducing transcription factors. It is suggested that acting alone, canonical TGFB signaling is not sufficient to permanently convert an epithelial into a mesenchymal phenotype. Moreover, it is hypothesized that EMT induction has to be promoted by additional signaling molecules including receptor tyrosine kinase ligands that triggers the canonical MAPK/RAS pathway and the Wnt ligand that triggers the canonical Wnt signaling pathway (Figure 3) (Crosstalk of the TGFB signaling pathway with MAPK and Wnt signaling pathways is reviewed in [Huber *et al.*, 2005; Xu *et al.*, 2009; Heldin *et al.*, 2012]).

In advanced cancers, TGFB is often overexpressed (reviewed in [Heldin *et al.*, 2012]), whereas the MAPK pathway is activated by oncogenic mutations in *RAS* and/or its downstream factor *BRAF* (reviewed in [Friday and Adjei, 2008]), as indicated for thyroid cancer (see Figure 1). Oncogenic Wnt pathway mutations were mostly observed in tissues that depend on Wnt for self-renewal and repair as e.g. colon mucosa, but have also been proposed for ATCs (see Figure 1). Inappropriate stabilization of the Wnt downstream coactivator CTNNB1, or the formation of constitutive complexes of CTNNB1 with transcription factors like LEF1 (lymphoid enhancer binding factor 1) or TCF4 (T-cell specific transcription factor 4) trigger malignant proliferation and support EMT (Figure 3) (reviewed in [Clevers and Nusse, 2012]). Additionally, the loss of *CDH1* expression as a consequence of EMT, leads to an increase of the cytoplasmic CTNNB1 pool due to the lack of binding at adherens junctions [Clevers, 2006].

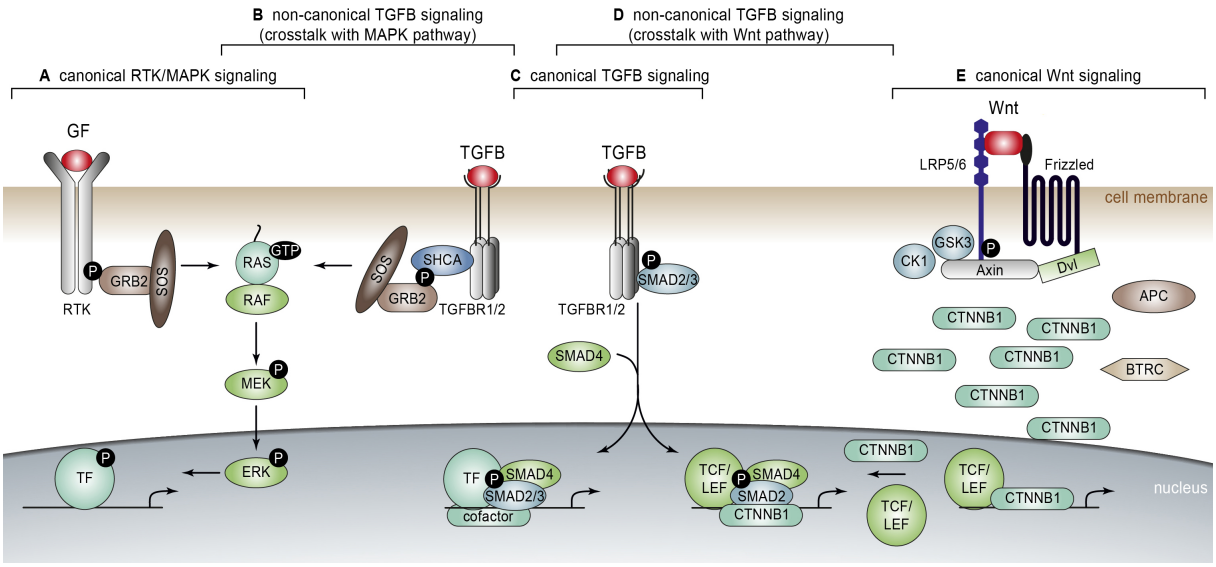

Figure 3 | Scheme of signal transduction pathways that crosstalk during EMT.
(A) The MAPK pathway is usually activated by auto-phosphorylation of receptor tyrosine kinase (RTK) complexes upon binding of growth factors (GF) like EFG (epidermal growth factor) or FGF (fibroblast growth factor). Then RTK phosphotyrosines recruit the GRB2/SOS/RAS complex, which activates the MAPK kinase kinase RAF (oncogene homologue of rodent raf (rapidly accelerated fibrosarcoma or rat fibrosarcoma)). SOS (son of sevenless) is the guanine exchange factor of RAS, and GRB2 (growth factor receptor-bound protein 2) is an adapter protein that binds phosphotyrosines. RAF then initiates a phosphorylation cascade involving MEK (MAPK/ERK kinase) and ERK (MAPK extracellular regulated kinase) resulting in the activation of transcription factors by phosphorylation (scheme modified from [Friday and Adjei, 2008]). (B, D) The non-canonical TGFB signaling results from the crosstalk with the MAPK or Wnt signaling pathway. (B) TGFB binds to the heterotetrameric complex of type I and type II serine/threonine kinase receptors (TGFBR1/2), which trigger the phosphorylation of SHCA (Src homology 2 domain containing). The SHCA phosphotyrosines allow docking of the GRB2/SOS/RAS complex, which activates the MAPK kinase cascade (scheme modified from [Chapnick *et al*., 2011; Gui *et al*., 2012]). (C) In canonical TGFB signaling, the activated TGFBR1 phosphorylates SMAD2 and SMAD3. Both form a complex with SMAD4. Through interactions with transcription factors like SNAIs and ZEBs, SMAD2/3/4 modulate transcription (scheme modified from [Xu *et al*., 2009; Xiao and He, 2010]). (D) TGFB signaling is also suggested to crosstalk with the Wnt signaling pathway through the transcription factors LEF1 (lymphoid enhancer binding factor 1) and TCF4 (T-cell specific transcription factor 4), as well as the coactivator CTNNB1. In this crosstalk, activated SMAD2/3/4 complexes would regulate gene expression in a complex with TCF4, LEF1 and/or CTNNB1 (scheme modified from [Xu *et al*., 2009]). (E) The canonical Wnt signaling pathway is initiated by the binding of the Wnt ligand to a receptor complex formed between the Frizzled receptor and its coreceptor LRP6 (low-density lipoprotein receptor related protein 6) or its close relative LRP5. Recruitment of the scaffold protein Dvl (Dishevelled) by Frizzled leads to LRP5/6 phosphorylation and Axin recruitment, which disrupts Axin-mediated phosphorylation and subsequent degradation of CTNNB1. The latter accumulates in the cell and serves as a coactivator for transcription factors like LEF1 or TCF4. In the absence of Wnt, cytoplasmic CTNNB1 is constantly degraded. A complex composed of Axin (scaffold protein), APC (adenomatous polyposis coli), CK1 (casein kinase 1) and GSK3 (glycogen synthase kinase 3) phosphorylates CTNNB1, which is then recognized by the E3 ubiquitin ligase BTRC (β-transducin repeat containing E3 ubiquitin protein ligase). Ubiquitinylation of CTNNB1 induces its proteasomal degradation (scheme modified from [MacDonald *et al*., 2009]).

In addition to the generation of highly invasive cells, the malignant EMT program was suggested to induce and/or promote a stemness-like phenotype [Mani *et al*., 2008; Morel *et al*., 2008; Scheel *et al*., 2011]. Like non-malignant stem cells, cancer stem cells (CSCs) are defined by their capability of renewing themselves unlimitedly through symmetrical and asymmetrical cell division. The latter generates a stem cell as well as a cell with the ability to differentiate into tumor tissue (reviewed in [Heldin *et al*., 2012; Scheel and Weinberg, 2012]). Hence, they have a high potential to generate a tumor, as demonstrated by studies with mice [Ishikawa *et al*., 2007; Quintana *et al*., 2008]. Therefore, CSCs are also termed tumor-initiating cells (TICs). The concept of migrating/invading cancer stem cells would explain how cancer cells survive and proliferate during dissemination and in the microenvironment of foreign tissues after extravasation (reviewed in [Brabletz *et al*., 2005; Heldin *et al*., 2012; Scheel and Weinberg,

2012]). CSCs represent a side population in solid tumors and cultured cancer-derived cell line populations (reviewed in [Lin, 2011; Scheel and Weinberg, 2012]), from which they can be isolated by cell-surface markers [O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007; Mani *et al.*, 2008]. Notably, carcinoma-originating CSCs were characterized by mesenchymal marker expression and epithelial dedifferentiation [Mani *et al.*, 2008].

Post-transcriptional gene regulation by microRNAs

MicroRNAs (miRNAs) are evolutionary conserved, small non-coding RNAs of 20-27 nucleotides in length. They serve as specificity guides for a protein complex termed miRNA-induced silencing complex (miRISC) that mediates post-transcriptional gene regulation. MiRNA genes are transcribed by RNA polymerase II into primary precursors (pri-miRNA) that fold into stem loop structures. The nuclear Drosha (class 2 ribonuclease type III)-containing microprocessor complex releases these stem loops and generates ~70-nucleotides long pre-mature miRNAs (pre-miRNAs). After transport to the cytoplasm, Dicer (class 3 ribonuclease type III) cleaves of their loop regions, and one strand of the resulting duplexes is incorporated into miRISC; the other strand is normally degraded. Apart from canonical miRNA biogenesis, pre-miRNAs can be generated from small nucleolar RNAs (snoRNAs) or short introns (mirtrons). The latter are Drosha-independent; instead the pre-miRNAs are liberated by the spliceosome. How snoRNAs are processed into pre-miRNAs remains largely elusive (MiRNA biogenesis is reviewed in [Pasquinelli, 2012; Meister, 2013]).

The miRISC minimally consists of an Argonaute (AGO) and a TNCR6 protein. Vertebrates contain four AGO (AGO1-4) and three TNRC6 paralogues (TNRC6A-C). TNRC6 that binds via AGO to the miRNA-mRNA hybrids, seems to play the central role in miRNA-mediated induction of mRNA decay and repression of mRNA translation. The protein was suggested to interact directly with the cytoplasmic poly(A)-binding protein (PABPC) and the deadenylase complexes CCR4-NOT and PAN2-PAN3. Both, the interaction with PABPC and the interaction with the CCR4-NOT complex, was suggested to result in the dissociation of PABPC from the poly(A)-tail of the mRNA, which increases the accessibility of deadenylases. As a consequence of ongoing deadenylation, the mRNA is decapped by the DCP1-DCP2 decapping complex and subsequently degraded by the 5’-to-3’ exonuclease XRN1 (5'-3' exoribonuclease 1) (Figure 4). However, in the case of fully complementary miRNA-mRNA duplexes these mechanisms seem dispensable, because AGO2 directs mRNA decay by endonucleolytic cleavage. (MiRNA-mediated mRNA degradation and miRISC components are reviewed

in [Tolia and Joshua-Tor, 2007; Huntzinger and Izaurralde, 2011; Pasquinelli, 2012; Braun *et al.*, 2013].)

Although the mechanism of miRNA-mediated mRNA decay is relatively well understood, the mechanism of miRNA-mediated translational repression is still controversially discussed. Conflicting data argue either for a repression of translation initiation or for post-initiation mechanisms. The latter was indicated by the association of miRNAs and target transcripts with polysomes in sucrose gradients [Olsen and Ambros, 1999; Maroney *et al.*, 2006; Nottrott *et al.*, 2006; Petersen *et al.*, 2006]. Additionally, cap-independent translation of reporters harboring an internal ribosome entry site (IRES) was shown to be repressed by miRNAs [Petersen *et al.*, 2006]. Two models of miRNA action after initiation were proposed: 1) MiRNAs reduce translation elongation rates, which results in a ribosome drop off [Petersen *et al.*, 2006]. 2) MiRNAs recruit proteases that degrade the growing protein chain [Nottrott *et al.*, 2006]. Several studies argued against these hypotheses, because they observed a shift of miRNA-targeted reporter RNAs towards lighter monosomal fractions, as well as miRNA-resistant IRES- or cap analog-driven translation [Humphreys *et al.*, 2005; Pillai *et al.*, 2005; Mathonnet *et al.*, 2007]. The hypothesis of a pre-initiation mechanism was further supported by the observation that *Drosophila melanogaster* AGO proteins bind directly to the m^7G cap [Djuranovic *et al.*, 2010]. However, the relevance of this association was questioned by tethering approaches [Eulalio *et al.*, 2008; Braun *et al.*, 2011]. Instead of AGO1, tethered *Drosophila melanogaster* TNRC6 was able to repress translation of a reporter RNA [Eulalio *et al.*, 2008; Braun *et al.*, 2011]. Translation repression by TNRC6 was suggested to essentially rely on the dissociation of the poly(A)-PABPC association, and thus the disruption of the translation promoting 'closed loop' configuration bringing the mRNA's 3'-end termination site in proximity to the 5'-Cap [Fabian *et al.*, 2009; Zekri *et al.*, 2009; Zekri *et al.*, 2013]. On the basis of this model, TNRC6 may mediate gene repression as a combination of mRNA degradation and translational repression by interacting with multiple components involved in translation and mRNA decay. Regarding the time course of these processes, different studies performed time-dependent analyses and suggested that miRNA-dependent silencing is initiated by translational repression, followed by deadenylation-induced mRNA decay [Selbach *et al.*, 2008; Fabian *et al.*, 2009; Djuranovic *et al.*, 2012].

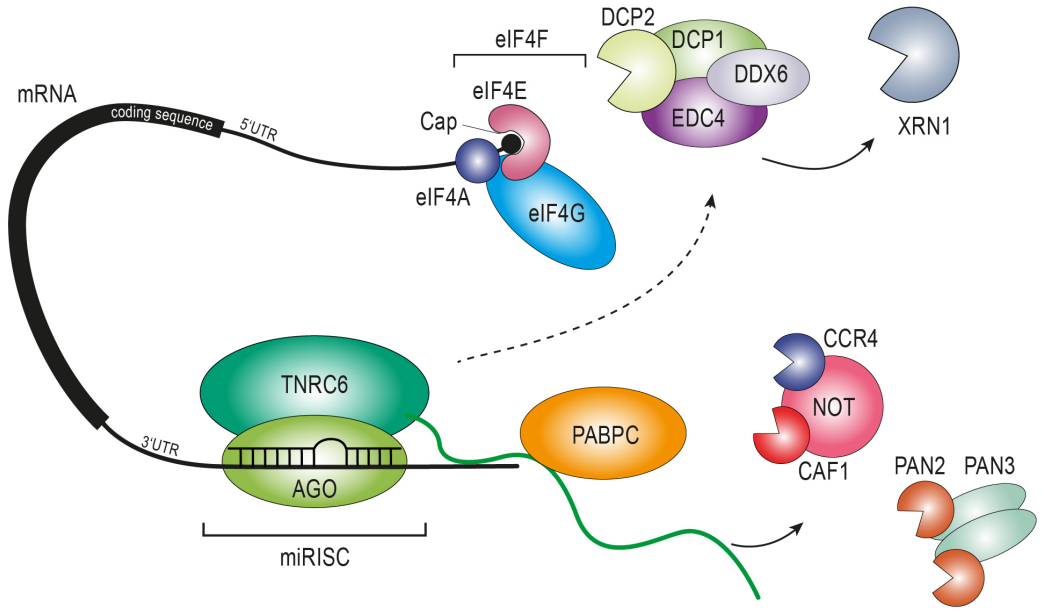

Figure 4 | Model of miRNA-mediated regulation of gene expression in animals.
MiRISCs, consisting of an miRNA, an AGO and a TNRC6 protein, bind to mRNA targets by incomplete base pairing. An mRNA consists of a 5'- and 3'-untranslated region (5'UTR and 3'UTR) as well as a coding sequence. TNRC6 interacts with PABPC leading to the release of PABPC from the poly(A)-tail, and the interference with the PABPC-eIF4G interaction resulting in an open loop configuration of the mRNA, which was suggested to contribute to a reduced translation efficiency. Moreover, TNRC6 interacts with the two cytoplasmic deadenylase complexes CCR4/NOT1/CAF1 and PAN2/PAN3, that deadenylate the mRNA; and TNRC6 promotes the association of the decapping complex (DCP1/2/DDX6/EDC4). EDC4, a component of the decapping complex, interacts with XRN1, which degrades the decapped mRNA from the 5'-end. (Illustration modified from [Huntzinger and Izaurralde, 2011].)

MicroRNA targeting site characteristics

Animal miRNA-target interactions are characterized by a discontinuous complementarity with one frequent feature: perfect pairing to the miRNA seed sequence comprising nucleotide 2 to 7 or 8, often supplemented with pairing to the 3'-end and a characteristic bulge in the center of the duplex (Figure 5A) (reviewed in [Bartel, 2009]). This so-called 'seed hypothesis' was supported by transcriptome-wide computational analyses that identified 6- or 7-mer seed-matching sequences as the most overrepresented sequences in 3'-untranslated regions (3'UTRs) of genes downregulated by ectopic miRNA expression [Grimson *et al.*, 2007]. Moreover, the seed sequence was considered the miRNA segment with the highest tendency to match multiple conserved elements within 3'UTRs [Lewis *et al.*, 2003; Lewis *et al.*, 2005]. In view of the 'seed hypothesis', miRNAs that comprise the same seed sequence predominantly regulate the same genes and are assigned to miRNA seed families (f) (Figure 5B). Nevertheless, miRNAs also exhibit non-canonical binding modes defined by discontinuous seed pairing with instead extensive complementarity to the miRNA center or 3'-end [Reinhart *et al.*, 2000; Shin *et al.*, 2010; Helwak *et al.*, 2013]. However, only a few of these 3'-compensatory, centered and 3'-end sites have been experimentally validated (Figure 5C-

E). This also applies for miRNA targeting sites (MTSs) with a near-perfect complementarity. To my knowledge, the only validated example is the *miR-196a-5p* MTS of *HOXB8* (*homeobox B8*) [Yekta *et al.*, 2004] (Figure 5F).

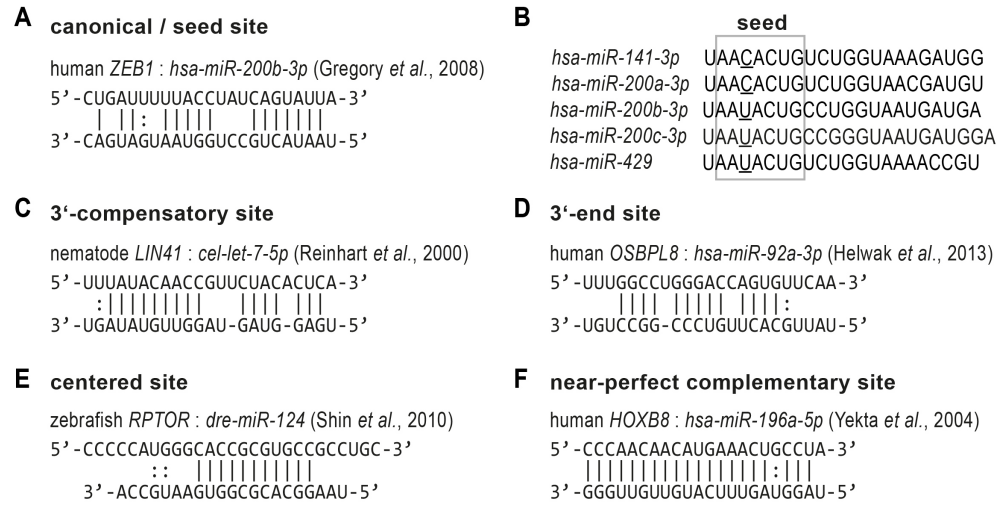

Figure 5 | Categories and functional examples of miRNA targeting sites.
(A) Canonical miRNA targeting sites are characterized by perfect complementarity to the miRNA seed sequence, and by complementarity to the 3'-end of the miRNA. The displayed example shows a *miR-200b-3p* targeting site within the *ZEB1* (zinc finger E-box binding homeobox 1)-3'UTR as a duplex with *miR-200b-3p*. (B) A miRNA seed family is characterized by the same seed sequence comprising nucleotide 2-7 or 8 (grey box). The human (hsa) *miR-200-3p* seed family comprises five members: *miR-141-3p*, *200a-3p*, *b-3p*, *c-3p*, *429*. Note although the seed sequences vary in one nucleotide (underlined), cytosine as well as uridine allow base pairing with guanosines. (C-E) Non-canonical MTSs are characterized by discontinuous seed-pairing, compensated by extensive 3'-end pairing (C, D), or extensive pairing to the miRNA center (E). Experimentally validated examples are (C) the nematode (*Ceanorhabditis elegans*, *cel*) *let-7-5p* MTS of *LIN41*, (D) the human *miR-92a-3p* MTS of *OSBPL8* (*oxysterol binding protein-like 8*) and (E) the zebrafish (*Danio rerio*, *dre*) *miR-124* MTS of *RPTOR* (*regulatory associated protein of MTOR, complex 1*). (F) A near-perfect complementary MTSs was identified for *miR-196a-5p* in the *HOXB8*-3'UTR.

The number of MTSs per transcript was suggested to correlate with the extent of miRNA-directed gene repression [Doench *et al.*, 2003; Grimson *et al.*, 2007]. Experimental data provide examples like the 3'UTRs of *IGF2BP1* (*IGF2 mRNA-binding protein 1*) or *ZEB1* that comprise multiple cognate MTSs for *let-7-5p* or *miR-200-3p* family members, which amplify miRNA-mediated repression resulting in a dramatic reduction of gene expression rather than fine-tuning [Boyerinas *et al.*, 2008; Gregory *et al.*, 2008a; Bartel, 2009]. These so-called 'switch interactions' were proposed to control cell fate decisions during development (reviewed in [Bartel, 2009]). Accordingly, target genes with roles in development and tissue-specific expression were suggested to preferentially harbor long 3'UTRs with evolutionary accumulated MTSs [Stark *et al.*, 2005]. In contrast, genes that are involved in basic cellular processes are co-expressed with multiple different miRNAs, and as a result of 'evolutionary avoidance' they were suggested to harbor short 3'UTRs with few single MTSs [Stark *et al.*, 2005]. In addition to multiple cognate targeting sites, other mRNA context features that enhance MTS efficiency were

hypothesized. These include: 1) MTS positioning within the 3'UTR at least 15 nucleotides from the stop codon; 2) MTS positioning away from the center of long UTRs; 3) MTS positioning in regions with a predicted accessible secondary structure; and 4) AU-rich nucleotide composition near the MTS [Grimson *et al.*, 2007; Bartel, 2009]. AU-rich sequences serve as binding sites for at least 20 different RNA-binding proteins known to be involved in mRNA degradation and/or suppression of translation [Meisner and Filipowicz, 2010]. Although, functional MTSs were also identified in 5'UTRs and coding sequences, these sites were suggested to rather enhance regulatory effects mediated by the 3'UTR than act on their own [Grimson *et al.*, 2007; Fang and Rajewsky, 2011].

The roles of microRNAs in cancer and EMT

By regulating multiple mRNAs, miRNAs are involved in a plethora of cellular processes including proliferation, apoptosis, differentiation, cell motility, and tissue invasion (reviewed in [Santarpia *et al.*, 2010; Kasinski and Slack, 2011]). These processes form the basis for cellular properties that define the hallmarks of cancer: 1) sustaining proliferative signaling, 2) evading antigrowth signals, 3) activating invasion and metastasis, 4) enabling replicative immortality, 5) inducing angiogenesis, and 6) resisting cell death (reviewed in [Hanahan and Weinberg, 2011]). The expressiveness of these characteristics was suggested to correlate with the differentiation stage of tumor cells (reviewed in [Jogi *et al.*, 2012]). A tumor composed of undifferentiated cells is more aggressive than its differentiated counterpart (reviewed in [Jogi *et al.*, 2012]). Although, differentiation denotes a developmental process whereby cells acquire the capacity for a specialized function, in cancer these processes can reverse (reviewed in [Jogi *et al.*, 2012]). The *let-7-5p* and *miR-200-3p* families have been identified as key regulators of general and epithelial differentiation processes, respectively. In many organisms including humans, *let-7-5p* family expression was reported to increase in late development to induce and sustain tissue differentiation (reviewed in [Boyerinas *et al.*, 2010]). A loss of their expression in turn marks tissue dedifferentiation in cancer. The *let-7-5p* family is expressed from eight different chromosomes and comprises twelve members (*let-7a-1, a-2, a-3, e, f-1, f-2, g, i,* and *miR-98*) that represent nine distinct *let-7-5p* sequences with identical seeds (reviewed in [Peter, 2009]). Major *let-7-5p f* targets include genes encoding for cell cycle regulators such as CDC25A (cell division cycle 25A) and CDK6 (cyclin-dependent kinase 6), promoters of proliferation including RAS and MYC (v-myc avian myelocytomatosis viral oncogene homologue), as well as genes involved in early development like *HMGA2* (*high mobility group AT-hook 2*) and *IGF2BP1.* Accordingly, *let-*

7-5p family members were established as common tumor-suppressive miRNAs. (The function of *let-7-5p* is reviewed in [Peter, 2009; Boyerinas *et al.*, 2010].)

The *miR-200-3p* family was shown to be a fundamental regulator of the epithelial phenotype and repressor of epithelial dedifferentiation. This miRNA family comprises five members (see Figure 3) that are expressed from two different gene clusters, *miR-141/200c* and *miR-200a/200b/429*. All members of the *miR-200-3p* family silence the expression of the EMT-inducing transcription factors ZEB1 and ZEB2. Like other epithelial genes, the promoter regions of the *miR-200* gene clusters comprise E-boxes, responsible for the repression by ZEB1 and ZEB2. Consistent with this double negative feedback loop, the expression of *miR-200-3p f* during development is restricted to epithelial cells of the endoderm and ectoderm, whereas *ZEB1*- and *ZEB2*-expressing mesoderm lacks *miR-200-3p f* expression. Similar expression patterns were hypothesized for solid tumors. Carcinoma-derived cell lines that underwent EMT also lack *miR-200-3p f* expression, but express *ZEB1* and *ZEB2*. (The role of *miR-200-3p* in EMT and cancer is reviewed in [Gregory *et al.*, 2008b; Peter, 2009; Brabletz, 2012; De Craene and Berx, 2013].)

Most recently, several other miRNAs with tumor- and EMT-suppressive functions have been identified. Two prominent examples are the *miR-34-5p* family and *miR-203a*, which were suggested to suppress the invasive and migratory potential, as well as stemness-like properties of cancer-derived cells in culture [Wellner *et al.*, 2009; Viticchie *et al.*, 2011; Ahn *et al.*, 2012; Wang *et al.*, 2013; Rokavec *et al.*, 2014]. Moreover, both miRNAs suppress *SNAI1* directly, and *ZEB1* indirectly [Siemens *et al.*, 2011; Moes *et al.*, 2012], potentially by the SNAI1-mediated repression of the *miR-141/200c* gene cluster [Burk *et al.*, 2008; Gill *et al.*, 2011; Siemens *et al.*, 2011]. Consistent with the reversibility of EMT and MET programs, SNAI1 and ZEB1 in turn directly and also indirectly inhibit *miR-203a* [Wellner *et al.*, 2009; Moes *et al.*, 2012] and *miR-34a-5p* [Siemens *et al.*, 2011; Ahn *et al.*, 2012] transcription, resulting in double negative feedback regulations.

Although it is suggested that miRNAs are generally downregulated in cancer [Lu *et al.*, 2005], some miRNAs are highly expressed in cancer and are defined as acting in an 'oncogenic' manner by targeting tumor-suppressor genes (reviewed in [Santarpia *et al.*, 2010; Kasinski and Slack, 2011]). Among those, the oncogenic *miR-21-5p* represents the best-studied candidate. It was found to be upregulated in various carcinomas, and suggested to mainly repress tumor-suppressor genes like *PTEN* (*phosphatase and tensin homologue*) and *PDCD4* (*programmed cell death 4*) (reviewed in [Selcuklu *et al.*, 2009; Liu *et al.*, 2010]). Moreover, *miR-21-5p* was proposed to promote mesenchymal marker

expression, stemness-like properties, *in vitro* cell migration and invasion [Asangani *et al.*, 2008; Yan *et al.*, 2011; Han *et al.*, 2012], as well as intravasation and metastasis formation as demonstrated in a mouse squamous cell carcinoma model [Bornachea *et al.*, 2012]. In accord to its pro-mesenchymal function, processing of *pre-miR-21-5p* was suggested to be stimulated by TGFB signaling [Davis *et al.*, 2008]. Other cellular mechanisms that cause deregulated miRNA expressions may involve genomic alterations like allelic amplification or deletion, transcriptional modulations like promoter methylation and an altered availability of transcription factors, as well as post-transcriptional mechanisms that affect miRNA biogenesis (reviewed in [Santarpia *et al.*, 2010; Kasinski and Slack, 2011]).

The comparative analyses of miRNA expression signatures from ATCs and non-transformed thyroid tissues by using microRNA microarrays determined a decreased expression of 20 and an elevated abundance of four miRNAs (*222-3p*, *198*, *let-7f-5p* and *let-7a-5p*) [Visone *et al.*, 2007]. The most severe decrease in expression was observed for a set of four miRNAs comprising *miR-30a-5p, -30d-5p, -125b-5p,* and *-26a-5p.* Another study demonstrated that this set of miRNAs is sufficient to discriminate ATCs from PTCs [Schwertheim *et al.*, 2009]. Although, gain-of-function studies in ATC-derived cells suggested a repressive function of *miR-125b-5p* and *-26a-5p* on ATC cell proliferation [Visone *et al.*, 2007], relevant target genes remain to be identified. The function of *miR-30a/d-5p* in ATCs was not determined. Another study focused exclusively on the determination and verification of miRNAs upregulated in all thyroid cancer, and identified the general upregulation of *miR-221-3p, -222-3p, -146b-5p, -155-5p, -181b-5p,* and *-187-3p* in all analyzed malignant subclasses of thyroid cancer, as well as the ATC-specific upregulation of *miR-204-5p, -137,* and *-214-3p* [Nikiforova *et al.*, 2008]. However, the role of these miRNAs was not analyzed.

Identification of miRNA-target interactions

In order to unravel the complexity and the physiological impact of post-transcriptional regulation by miRNAs, the reliable identification of functional miRNA-target interactions in specific biological contexts is necessary. A variety of algorithms has been developed to predict such interactions *in silico* (Table 1). These prediction tools mainly suggest MTSs according to seed complementarities, evolutionary conservation, target secondary structure, presence of multiple cognate sites, and thermodynamic properties of potential miRNA-target RNA duplexes (reviewed in [Min and Yoon, 2010; Thomson *et al.*, 2011]). Due to the weighting of these features, distinct prediction

platforms suggest substantially different MTSs and targeting miRNAs for some transcripts. To give an example, the experimentally verified targeting of *MYC* by the *let-7-5p* family [Sampson *et al.*, 2007; Shah *et al.*, 2007; Bueno *et al.*, 2011] is predicted by miRANDA [Enright *et al.*, 2003; John *et al.*, 2004; Betel *et al.*, 2008; Betel *et al.*, 2010], but cannot be identified by TargetScan [Lewis *et al.*, 2005; Grimson *et al.*, 2007; Friedman *et al.*, 2009; Garcia *et al.*, 2011]. However, both algorithms suggest reported targeting of *MYC* by the *miR-34-5p* family [Kong *et al.*, 2008; Kress *et al.*, 2011]. Hence, experimental identification and verification of miRNA-target interactions is indispensable.

Table 1 | List of accessible miRNA target prediction algorithms.

name	uniform resource locator	references
TargetScan	genes.mit.edu/targetscan	Lewis *et al.*, 2005 Grimson *et al.*, 2007; Friedman *et al.*, 2009; Garcia *et al.*, 2011
PicTar	pictar.mdc-berlin.de	Krek *et al.*, 2005; Grün *et al.*, 2005; Lall *et al.*, 2006; Chen & Rajewsky, 2006
miRANDA	microrna.org	Enright *et al.*, 2003; John *et al.*, 2004; Betel *et al.*, 2008; Betel *et al.*, 2010
DIANA-microT	http://diana.cslab.ece.ntua.gr/microT/	Kiriakidou *et al.*, 2004; Maragkakis *et al.*, 2009
EIMMo	http://www.mirz.unibas.ch/EIMMo3/	Gaidatzis *et al.*, 2007
MirTarget 2	mirdb.org	Wang and El Naqa, 2008; Wang, 2008
rna22	cbcsrv.watson.ibm.com/rna22.html	Miranda *et al.*, 2006
RNAhybrid	bibiserv.techfak.uni-bielefeld.de/rnahybrid	Rehmsmeier *et al.*, 2004
miRcode	http://www.mircode.org/mircode/	Jeggari *et al.*, 2012

Several high-throughput methods have been developed to identify miRNA-target interactions experimentally. Some of these aimed at the identification of genes regulated by specific, ectopically expressed miRNAs or miRISC components (Table 2). Global target determination was then achieved either by gene expression analyses at the mRNA (gene expression analyses, [Lim *et al.*, 2005; Grimson *et al.*, 2007]) or protein level (SILAC, [Baek *et al.*, 2008; Selbach *et al.*, 2008]; translational profiling [Hendrickson *et al.*, 2009; Guo *et al.*, 2010]), or RNA co-immunoprecipitation (co-IP) with miRISC components [Easow *et al.*, 2007; Orom and Lund, 2007; Chi *et al.*, 2009; Hafner *et al.*, 2010; Cambronne *et al.*, 2012]. Although all approaches identified multiple miRNA targets, it remained elusive whether they are physiologically relevant, because the findings could potentially be biased by the severe overexpression of miRNAs or miRISC components. Another disadvantage of these methods is that one can hardly discriminate between direct and indirect miRNA targets unless a functional validation is performed. To overcome this limitation a most recent approach combined crosslinking and IP (CLIP) of ectopically expressed AGO1 with ligation and sequencing of purified miRNA-mRNA hybrids (CLASH) [Helwak *et al.*, 2013]. However, in addition to a potential bias by AGO1 overexpression, CLASH is apparently biased by the abundance of endogenous mRNAs. The sequenced CLASH hits were enriched in coding sequences of highly abundant

transcripts like the overexpressed AGO1 transcript or genes encoding for housekeeping or ribosomal proteins. Thus, strongly regulated miRNA targets expressed at low abundance, due to mRNA destabilization, were most likely missed by CLASH.

On the other hand, two types of methods allow the identification of regulatory miRNAs for a target RNA of interest: high-throughput reporter assays [Wu *et al.*, 2010; Gaken *et al.*, 2012] and RNA affinity purifications [Vo *et al.*, 2010; Yoon *et al.*, 2012; Hassan *et al.*, 2013] (Table 2). Although, the use of high-throughput luciferase reporter assays identified for instance the regulation of *CDKN1A* (*cyclin-dependent kinase inhibitor 1A, p21)* by 28 distinct miRNAs [Wu *et al.*, 2010], these approaches are biased by the choice of tested miRNA candidates. RNA affinity purification approaches circumvent this issue by globally screening for RNA-interacting miRNAs. Capturing endogenous *AAT* (*serpin peptidase inhibitor, clade A, member 1*) mRNAs with a complementary, immobilized DNA oligonucleotide identified a total of 140 mRNA-associated miRNAs from three different cell lines [Hassan *et al.*, 2013]. Although, such a high number of candidates provided evidence that mRNAs can be regulated by dozens of different miRNAs, co-purification of about 40 - 60% of all miRNAs expressed in the used cell models suggests a potential bias by false-positives. In contrast, affinity purifications of ectopically expressed MS2-tagged reporter RNAs encoding the mouse *Hand2 (heart and neural crest derivatives expressed 2*)-3'UTR or the *lincRNA-p21* captured only *mmu-miR-1-3p* and *mmu-miR-133a-3p*, or respectively *mmu-let-7-5p* family members, which were among the most abundantly expressed miRNAs [Vo *et al.*, 2010; Yoon *et al.*, 2012]. Low abundant candidates were potentially missed because of low signal-to-noise ratios, presumably due to exceedingly low RNA purification rates.

Table 2 | Summary of available experimental approaches to identify miRNA-mRNA interactions.

name	methodology	reference
gene expression analysis	ectopic miRNA expression followed by detection of mRNA abundance	Lim *et al.*, 2005; Grimson *et al.*, 2007
SILAC (stable isotope labelling with amino acids in cell culture)	ectopic miRNA expression in cells with stable isotope labeled amino acids followed by mass spectrometry	Selbach *et al.*, 2008; Baek *et al.*, 2008
translation profiling	ectopic miRNA expression; cyloheximide treatment; cell lysis and sucrose gradient; analysis of ribosome-bound mRNAs	Hendrickson *et al.*, 2009; Guo *et al.*, 2010
RNA-co-IP with RISC components	ectopic miRNA expression together with expression of epitope tagged RISC components (AGO or TNRC6)	Easow *et al.*, 2007; Cambronne *et al.*, 2012
HITS-CLIP (high-throughput sequencing of RNA isolated by crosslinking IP)	UV crosslink prior to IP of RISC components followed by deep sequencing of crosslinked sequences, also coupled with ectopic miRNA expression	Chi *et al.*, 2009
PAR-CLIP (Photoactivatable-ribonucleoside-enhanced crosslinking and IP)	incubation of cells with photoactivatable nucleotide to offer more efficient UV crosslinking prior to IP of RISC components followed by deep sequencing, may be coupled with miRNA gain- or loss-of-function	Hafner *et al.*, 2010
pulldown of biotin-tagged miRNAs	transfection of biotinylated miRNA duplexes followed by miRNA-mRNA complex capture with streptavidin	Orom *et al.*, 2007
high-throughput reporter assay	ectopic expression of many different miRNAs together with specific 3'UTR-reporter	Wu *et al.*, 2010; Gäken *et al.*, 2012
affinity purification of overexpressed reporter	ectopic expression of 3'UTR-reporter with MS2-tag followed by RNA purification and miRNA co-purification	Vo *et al.*, 2010; Yoon *et al.*, 2012
affinity purification of endogenous RNA	formaldehyde crosslink followed by mRNA-miRISC capture with biotinylated antisense DNA oligonucleotide	Hassan *et al.*, 2013
CLASH (crosslinking, ligation and sequencing of hybrids)	UV crosslink of RNA to bound proteins prior to IP of epitope tagged AGO components followed by ligation of miRNA-mRNA duplexes prior deep sequencing	Helwak *et al.*, 2013

The aim of the study

The aggressive behavior of anaplastic thyroid cancer results from the high metastatic potential and the rapidly growing tumor mass [O'Neill *et al.*, 2010]. Genetic alterations in genes encoding signaling transducers and cell cycle regulators, as well as aberrant expression of miRNAs had been associated with a status of maximal signal transduction and cell proliferation in ATCs [Visone *et al.*, 2007; O'Neill *et al.*, 2010; Schmid, 2010]. In contrast, at the beginning of this study the molecular determinants that direct ATC invasiveness were essentially unknown. The observations that ATCs show a reduced or lost *CDH1* expression [Brabant *et al.*, 1993; Scheumman *et al.*, 1995; Naito *et al.*, 2001; Rocha *et al.*, 2003; Brecelj *et al.*, 2005; Wiseman *et al.*, 2006], and that this coincides with metastatic spread [Scheumman *et al.*, 1995] suggested the involvement of malignant EMT processes in thyroid cancer progression. On the basis of this suggestion, one of the main subjects of the study was to identify the molecular determinants that direct EMT in thyroid cancer.

In addition to protein-coding genes, miRNAs had been suggested as molecular markers of advanced cancers as well as crucial gatekeepers of tissue homeostasis and differentiation (reviewed in [Croce, 2009; Peter, 2009]). Accordingly, aberrant miRNA expression signatures were hypothesized to distinguish ATCs from well-differentiated subclasses of thyroid cancer, and to direct malignant EMT. Molecules that mark the

transformation from non-metastatic to aggressive anaplastic thyroid carcinomas would provide a valuable tool for diagnosis and the assessment of treatment effects. In contrast to mRNAs, studying miRNAs expression signatures are more manageable. MiRNA expression signatures consider hundreds of molecules; those for protein-coding genes consider thousands. Upon identification of candidate miRNAs, studying their downstream effectors might also allow a detailed view if, how and to what extend miRNAs modulate EMT in ATCs.

The second major aim of this study was to develop an experimental approach to identify regulatory miRNAs of an RNA of choice. This exhibits a prerequisite to study miRNA-mediated post-transcription control of mRNAs, long noncoding RNAs and also viruses. Incomplete base pairing between miRNAs and their target transcripts often impedes reliable suggestion of miRNA-target interactions by *in silico* predictions. However, at the beginning of this doctoral study, experimental high-throughput approaches aimed exclusively at the identification of target transcripts of a specific miRNA [Lim *et al.*, 2005; Easow *et al.*, 2007; Grimson *et al.*, 2007; Orom and Lund, 2007; Baek *et al.*, 2008; Selbach *et al.*, 2008; Chi *et al.*, 2009; Hendrickson *et al.*, 2009]. In contrast to miRNAs, regulatory RNA-binding proteins had been successfully identified by affinity purifications of *in vitro* transcribed RNAs from cellular extracts [Czaplinski *et al.*, 2005; Duncan *et al.*, 2006]. These results proposed *in vitro* RNA affinity purifications as promising approaches for the comprehensive identification of regulatory miRNAs. Moreover, studies demonstrating RNA interference [Tuschl *et al.*, 1999] and miRNA-mediated regulation in cell-free systems [Mathonnet *et al.*, 2007] hypothesized preserved binding specificities of siRNAs or miRNAs to *in vitro* transcribed bait RNAs. The *MYC*-3'UTR was used as an RNA testing bait, because post-transcriptional control of *MYC* expression by miRNAs was proposed before this study started (*let-7-5p* [Sampson *et al.*, 2007; Shah *et al.*, 2007], *miR-34-5p* [Kong *et al.*, 2008]). Hence, testing co-purification of these miRNAs would allow evaluation of experimental conditions.

3 RESULT SUMMARY & DISCUSSION

In the context of this doctoral study molecular determinants involved in the aggressive behavior of anaplastic thyroid carcinomas were investigated. New insights were obtained regarding transcriptional and post-transcriptional regulators involved in EMT processes of thyroid cancer ([Braun *et al.*, 2010; Braun and Huttelmaier, 2011]; ADDITIONAL RESULTS). Moreover, by improving an *in vitro* RNA affinity purification protocol, a new approach for the comprehensive identification of regulatory miRNAs was developed [Braun *et al.*, 2014].

Post-transcriptional gene regulation modulates epithelial-to-mesenchymal transition of anaplastic thyroid carcinomas

Due to a high metastatic potential and a rapidly growing tumors mass, ATCs represent highly aggressive and lethal cancers. Before this study started, a positive correlation between the metastatic spread of ATCs and *CDH1* expression was observed [Scheumman *et al.*, 1995; Naito *et al.*, 2001]. Consistent with these observations, the majority of ATC cases analyzed in this study showed a decreased or lost *CDH1* expression (Figure 2d in [Braun *et al.*, 2010]; Figure 9C in ADDITIONAL RESULTS, p. 98). From a total of 29 ATC tumor tissues analyzed only 10 exhibited *CDH1* expression, whereas five of these 10 tumors expressed *CDH1* at low levels (compare A1, A6, A11, A14, A15 with A2-5, A7 in Figure 9C in ADDITIONAL RESULTS, p. 98). In contrast to previous studies that determined the reduction of *CDH1* expression in ATCs by means of immunohistochemistry (IHC), the findings of this study were based on Western blot analyses of protein extracts from tumor tissues or non-transformed tissues (NTs). Although the evaluation of protein expression in tumor tissues by IHC is superior to Western blot analyses, paraffin-embedded tissues were not available. The lack of IHC evaluation harbors the risk that tumor tissue is partially mixed with *CDH1*-expressing non-transformed tissue or concurrent differentiated variants of thyroid cancer that have been suggested as ATC precursor [Kondo *et al.*, 2006; Pallante *et al.*, 2014]. In this respect, it also has to be mentioned that immune cells, fibroblasts as part of tumor stroma or even cancer cells could have infiltrated the non-transformed tissues used for Western blot analyses. In view of these limitations it was quite surprising that the loss of *CDH1* expression correlated well to the gain of *ZEB1* expression in ATCs (Figure 2d in [Braun *et al.*, 2010]; Figure 9C in ADDITIONAL RESULTS, p. 98). This finding was of particular

interest, because ZEB1 is a known transcriptional repressor of *CDH1* [Eger *et al.*, 2005] and a potent inducer and sustainer of the mesenchymal cell phenotype [Eger *et al.*, 2005; Aigner *et al.*, 2007b; Burk *et al.*, 2008]. In contrast, ZEB2, another reported repressor of *CDH1* [Comijn *et al.*, 2001] was in addition to its re-expression in ATCs also detectable in *CDH1*-expressing NTs, FTCs and PTCs, although at lower levels (Figure 2d in [Braun *et al.*, 2010]; Figure 9C in ADDITIONAL RESULTS, p. 98). Such co-existence of ZEB2 with CDH1 was also suggested for normal epithelial cells of kidney, stomach, colon, rectum and oesophagus [Oztas *et al.*, 2010]. Thus, ZEB1 is, rather than ZEB2, a promising candidate responsible for *CDH1* repression as well as the loss of epithelial differentiation in ATCs. Nevertheless, high levels of ZEB2 may support the function of ZEB1. The potential of ZEB1 to repress *CDH1* was confirmed by the induction of *CDH1* expression upon *ZEB1* knockdown in 8505C and HeLa cells (Figure 14 in ADDITIONAL RESULTS, p. 109). In contrast, the knockdown of *ZEB2* in both cell lines did not increase CDH1 levels (data not shown). However, in addition to ZEB1 and ZEB2, the presence of other EMT-inducing transcription factors remains to be analyzed in ATCs. It is most likely that factors like SNAI1/2 or TWIST1/2 also play a role in thyroid cancer progression.

What are the reasons for the gain of *ZEB1* expression? To answer this question a multitude of events that most likely depend on each other have to be considered. One crucial event is supposed to be the identified loss of *miR-200-3p f* expression in ATCs (Figure 1a in [Braun *et al.*, 2010]; Figure 9F in ADDITIONAL RESULTS, p. 98). This miRNA family efficiently decreased the expression of *ZEB1*, and potentially as a result of this, it increased the expression of *CDH1* in ATC-derived 8505C cells (Figure 2b, c in [Braun *et al.*, 2010]). Whether the loss of *miR-200-3p f* induces *ZEB1* transcription in ATCs, or whether *ZEB1* mRNA is produced and post-transcriptionally regulated by *miR-200-3p f* in normal thyrocytes remains to be tested.

Concomitantly with the loss of *miR-200-3p* expression in ATCs, accelerated TGFB signaling may trigger *ZEB1* expression. TGFB signaling is a strong inducer of *ZEB1* expression [Shirakihara *et al.*, 2007; Gregory *et al.*, 2008a], and in ATC-derived cells the inhibition of the TGFB signaling pathway via blocking TGFBR1 reduced *ZEB1* expression and accelerated *CDH1* expression, however only detectable at the mRNA level (Figure 3f, g in [Braun *et al.*, 2010]). In advanced-stage tumors several studies suggested TGFB signaling as an inducer of EMT, and subsequent tumor cell dissemination (reviewed in [Heldin *et al.*, 2012; Morrison *et al.*, 2013]). The increased expression of TGFBR1 in ATCs compared to NTs, FTCs and PTCs (Figure 3a in [Braun *et al.*, 2010], Figure 9C in ADDITIONAL RESULTS, p. 98) may sensitize ATC tumors to

TGFB present in the microenvironment. However, it remains to be verified via IHC of ATC specimen whether TGFB is available in ATCs. Moreover, to transduce TGFB signals TGFBR1 needs to heterodimerize with TGFBR2. To finally clarify whether accelerated TGFB signaling induces EMT in ATCs, *TGFBR2* expression has to be analyzed, as well as the IHC co-staining of ZEB1, TGFBRs and phospho-SMAD2/3 in tissue specimen negative for *CDH1* expression.

Two other potential mechanisms that may modulate *ZEB1* expression in ATCs involve TP53 and integrin-linked kinase (ILK). The latter was found significantly overexpressed in ATC tissues [Younes *et al.*, 2005]. ILK interacts with the cytoplasmic tail of integrins, transmembrane receptors responsible to transduce mechanical and biochemical changes of the extracellular matrix into the cell (reviewed in [Kim *et al.*, 2011a; Qin and Wu, 2012]). In bladder cancer cells, the depletion of *ILK* downregulated *ZEB1* and upregulated *CDH1* expression, whereas the overexpression of constitutive active ILK increased *ZEB1* and decreased *CDH1* expression [Matsui *et al.*, 2012]. Inactivating mutations of *TP53* were identified with high frequency in ATCs and suggested to be associated with tissue dedifferentiation [Ito *et al.*, 1992; Donghi *et al.*, 1993; Fagin *et al.*, 1993; Antico Arciuch *et al.*, 2011]. TP53 was shown to activate *miR-200c-3p* expression by directly binding to its promoter [Chang *et al.*, 2011; Kim *et al.*, 2011b]. Depletion of *TP53* in mammary epithelial cells or colon carcinoma-derived cells resulted in the induction of EMT and *ZEB1* expression due to the decrease of *miR-200c-3p* expression [Chang *et al.*, 2011; Kim *et al.*, 2011b]. Accordingly, it will be interesting to analyze whether *TP53* loss-of-function mutations and/or *ILK* overexpression correlate with the gain of *ZEB1* expression in ATC, and the onset of EMT.

ZEB1 promotes several hallmarks of cancer that have been associated with ATCs

The expression of *ZEB1* in *CDH1*-negative ATC tumors may also explain the aggressive behavior of these tumor types [Scheumman *et al.*, 1995]. A role of ZEB1 in directing thyroid cancer progression is given by the fact that ZEB1 is an extremely potent inducer and sustainer of the mesenchymal cell phenotype, which is for instance characterized by a high cell migration and invasion capacity [Eger *et al.*, 2005; Aigner *et al.*, 2007a; Bracken *et al.*, 2008; Burk *et al.*, 2008; Spaderna *et al.*, 2008; Ahn *et al.*, 2012]. Accordingly, *ZEB1* expression contributed to tumor cell dissemination in a mouse xenograft model [Wellner *et al.*, 2009]. EMT induction by ZEB1 is facilitated by repressing a plethora of protein-coding and miRNA genes that control epithelial cell-cell adhesion, apicobasolateral cell polarity and cell-basement membrane interactions [Aigner *et al.*, 2007a; Burk *et al.*, 2008;

Spaderna *et al.*, 2008; Wellner *et al.*, 2009; Ahn *et al.*, 2012; Horiguchi *et al.*, 2012]. The fundamental role of ZEB1 in inducing and sustaining the mesenchymal phenotype is also indirectly given by the fact that it represents, next to ZEB2, the main target of the *miR-200-3p* family [Burk *et al.*, 2008; Gregory *et al.*, 2008a]. This finding is important, because the expression of *miR-200-3p f* is strongly associated with the epithelial phenotype (Figure 2 in [Park *et al.*, 2008], Figure 1a in [Braun *et al.*, 2010]; Figure 10B in ADDITIONAL RESULTS, p. 100). Moreover, *miR-200-3p f* is a crucial suppressor of EMT (Figure 2b, c in [Braun *et al.*, 2010], [Bracken *et al.*, 2008; Gregory *et al.*, 2008a; Park *et al.*, 2008]). The strong EMT-suppressing effect of *miR-200-3p* was suggested to be essentially due to the repression of *ZEB1* and *ZEB2* [Brabletz and Brabletz, 2010]. However, the identification of *ZEB2* expression in less aggressive, differentiated subclasses of thyroid cancer (FTCs, PTCs) and even in tumor surrounding tissue argues against ZEB2 as a key driver of malignant EMT in ATCs. In contrast, *ZEB1* expression was until now not identified in normal epithelium, but in invading dedifferentiated cancers of colon, breast, liver, ovary, lung, prostate, and pancreas (reviewed in [Sanchez-Tillo *et al.*, 2012]).

Beyond EMT induction, *ZEB1* expression was suggested to promote other hallmarks of cancer (reviewed in [Sanchez-Tillo *et al.*, 2012]), which have been associated with ATC carcinogenesis (reviewed in [O'Neill *et al.*, 2010]). Findings from mouse development as well as studies in esophageal cancer cells and endothelial cells indicated that ZEB1 sustains cell proliferation by directly repressing the cyclin-dependent kinase inhibitors 1a (p21), 2a (p16), and 2b (p15) [Liu *et al.*, 2008; Ohashi *et al.*, 2010; Magenta *et al.*, 2011]. *ZEB1* loss-of-function also led to cellular senescence, and in the case of esophageal cancer cells impaired sensitivity for TGFB to induce EMT [Ohashi *et al.*, 2010]. Hence, one can imagine that *ZEB1* expression accelerates ATC cell proliferation and sensitivity to TGFB. ZEB1 may also promote tumor growth and dissemination through increased resistance to apoptosis. The knockdown of *ZEB1* in non-small cell lung cancer cells reduced colony formation under anchorage-independent growth conditions in soft agar assays [Takeyama *et al.*, 2010]. Anchorage-independent growth is a feature of aggressive cancer cells, which enables survival during transport through lymph or blood vessels as well as in new environments. In normal cells, these conditions trigger caspase-dependent apoptosis [Sanchez-Tillo *et al.*, 2012].

In addition to its function in cancer cell proliferation and resistance to apoptosis, *ZEB1* expression was connected to cancer stem cell properties, namely sphere formation, stem cell marker expression, drug resistance and the potential to induce tumors for instance in mice [Wellner *et al.*, 2009]. These effects were demonstrated to be partially due to the

ability of ZEB1 to suppress the stemness-inhibiting miRNAs *200-3p f, 203a* and *183-5p* [Wellner *et al.*, 2009]. Like for other cancers, CSCs were identified in thyroid carcinomas [Todaro *et al.*, 2010]. Consistent with the high recurrence potential of ATCs, these tumors harbored more than double the amount of CSCs (14 ± 3%) compared to PTCs (5 ± 2%) and FTCs (2 ± 1.2%) [Todaro *et al.*, 2010]. Whether ZEB1 sustains the replicative immortality of ATC stem cells or modulates the balance between tumorigenic and non-tumorigenic CSC states would be worth to analyze. Moreover, the connection of EMT and/or *ZEB1* expression with resistance to chemotherapeutics (reviewed in [Sanchez-Tillo *et al.*, 2012]), a main characteristic of cancer stem cells, proposes *ZEB1* as a potential target to decrease ATC recurrence. Usually, ATCs are highly resistant to conventional cancer therapies (radiotherapy, chemotherapy), and 90% of patients die within six months after diagnosis through recurrence or distant metastasis [Lin, 2011]. Therefore, methods to reduce ATC stem cells offer a great potential to manage ATCs.

The *miR-200-3p* family regulates TGFB signaling pathway at multiple levels

TGFB-mediated SMAD signaling was proposed to be essential to induce EMT, invasion and metastasis in late-stage cancers (reviewed in [Heldin *et al.*, 2012; Morrison *et al.*, 2013]). ZEB1 and ZEB2 are two of several EMT-inducing transcription factors that are expressed in response to TGF signals [Shirakihara *et al.*, 2007; Gregory *et al.*, 2008a; Gregory *et al.*, 2011]. In addition to inducing their expression, TGFB also mediates their transcriptional activity [Gregory *et al.*, 2008a; Gregory *et al.*, 2011]. In the nucleus, ZEB1 and ZEB2 were suggested to interact with the trimeric SMAD2/3/4 complex [Postigo, 2003; Xu *et al.*, 2009]. This was evidenced for instance in kidney-derived MDCK cells, in which TGFB-induced ZEB1/2-dependent induction of EMT was impaired upon the knockdown of SMAD4 [Gregory *et al.*, 2011]. In accord to the crucial role of the TGFB pathway in EMT induction, it appeared tempting to hypothesize that the *miR-200-3p* family does not only control *ZEB1* and *ZEB2* expression, but also genes encoding effectors of the TGFB signaling pathway.

The miRANDA [Enright *et al.*, 2003; John *et al.*, 2004; Betel *et al.*, 2008; Betel *et al.*, 2010] or RNAhybrid [Rehmsmeier *et al.*, 2004] algorithms predict several target sites for members of the *miR-200-3p* family in the 3'UTRs of *TGFBR1* (Figure 3b in [Braun *et al.*, 2010]) and *TGFBR2*, as well as in the *SMAD2* (Figure 3b in [Braun *et al.*, 2010]), *SMAD3*, *SMAD4* and *TGFB2* 3'UTRs. Accordingly, *miR-200-3p f* may post-transcriptionally control the TGFB signaling pathway at multiple levels (Figure 6). The reduction of *TGFB2* expression upon *miR-141-3p* overexpression in colorectal and pancreas

carcinoma-derived cells supported this notion [Burk *et al.*, 2008]. Additionally, in the context of this doctoral study, the repression of *TGFBR1* and *SMAD2* expression by *miR-200-3p* family members was confirmed. Ectopic expression of *miR-141-3p* and *miR-200a-3p* in 8505C cells decreased *TGFBR1* and *SMAD2* expression at both, the mRNA and protein level (Figure 3c, d in [Braun *et al.*, 2010]). Moreover, *SMAD2* expression was also reduced by *miR-200c-3p* overexpression (Figure 3c, d in [Braun *et al.*, 2010]). To test direct targeting of *miR-200-3p* family members to the *TGFBR1* and *SMAD2* 3'UTRs, luciferase reporter assays were performed (Supplementary Figure 1, 2 in [Braun *et al.*, 2010]). Luciferase reporters harbored either two fragments (nt 1-1500; 1500-4887) of the 4887 nucleotide long *TGFBR1*-3'UTR or the 1000 nucleotide long 5'-end fragment of the 8732 nucleotide long *SMAD2*-3'UTR. Co-transfection of *miR-141-3p* with the two TGFBR1-reporters resulted in an approximately 40-50% decreased reporter activity. The mutation of potential *miR-141-3p* targeting sites in the *TGFBR1*-3'UTR abolished the miRNA effect, which suggested 3'UTR-directed targeting of *miR-141-3p*. Co-transfections with *cel-miR-239b-5p* served as a negative control; as well as co-transfections of *miR-200c-3p* with the TGFBR1-reporter, which did not alter reporter activity. In contrast, the SMAD2-reporter responded to the transfection of *miR-200c-3p*. Again, reporter expression was rescued by the mutation of potential miRNA target sites. Unfortunately, these studies did not completely elucidate whether all *in silico* predicted miRNA target sites (MTSs) are responsible for miRNA-mediated repression, because mutant reporters harbored concurrent mutations in several MTSs predicted for one specific miRNA. To fully characterize the competence of individual MTSs, 3'UTR-reporters with mutations in individual MTSs have to be tested. In addition, miRNA-MTS interaction could be verified by using isolated MTSs as miTRAP bait RNAs. Similar experiments were carried out to confirm the interaction of *miR-455-3p* and *miR-125a-3p* with MTSs from the *MYC*-3'UTR (Figure 4E in [Braun *et al.*, 2014]). MiTRAP would also be a valuable method for future studies aiming at verifying *in silico* predicted *miR-200-3p f* targeting of additional effectors of the TGFB signaling pathway, namely *TGFBR2*, *SMAD3* and *SMAD4* (Figure 6).

The slight reduction of endogenous *TGFBR1* and *SMAD2* protein and mRNA levels suggests that the *miR-200-3p* family dampens TGFB signaling. Fine-tuning of *SMAD2* and *TGFBR1* contrasts the severe repression of *ZEB1* and *ZEB2* upon overexpression of *miR-200-3p* family members (compare Figure 2b with 3c in [Braun *et al.*, 2010]). This difference may be explained by the "TGFB paradox": In normal cells and early-stage cancers TGFB functions as a tumor suppressor by inducing growth arrest and promoting

apoptosis, whereas in late-stage cancers it promotes tumorigenesis by inducing EMT (reviewed in [Morrison *et al.*, 2013]). Hence, a strong repression of *TGFBR1* and *SMAD2* would impair the tumor-suppressive function of TGFB signaling in epithelia. Accordingly, SMAD2 and TGFBR1 were detectable in non-transformed tissues of thyroid cancer patients, as well as in less aggressive subclasses of thyroid cancer (FTCs, PTCs) (Figure 3a in [Braun *et al.*, 2010], Figure 9C in ADDITIONAL RESULTS, p. 98). However, elevated expression levels of *TGFBR1* and *SMAD2* in ATCs potentially trigger TGFB stimulation of EMT. This hypothesis is supported by studies in mammary epithelial cells. The overexpression of constitutive active *TGFBR1* at high doses induced a mesenchymal-like phenotype, whereas low doses had to be supplemented with *SMAD2* and *SMAD4* overexpression to induce EMT [Piek *et al.*, 1999]. Taken together, fine-tuning of TGFB signaling effectors in addition to the severe repression of ZEB1 and ZEB2 may represent a fail-safe mechanism of the *miR-200-3p* family to prevent induction of the mesenchymal phenotype in epithelial cells. Moreover, repression of multiple genes that sustain the mesenchymal phenotype allows rapid induction of an MET by *miR-200-3p f*. Fine-tuning of TGFB signaling effectors would also decrease the crosstalk with other signaling pathways (see Figure 3 in INTRODUCTION, p. 9), which was suggested to be indispensible to permanently convert an epithelial cancer cell into a mesenchymal-like phenotype [Parvani *et al.*, 2011].

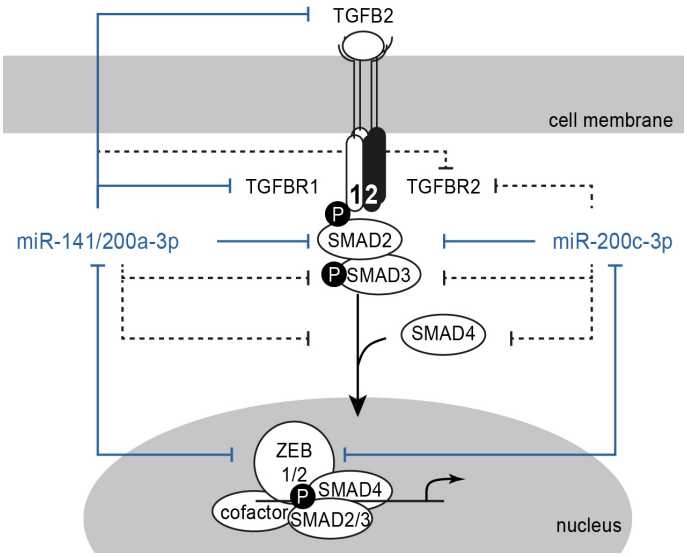

Figure 6 | Post-transcriptional control of genes involved in the TGFB signaling pathway by *miR-200-3p* family members.
TGFB signals by binding to type I and type II TGFB receptor (TGFBR1/2) complex. TGFBR1 phosphorylates SMAD2 and SMAD3, which subsequently form a complex with SMAD4. In the nucleus, the SMAD2/3/4 complex interacts with cofactors and transcription factors like ZEB1 or ZEB2 and modulates transcription [Piek *et al.*, 1999; Postigo, 2003]. Members of the *miR-200-3p* family have been shown to simultaneously modulate expression of genes encoding TGFB2 [Burk *et al.*, 2008], TGFBR1, SMAD2 [Braun *et al.*, 2010], ZEB1 and ZEB2 [Burk *et al.*, 2008; Gregory *et al.*, 2008a] (blue lines). Moreover, *in silico* predictions suggest MTSs for *miR-200-3p* family members in the 3'UTRs of genes encoding TGFBR2, SMAD3 and SMAD4 (dashed lines). Note, although *miR-141/200a-3p* and *miR-200c-3p* belong to the *miR-200-3p* seed family, their seed differs in one nucleotide (see Figure 5 in INTRODUCTION, p. 13), which results in a slightly different binding behavior.

Interestingly, miRNA-mediated co-downregulation of genes from the same functional module was also proposed for *miR-33a/b-5p* and *miR-124-3p*. The latter simultaneously repressed *AKT2* (*v-akt murine thymoma viral oncogene homologue 2*), *MAPK14* (*p38*), and *STAT3* (*signal transducer and activator of transcription 3*) to inhibit the EGFR (epidermal growth factor receptor)-driven signaling pathway, leading to a cell cycle inhibition [Uhlmann *et al.*, 2012]. On the other hand, *miR-33a/b-5p* controlled the expression of genes encoding key enzymes involved in fatty acid metabolism [Davalos *et al.*, 2011]. Whether other miRNAs regulate signaling pathways or metabolic pathways at multiple levels remains to be investigated. Nevertheless, it would be a very efficient mechanism to control cellular homeostasis at the post-transcriptional level.

ATCs are characterized by a downregulation of miRNAs rather than an upregulation

The observation that miRNAs are mainly downregulated in tumor tissues [Lu *et al.*, 2005] suggested that global miRNA expression reflects the state of cellular differentiation [Peter, 2009; Blandino *et al.*, 2014]. Consistent with this hypothesis, the comparative expression analysis of ATC tisssues and non-transformed tissues by using microRNA microarrays identified a higher number of downregulated than upregulated miRNAs (Table 1 in [Braun and Huttelmaier, 2011]). Regarding a threshold of two, 62 miRNAs were downregulated, and 21 miRNAs were upregulated in at least two of three ATC/NT pairs (Table 1 in [Braun *et al.*, 2010]). Notably, the downregulated population covered more than half of the miRNAs (13 of 20) identified as downmodulated by Visone *et al.*, namely *miR-30a/d-5p*, *miR-125a-5p/125b-1/2-5p*, *miR-26a-5p*, *miR-138-5p*, *miR-29b-3p*, *miR-99a/b-5p*, *miR-145-5p*, *let-7c-5p,* and *miR-151* [Visone *et al.*, 2007]. In total, Visone *et al.* identified 20 downregulated and four upregulated miRNAs regarding a threshold of -/+ 1.25 fold [Visone *et al.*, 2007]. The lower number of affected miRNA candidates may be due to lower probe content of the used microarray platform. In contrast to 773 miRNAs analyzed in this study, Visone and colleagues used microarrays, which harbored only 245 probes. From the upregulated miRNA population, only *miR-222-3p* was identified by Visone *et al*.. The upregulation of *miR-222-3p* and *miR-221-3p*, both expressed from the same genomic cluster, seems to be a hallmark of thyroid carcinomas of follicular origin [Braun and Huttelmaier, 2011]. All studies that analyzed miRNA expression in either FTCs, PTCs, PDTCs or ATCs identified this miRNA cluster to be upregulated [He *et al.*, 2005; Pallante *et al.*, 2006; Visone *et al.*, 2007; Nikiforova *et al.*, 2008; Schwertheim *et al.*, 2009; Braun *et al.*, 2010]. Only the study by Schwertheim *et al*. contradicted this

hypothesis. Although Nikiforova *et al*. identified *miR-221-/222-3p* upregulated in PDTCs, Schwertheim *et al.* observed negligible changes.

The finding that 29 of the 62 downregulated miRNAs are expressed from 12 genomic clusters as well as the identification of four downregulated miRNA pairs processed from the same precursor (*199a-5p/-3p; 29c-5p/-3p; 30a-5p/-3p; miR-361-5p/-3p*) suggests a high reliability of the results (Table 1 in [Braun *et al*., 2010]). Moreover, this observation proposes a selective downmodulation of miRNA transcription units in ATCs via oncogenic transcription factors, promoter methylation [Lopez-Serra and Esteller, 2012] and/or from genomic deletions [Calin and Croce, 2006; Rossi *et al*., 2008]. As mutations, deletions, or amplifications have been shown to alter the expression of protein-coding genes in human cancers, Calin *et al*. analyzed the location of miRNA genes in regard to reported cancer-associated genomic regions (CAGRs) [Calin *et al*., 2004]. Interestingly, five of the twelve downregulated miRNA gene clusters in ATCs (*let-7a/f/d; let-7c/miR-99a; miR-24/27b/23b; miR-143/145; miR-29a/b*) were located close to fragile sites reported to be affected by deletion in various cancers [Calin *et al*., 2004]. However, up until now no study directly linked miRNA expression to genomic alterations. Instead, DNA hypermethylation was suggested to be the most common cause of miRNA gene promoter shutdown in cancer [Lopez-Serra and Esteller, 2012]. Hypermethylation-associated silencing of both *miR-200* clusters (*miR-141/200c; miR-200a/200b/429*) was reported for different cancer cell lines [Ceppi *et al*., 2010; Vrba *et al*., 2010; Wiklund *et al*., 2010; Eades *et al*., 2011]. TGFB treatment of mammary epithelial cells was proposed to mediate *miR-200a/200b/429* promoter methylation via upregulation of *SIRT1* (*sirtuin 1*) expression [Eades *et al*., 2011]. SIRT1 is a histone deacetylase (HDAC) that was also shown to silence the *CDH1* promoter by interacting with ZEB1 [Byles *et al*., 2012]. An association between *miR-141/200c* histone deacetylation and promoter methylation was identified in human mammary [Vrba *et al*., 2010] and bladder cells lines [Wiklund *et al*., 2010]. In addition to SIRT1, ZEB1 was proposed to interact with HDAC1 and HDAC2 to silence *CDH1* expression [Aghdassi *et al*., 2012]. Taken together, accelerated TGFB signaling and increased *ZEB1* expression in ATCs may reduce *miR-200 f* transcription, potentially by promoter hypermethylation as a result of histone deacetylation. The repressive function of the TGFBR1 and of ZEB1/2 was supported by the observed increase of *miR-200-3p f* expression upon *TGFBR1* knockdown and inhibition, as well as *ZEB1/2* depletion in 8505C cells (Figure 3h and Supplementary Figure 4A in [Braun *et al*., 2010]). On the contrary, TGFB signaling may enhance *miR-21-5p* expression in ATCs (Figure 3h in [Braun *et al*., 2010]) by promoting its maturation [Davis *et al*., 2008]. This

oncogenic miRNA [Selcuklu *et al.*, 2009] was found dramatically upregulated in ATC tissues analyzed by this study (Table 1 in [Braun *et al.*, 2010], Figure 9G in ADDITIONAL RESULTS, p. 98) and by another study [Frezzetti *et al.*, 2011]. In addition to the TGFB signaling pathway, also the MAPK pathway was suggested to alter miRNA expression patterns. ERK-mediated phosphorylation of the Dicer cofactor TARBP2 selectively enhanced Dicer-mediated processing of pro-proliferative miRNAs and inhibited processing of anti-proliferative miRNAs [Paroo *et al.*, 2009].

To validate the microarray analysis and to compare miRNA expressions in ATC tissues with those in FTC and PTC tissues, qRT-PCR analyses were performed for 14 of the 62 downmodulated miRNAs, which involved two *let-7-5p* family members, as well as all members of the *miR-200-3p, miR-30-5p*, and *miR-26-5p* family (Figure 1 in [Braun *et al.*, 2010]). For internal normalization, the snRNA U18 was validated to barely vary in their abundance (data not shown). Consistent with the findings from the microarray, the relative expression of all analyzed miRNAs was significantly downregulated in ATCs compared to non-transformed tissues. This was further confirmed for *miR-141-3p* and *miR-200c-3p* by using a larger cohort of samples (Figure 9F in ADDITIONAL RESULTS, p. 98). The comparison of the miRNA expression between FTCs, PTCs and ATCs uncovered *miR-200-3p* family members as preferentially downregulated in ATCs. This finding supported the role of *miR-200-3p f* in sustaining the epithelial phenotype of ATCs. In addition to ATCs, reduced expression of *let-7-5p, miR-26-5p* and *miR-30-5p* family members was also detectable in differentiated subclasses of thyroid cancer. However, the reduction of *miR-30-5p f* expression was less pronounced in FTCs and PTCs suggesting a role of *miR-30-5p f* in sustaining the epithelial phenotype. The tumor-suppressive functions of *miR-26-5p f* and *let-7-5p f* were previously connected to thyroid cancer cell proliferation [Visone *et al.*, 2007; Ricarte-Filho *et al.*, 2009].

The differential expression of the *miR-200-3p* family is of particular interest since ATCs often emerge from manageable differentiated thyroid carcinomas (see INTRODUCTION, p. 5). It could be a useful biomarker to distinguish between ATCs and ATC precursors. An early ATC-diagnosis displays an essential prerequisite for therapeutic strategies involving neo-adjuvant, additive or palliative concepts (personal communication of Stefan Hüttelmaier and Kerstin Lorenz (surgeon university hospital of Halle)). Usually, ATC diagnosis relies exclusively on the preoperative, cytologic examination of fine-needle aspiration biopsy (FNAB) samples [Cornett *et al.*, 2007; Pallante *et al.*, 2014]. However, the feasibility to use *miR-200-3p f* as a diagnostic marker has to be evaluated by analyzing a substantially larger cohort of samples including also

PDTCs in addition to FTCs, PTCs and ATCs. Beyond the use as biomarkers, *miR-200-3p* family members repletion therapy may reverse malignant EMT of ATCs and prevent cancer cell invasion. Lipid-based delivery of for instance *let-7b-5p* has been successfully used to decrease tumor burden in an orthotopic mouse model of non-small cell lung cancer [Trang *et al.*, 2011].

The role of the tumor-suppressive *miR-30-5p* family in the context of anaplastic thyroid carcinomas

The significant downregulation of *miR-30-5p f* in ATC tissues compared to non-transformed thyroid and well-differentiated thyroid tumor tissues (Figure 1b in [Braun *et al.*, 2010]) proposed a tumor-suppressive function of *miR-30-5p f* in thyroid follicular cells. This suggestion was supported by studies in 8505C cells (Figure 2b, c, f in [Braun *et al.*, 2010]) and several recent reports demonstrating a tumor-suppressive role of the *miR-30-5p* family. Ectopic expression of *miR-30d/e-5p* in 8505C cells reduced *in vitro* cell invasion (Figure 2f in [Braun *et al.*, 2010]), which was potentially linked to the repression of *vimentin* (*VIM*), *ZEB2* and *SMAD2* expression (Figure 2b, c, Figure 3c, d in [Braun *et al.*, 2010]). In breast cancer-derived cells, another *miR-30-5p* family member (*miR-30a-5p*) decreased the abundance of VIM, which was suggested to modulate *in vitro* cell invasion [Cheng *et al.*, 2012]. Based on luciferase reporter studies, the direct targeting of *VIM* [Cheng *et al.*, 2012], *SMAD2* (Supplementary Figure 2 in [Braun *et al.*, 2010]), and *ZEB2* 3'UTRs (Supplementary Figure 3 in [Braun *et al.*, 2010]) by members of the *miR-30-5p* family was hypothesized. Targeting to the *ZEB2*-3'UTR was further supported by the affinity purification of *miR-30c-5p* and *miR-30e-5p* with a bait RNA comprising the *ZEB2*-3'UTR (Figure 1E in [Braun *et al.*, 2014]). In addition to *ZEB2*, the *miR-30-5p* family was shown to directly target *SNAI1*, which encodes another EMT-inducing transcription factor [Kumarswamy *et al.*, 2012].

The suppressive function of *miR-30-5p f* on cell invasiveness and, in addition to this study, cell motility was shown for breast cancer cells [Cheng *et al.*, 2012], non-small cell lung cancer cells [Kumarswamy *et al.*, 2012], and prostate cancer cells [Kao *et al.*, 2013]. Consistent with these findings, *miR-30-5p f* overexpression in mouse xenografts generated from multiple myeloma cells [Zhao *et al.*, 2014] and breast cancer cells [Yu *et al.*, 2010] reduced metastatic spread. Additionally, *miR-30-5p f* was suggested to decrease the self-renewal capacity of cancer stem cells from multiple myeloma [Zhao *et al.*, 2014] and breast cancer cell populations [Yu *et al.*, 2010; Ouzounova *et al.*, 2013], which may be linked to the reduced growth of *miR-30-5p f* overexpressing xenografts [Yu *et al.*, 2010;

Ouzounova *et al.*, 2013; Zhao *et al.*, 2014]. Taken together, the plethora of functions exhibit *miR-30-5p f* as essential tumor-suppressive miRNAs in the thyroid. In addition to the decrease of *miR-200-3p f* expression, *miR-30-5p f* downmodulation may be a crucial step in thyroid cancer progression.

Identification of regulatory microRNAs by miTRAP

Studying post-transcriptional control by miRNAs presupposes the identification of physiological relevant miRNA-target interaction. Incomplete base pairing between miRNAs and target RNAs however challenges the identification of these interactions. Although a variety of *in silico* prediction tools (see Table 1 in INTRODUCTION, p. 17) have been developed to identify these interactions, different output lists limit reliable determination. Therefore, in the context of this doctoral study an *in vitro* RNA affinity purification protocol initially developed to identify regulatory RNA-binding proteins [Czaplinski *et al.*, 2005; Duncan *et al.*, 2006] was adapted and tested as an approach for the comprehensive identification of regulatory miRNAs. The protocol was termed miTRAP (microRNA trapping by RNA *in vitro* affinity purification) and the *MYC*-3'UTR was used as the major testing bait. The large repertoire of documented regulatory miRNAs, as well as the reported interaction with the RNA-binding protein ELAVL1 (embryonic lethal abnormal vision like RNA-binding protein 1) [Kim *et al.*, 2009a] qualified this 3'UTR as an optimal RNA bait for the analysis and evaluation of purification results. MiRNA-mediated post-transciptional control of *MYC* expression was suggested to involve the following miRNAs or miRNA families: *let-7-5p f* [Sampson *et al.*, 2007; Shah *et al.*, 2007; Bueno *et al.*, 2011], *miR-34-5p f* [Kong *et al.*, 2008; Kress *et al.*, 2011] (Supplementary Figure 1A in [Braun *et al.*, 2014]), *miR-33-5p f* [Takwi *et al.*, 2012], *miR-92-3p f* (also known as *miR-25-3p f*) [Bueno *et al.*, 2011], *miR-331-3p* [Bueno *et al.*, 2011], *miR-24-3p* [Lal *et al.*, 2009], *miR-145-5p* [Sachdeva *et al.*, 2009], and *miR-185-3p* [Liao and Lu, 2011].

The *MYC*-3'UTR bait was tagged with four MS2 stem-loops, which facilitated immobilization to amylose resin via recombinant maltose-binding protein (MBP)-fused MS2-coat protein (CP) (Figure 1A, B in [Braun *et al.*, 2014]). Incubation of immobilized bait RNA with U2OS as well as HEK293 cell lysates allowed co-purification of *let-7-5p* and *miR-34-5p* family members, as determined by means of qRT-PCR (Figure 1C, Supplementary Figure 2A in [Braun *et al.*, 2014]). Demonstrating miTRAP specificity, the substitution of two cytosines by guanines within the overlapping seed-matching region (Figure 1B in [Braun *et al.*, 2014], lower panel) abolished specific miRNA co-purification,

and miTRAP ratios (Figure 1C in [Braun *et al.*, 2014], upper panel) dropped to MS2 control bait levels (Figure 1C in [Braun *et al.*, 2014], lower panel; Supplementary Figure 2A in [Braun *et al.*, 2014]). Analyzing co-purification of non-reported, non-predicted control miRNAs further controlled selective co-purification. In contrast to reported *MYC*-regulatory miRNAs, the five control miRNAs including high abundant *miR-21-5p* (Supplementary Figure 1B in [Braun *et al.*, 2014]) co-purified at miTRAP ratios indistinguishable for all three bait RNAs. Consistent with miRNA co-purification, the miRISC component AGO2 was co-purified with the wild type and to a lesser extend with the mutant *MYC*-3'UTR bait (Figure 1D, Supplementary Figure 2B in [Braun *et al.*, 2014]). In contrast to AGO2, ELAVL1 proteins co-purified at indistinguishable amounts with wild type and mutant *MYC*-3'UTR baits, but not with the bead control or the MS2 control bait (Figure 1D, Supplementary Figure 2B in [Braun *et al.*, 2014]).

The analyses of co-purified miRNAs by next generation sequencing determined the selective co-purification of 36 miRNAs including 18 reported *MYC*-regulatory miRNAs (Figure 2E, Supplementary Table 1 in [Braun *et al.*, 2014]). These 18 reported miRNAs comprised members of the *let-7-5p*, *miR-34-5p*, *miR-92-3p* and *miR-33-5p* families. In contrast, eight miRNAs previously suggested to control *MYC* expression were not specifically co-purified with the *MYC*-3'UTR bait. However, one miRNA (*miR-185-3p*) was proposed to target the coding sequence (CDS) of *MYC* [Liao and Lu, 2011], and six other were expressed at low levels in U2OS cells (see input reads in Supplementary Table S1 in [Braun *et al.*, 2014]). Low expression and a potential low affinity of these miRNAs to the *MYC*-3'UTR may have reduced the amount of co-purification. The positive correlation between miRNA abundance in miTRAP eluates to input levels was visualized in Figure 2G in [Braun *et al.*, 2014]. *MiR-24-3p* was the only reported *MYC*-regulatory miRNA that was expressed at high levels in U2OS cells (Supplementary Table S1 in [Braun *et al.*, 2014]). However, it has to be noted that *miR-24-3p* targeting was validated on the basis of gain-of-function studies with luciferase reporters comprising isolated wild type or mutant *miR-24-3p* targeting sites, whereas one of two functional sites was encoded by a region downstream of the last poly(A)-signal [Lal *et al.*, 2009]. Hence, for this miRNA it remains to be addressed whether it regulates *MYC* expression in a direct and 3'UTR-dependent manner.

Nevertheless, the specific co-purification of 18 reported *MYC*-regulatory as well as their significant enrichment within the MYC sequencing libraries compared to the MS2 control libraries (Figure 2B in [Braun *et al.*, 2014]) suggested miTRAP as a reliable tool for the identification of novel *MYC*-regulatory miRNAs. To test this notion, the regulatory

potency of 10 non-reported, but selectively co-purified miRNAs was analyzed *in vivo*. Strikingly, silencing of nine candidates increased endogenous *MYC* expression in U2OS cells (Figure 3 in [Braun *et al.*, 2014]), as well as the expression of a luciferase reporter comprising the *MYC*-3'UTR (Figure 4A, B in [Braun *et al.*, 2014]). Moreover, ectopic expression of these miRNAs in HEK293 cells decreased *MYC*-3'UTR reporter activity (Figure 4C in [Braun *et al.*, 2014]). One of the ten tested candidates, *miR-1294*, did not regulate endogenous *MYC* expression, however, it affected luciferase expression in both loss- and gain-of-function analyses. This discrepancy may be due to additional sequence elements present in the full length, endogenous *MYC* transcript. It remains to be tested by future studies, whether *miR-1294* co-purifies less efficiently with full-length *MYC* miTRAP bait RNAs comprising 5'UTR, CDS, and 3'UTR.

The surprisingly low false-positive rate determined by the *in vivo* validation studies suggests that miRNAs of cell lysates specifically associate with *in vitro* transcribed RNAs lacking 5'- or 3'-modifications. Several previous observations supported the hypothesis of preserved targeting of miRNAs or RNA-binding proteins (RBPs) to *in vitro* transcribed RNAs: 1) The formation of miRNA-AGO complexes was observed after cell lysis without precedent chemical or UV-based stabilization of protein-RNA complexes [Zekri *et al.*, 2009; Riley *et al.*, 2012]. 2) The regulatory role of miRNAs or siRNAs was successfully recapitulated *in vitro* [Tuschl *et al.*, 1999; Mathonnet *et al.*, 2007]. 3) RBPs were co-purified with *in vitro* transcribed bait RNAs [Zhou *et al.*, 2002; Czaplinski *et al.*, 2005; Duncan *et al.*, 2006] or pre-miRNAs [Lee *et al.*, 2013] at high specificity. Additionally, the identification of *MYC*-regulatory miRNAs by miTRAP suggests that an overrepresentation of bait RNAs did not significantly bias miRNA-targeting specificity. Instead, it increased signal-to-noise ratios by enhancing purification yields. Accordingly, miTRAP also identified low abundant *MYC*-targeting miRNAs (Figure 2G in [Braun *et al.*, 2014]). Low purification rates of endogenously expressed MS2-tagged bait transcripts may be one explanation, why previously reported affinity purifications of endogenously expressed reporter RNAs did exclusively identify high abundant regulatory miRNAs [Vo *et al.*, 2010; Yoon *et al.*, 2012]. However to evaluate this hypothesis, the bait RNAs used in those studies remain to be analyzed by miTRAP. Nevertheless, although different in the set up, these approaches also demonstrated the suitability of RNA affinity purifications to identify regulatory miRNAs. Moreover, they confirmed *in vitro* targeting of miRNAs, because the endogenously expressed bait RNAs were purified from cell lysates without covalent stabilization of RNA-complexes prior to cell lysis. In accord with the identified *de novo* formation of miRISC-mRNA complexes after cell lysis

[Zekri *et al.*, 2009; Riley *et al.*, 2012], miRNA targeting may be a highly dynamic process, which can be rapidly affected by an altered stoichiometry of target RNAs. In the cell, this may be necessary to rapidly adjust gene expression to different conditions of gene expression like entry into cell cycle or response to altered nutrition.

In addition to higher purification rates, *in vitro* systems allow the investigation of isolated RNA *cis*-elements, and can be easily supplemented with reagents, such as recombinant proteins or even miRNAs. Up until now, miTRAP was applied for the *MYC, ZEB2* and *IGF2BP1* 3'UTRs, as well as for isolated miRNA targeting sites. In addition to these *cis*-elements, miTRAP would allow the analyses of miRNA targeting to isolated CDSs or 5'UTRs. MiRNA-mediated repression via CDSs [Forman *et al.*, 2008; Elcheva *et al.*, 2009] and 5'UTRs [Lee *et al.*, 2009] was reported, however, it was proposed that e.g. MTSs within the CDSs enhance miRNA-mediated repression via the 3'UTR instead of acting independently [Fang and Rajewsky, 2011]. Therefore, it will be interesting to analyze whether the miRNA interactomes of CDSs predominantly resemble 3'UTR-targeting miRNA populations.

The successful co-purification of reported and verified miRNAs with bait RNAs ranging from 280 nucleotides (MTS plus 4MS2 tag) (Figure 4D in [Braun *et al.*, 2014]) to approximately 6700 nucleotides (IGF2BP1-3'UTR bait) (Figure 16A in ADDITIONAL RESULTS, p. 113) suggests that miTRAP specificity is independent of bait RNA length. In regard of this bait length independence, miTRAP may also allow mapping of targeting miRNAs to full-length mRNAs, long noncoding RNAs as well as to viral RNA genomes. The clinical relevance of viral-miRNA interactions was demonstrated for the binding of host *miR-122-5p* to the hepatitis C virus RNA [Henke *et al.*, 2008]. Silencing of *miR-122-5p* with locked nucleic acids in chimpanzee strongly reduced blood HCV concentration [Lanford *et al.*, 2010].

Most strikingly, miTRAP identified five *MYC*-regulatory miRNAs that could not be predicted by the classical prediction algorithms (see Table 1 in INTRODUCTION, p. 17). Consistent with these findings, the recently reported CLASH approach (Table 2 in INTRODUCTION, p. 19) also identified a high number of seedless miRNA-mRNA interactions [Helwak *et al.*, 2013]. Although, the extensive list of putative miRNA-target interactions still awaits thorough evaluations, it further supported the notion that non-canonical miRNA targeting modes exist. To confirm *MYC*-3'UTR-dependent targeting of five 'unpredictable' miRNAs, putative targeting sites within the *MYC*-3'UTR were mutated. Although barely matching with previously described miRNA binding patterns (Figure 5 in INTRODUCTION, p. 13), the mutation of these MTSs abolished miRNA-

mediated regulation of the *MYC*-3'UTR comprising luciferase reporter (Figure 4A-C [Braun *et al.*, 2014]). These analyses provided strong evidence that miTRAP identified miRNAs regulate *MYC* expression in a 3'UTR-dependent manner. However, it has to be mentioned that these analyses cannot exclude bias by structural constraints, or targeting via additional sites, nor do they clarify MTS base pairing thoroughly. To further evaluate miRNA targeting via the putative non-canonical MTSs, *in vitro* binding of *miR-125a-3p* and *miR-455-3p* was analyzed by using the respective MTSs as miTRAP bait RNAs (Figure 4D in [Braun *et al.*, 2014]). Future studies remain to evaluate binding of the yet not analyzed *MYC*-regulatory non-canonical miRNAs (*miR-1248*, *miR-375* and *miR-4677-3p*) to their respective MTSs.

Interestingly, for the majority of miTRAP-identified *MYC*-regulatory miRNAs targeting was suggested within the first 170 nucleotides of the *MYC*-3'UTR (Figure 4A in [Braun *et al.*, 2014]). Although this needs experimental validation, such MTS positioning away from the center of the 3'UTR and in proximity to AU-rich regions was previously hypothesized [Grimson *et al.*, 2007; Bartel, 2009]. Moreover, HITS-CLIP studies experimentally identified AGO2 binding preferences in 3'UTRs mainly around the stop codon and at the 3'-ends [Chi *et al.*, 2009]. Based on the binding specificity of ELAVL1, two AU-rich elements have been identified within the *MYC*-3'UTR [Kim *et al.*, 2009a]. Both elements comprise approximately 50 nucleotides and are located at the very 3'-end and downstream of the miRNA targeting hotspot at the 5'-end of the *MYC*-3'UTR. In regard of the suggested stimulatory effect of ELAVL1 on *let-7-5p f* binding to the *MYC*-3'UTR [Kim *et al.*, 2009a] the question arises whether ELAVL1 or other AU-rich element-binding proteins modulate post-transcriptional regulation of *MYC* by one or several of the miTRAP-identified miRNAs. To address this question future studies will analyze the regulatory potency of these miRNAs in the absence of these proteins.

Relevance of miTRAP results in context of the post-transcriptional control of *MYC* and *IGF2BP1* expression

The miTRAP results for *MYC* as well as for *IGF2BP1* (miTRAP experiment conducted by Bianca Busch) suggest post-transcriptional regulation by multiple miRNAs ([Braun *et al.*, 2014], Figure 16A in ADDITIONAL RESULTS, p. 113). To some extend, this notion was in agreement with previous studies that identified several human and murine *MYC*-regulatory miRNAs [Sampson *et al.*, 2007; Shah *et al.*, 2007; Kong *et al.*, 2008; Lal *et al.*, 2009; Sachdeva *et al.*, 2009; Bueno *et al.*, 2011; Liao and Lu, 2011; Takwi *et al.*, 2012]. However, experimental data provided only *CDKN1A* as another example for such a

multilayered regulation by more than ten different miRNAs [Wu *et al.*, 2010]. In contrast to experimental data, *in silico* predictions like TargetScan [Lewis *et al.*, 2005; Grimson *et al.*, 2007; Friedman *et al.*, 2009; Garcia *et al.*, 2011] or PicTar [Grun *et al.*, 2005; Krek *et al.*, 2005; Chen and Rajewsky, 2006; Lall *et al.*, 2006] (Table 1 in INTRODUCTION, p. 17) support the view that the human genome encodes thousands of so-called target hubs, genes with more than 15 unrelated potential MTSs [Shalgi *et al.*, 2007]. Particularly, genes associated to development and transcription factors were enriched among these computationally identified target hubs [Shalgi *et al.*, 2007]. The tight control of transcription factor expression most likely corresponds to their crucial function in regulating a variety of cellular processes. On the other hand, genes expressed exclusively during development may evolutionary accumulate MTSs for miRNAs expressed post-developmentally in differentiated cells [Stark *et al.*, 2005; Bartel, 2009]. In accord with their function, *IGF2BP1* and *MYC* belong to the respective gene classes: *IGF2BP1* plays an crucial role during development, but its expression is essentially lost in adult tissues (reviewed in [Bell *et al.*, 2013]), whereas *MYC* is a transcription factor with broad impact (reviewed in [Eilers and Eisenman, 2008; Meyer and Penn, 2008]). Hence, the high number of regulatory miRNAs identified for these transcripts supports the target hub hypothesis [Shalgi *et al.*, 2007]. However, to further evaluate this hypothesis as well as the reliability of miTRAP-identified miRNAs, a larger set of transcripts remains to be analyzed. Moreover, it has to be demonstrated that miTRAP bait RNAs co-purify an essentially lower number of miRNAs when encoding 3'UTRs of e.g. housekeeping genes. To complete the picture, future studies remain to verify the regulatory potency of all miTRAP-identified *MYC*- and *IGF2BP1*-interacting miRNAs.

In addition to the reported autoregulation of *MYC* by *miR-185-3p* [Liao and Lu, 2011], the miTRAP analyses uncovered an autoregulatory feedback loop involving *MYC* and the *miR-17/92* genomic cluster. Consistent with previous findings that suggested the regulation of *MYC* by *miR-363-3p* [Bueno *et al.*, 2011], miTRAP identified the interaction of additional members of the *miR-92-3p* family (*miR-92a/b-3p*, *miR-25-3p*) with the *MYC*-3'UTR [Braun *et al.*, 2014]. Moreover, silencing of *miR-92a-3p* in U2OS cells increased the expression of *MYC* (Figure 3 in [Braun *et al.*, 2014]). The majority of *miR-92-3p* family members (*miR-92a-3p*, *miR-25-3p* and *miR-363-3p*) is expressed from three paralogues gene clusters (Figure 7) (reviewed in [Mogilyansky and Rigoutsos, 2013]), from which one *miR-92a-3p*-expressing cluster was shown to be directly activated by MYC [O'Donnell *et al.*, 2005]. Hence, MYC potentially autoregulates its expression by inducing *miR-92a-3p* expression (Figure 7). Furthermore, miTRAP studies by Bianca Busch

identified *in vitro* binding of *miR-17-5p*, *-20a-5p*, *-93-5p* and *-106b-3p* to the *IGF2BP1*-3'UTR (Figure 16A in ADDITIONAL RESULTS, p. 113). Like *miR-92a-3p*, *miR-17-5p* and *-20a-5p* are encoded by the *miR-17/92* cluster, whereas *miR-93-5p* and *-106b-3p* are encoded by its paralogue, the *miR-106b/25* cluster (Figure 7). In view of the role of IGF2BP1 in preventing *MYC* mRNA degradation [Lemm and Ross, 2002; Weidensdorfer *et al.*, 2009], these findings add an additional protagonist to the autoregulatory network of *MYC*. Induction of *miR-17/20a-5p* expression by MYC would decrease IGF2BP1 levels, which results in reduced *MYC* mRNA levels.

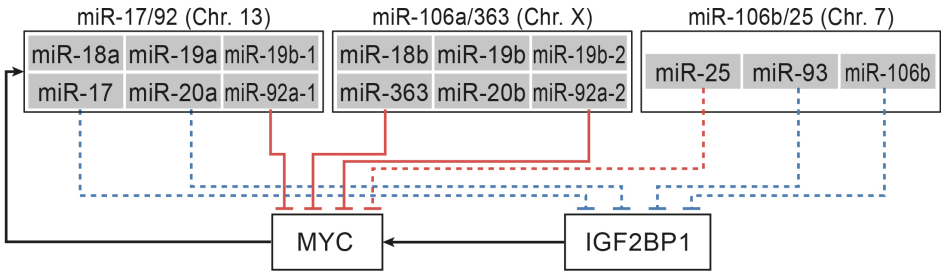

Figure 7 | Model of potential autoregulation of *MYC* by the *miR-17-92* cluster.
MYC transcriptionally activates the miR-17/92 cluster [O'Donnell *et al.*, 2005], which is located on chromosome 13 (Chr. 13) and encodes *miR-18a*, *-19a/b-1*, *-17*, *-20a* and *-92a-1*. The RNA-binding protein IGF2BP1 promotes *MYC* expression by stabilizing its mRNA [Lemm and Ross, 2002; Weidensdorfer et al., 2009]. MiTRAP studies suggested *MYC* and *IGF2BP1* as targets of several miRNAs (red and dashed blue lines) expressed by the miR-17/92 cluster or its paralogues located on chromosome X and 7. Regulation of *MYC* expression by *miR-363-3p*, a *miR-92-3p* family member was previously shown by gain-of-function analyses [Bueno *et al.*, 2011]. Moreover, in U2OS cells, silencing of *miR-92a-3p* increased *MYC* expression. The regulation of *MYC* and *IGF2BP1* expression by *miR-25-3p* and respectively *miR-17/20/93-5p* and *miR-106b-3p* remains to be validated *in vivo* (dashed lines).

What may be the physiological role of this regulatory feedback loop? MYC has the ability to promote cell growth, cell proliferation and to inhibit cell differentiation (reviewed in [Dang, 2013]). It was suggested that MYC-mediated gene regulation pushes growing cells to a critical mass until cells are pulled into S (synthesis) phase of cell cycle. Moreover, MYC directly activates expression of genes like *E2F1* (*E2F transcription factor 1*), which are involved in DNA replication (reviewed in [Dang, 2013]). By inducing the *miR-17/92* cluster, it was proposed that MYC tempers *E2F1* expression through G1 phase until cells enter S phase [Pickering *et al.*, 2009]. Premature accumulation of E2F1 by blocking *miR-17-5p* and *miR-20a-5p* led to a DNA-damage-induced G1 checkpoint, and as a result of this, induced cell cycle arrest [Pickering *et al.*, 2009]. High [Felsher *et al.*, 2000] as well as low levels of MYC [Trumpp *et al.*, 2001] have also been associated with cell cycle arrest. The overexpression of *MYC* in fibroblasts induced a G2 cell cycle arrest [Felsher *et al.*, 2000]. Interestingly, *pri-miR-17/92* levels were identified to reach a maximum at the G2/M (mitosis) checkpoint [Cloonan *et al.*, 2008]. Hence, fine-tuning *MYC* expression by *miR-92a-3p* potentially fine-tunes normal cell cycle progression, which was shown to be the major function of the *miR-17/92* cluster [Concepcion *et al.*,

2012]. All players of the suggested MYC autoregulatory loop were shown to be crucial promoters of cell proliferation during development. Knockout of *MYC*, *IGF2BP1* or the *miR-17/92* cluster significantly reduced mouse organ and body size [Trumpp *et al.*, 2001; Hansen *et al.*, 2004; Lu *et al.*, 2007], as well as neural stem cell proliferation [Kerosuo *et al.*, 2008; Bian *et al.*, 2013; Nishino *et al.*, 2013]. Future studies may shed light in the physiological relevance of this feedback loop by investigating expression levels of *MYC*, *IGF2BP1* and/or *miR-17/92* cluster miRNAs in mice lacking individual protagonists.

4 PUBLICATIONS & ADDITIONAL RESULTS

Article: Downregulation of microRNAs directs the EMT and invasive potential of anaplastic thyroid carcinomas

Oncogene (2010), 1–8

www.nature.com/onc

SHORT COMMUNICATION

Downregulation of microRNAs directs the EMT and invasive potential of anaplastic thyroid carcinomas

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Anaplastic thyroid carcinomas (ATCs) arise from epithelial thyroid cells by mesenchymal de-/transdifferentiation and rapidly invade the adjacent tissue. Specific microRNA signatures were suggested to distinguish ATCs from normal thyroid tissue and other thyroid carcinomas of follicular origin. Whether distinct microRNA patterns correlate with de-/transdifferentiation and invasion of ATCs remained elusive. We identified two significantly decreased microRNA families that unambiguously distinguish ATCs from papillary and follicular thyroid carcinomas: miR-200 and miR-30. Expression of these microRNAs in mesenchymal ATC-derived cells reduced their invasive potential and induced mesenchymal–epithelial transition (MET) by regulating the expression of MET marker proteins. Supporting the role of transforming growth factor (TGF)β signaling in modulating MET/epithelial–mesenchymal transition (EMT), expression of SMAD2 and TGFBR1, upregulated in most primary ATCs, was controlled by members of the miR-30 and/or miR-200 families in ATC-derived cells. Inhibition of TGFβ receptor 1 (TGFBR1) in these cells induced MET and reduction of prometastatic miR-21, but caused an increase of the miR-200 family. These findings identify altered microRNA signatures as potent markers for ATCs that promote de-/transdifferentiation (EMT) and invasion of these neoplasias. Hence, TGFBR1 inhibition could have a significant potential for the treatment of ATCs and possibly other invasive tumors.
Oncogene advance online publication, 24 May 2010; doi:10.1038/onc.2010.169

Keywords: ATC; microRNA; TGFβ; EMT; TGFBR1; SMAD2

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Received 14 September 2009; revised 8 March 2010; accepted 16 March 2010

Article: Pathogenic mechanisms of deregulated microRNA expression in thyroid carcinomas of follicular origin

Braun and Hüttelmaier *Thyroid Research* 2011, **4**(Suppl 1):S1
http://www.thyroidresearchjournal.com/content/4/S1/S1

REVIEW Open Access

Pathogenic mechanisms of deregulated microRNA expression in thyroid carcinomas of follicular origin

Juliane Braun, Stefan Hüttelmaier*

Abstract

Thyroid cancer is one of the most common malignancies of the endocrine system with increasing incidence. The vast majority of thyroid carcinomas derive from thyroid hormone producing follicular cells. Carcinomas of follicular origin are classified as follicular (FTCs), papillary (PTCs), partially differentiated (PDTCs) or anaplastic (ATCs) thyroid carcinomas. While FTCs and PTCs can be managed effectively, ATCs are considered one of the most lethal human cancers. Despite the identification of various genetic alterations, pathogenic mechanisms promoting the progression of thyroid carcinomas are still largely elusive. Over the recent years, aberrant microRNA expression was revealed in all as yet analyzed human cancers, including thyroid carcinomas. In view of the rapidly evolving perception that deregulated microRNA expression serves a pivotal role in tumor progression, microRNAs provide powerful tools for the diagnosis of thyroid carcinomas as well as the identification of potential therapeutic targets. Here, we summarize recent findings on microRNA signatures in thyroid carcinomas of follicular origin and discuss how deregulated microRNA expression could promote cancer progression.

Article: Rapid identification of regulatory microRNAs by miTRAP (miRNA trapping by RNA *in vitro* affinity purification)

Nucleic Acids Research Advance Access published February 7, 2014

Nucleic Acids Research, 2014, 1–14
doi:10.1093/nar/gku127

Rapid identification of regulatory microRNAs by miTRAP (miRNA trapping by RNA *in vitro* affinity purification)

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Received August 31, 2013; Revised January 15, 2014; Accepted January 20, 2014

ABSTRACT

MicroRNAs (miRNAs) control gene expression at the post-transcriptional level. However, the identification of miRNAs regulating the fate of a specific messenger RNA remains limited due to the imperfect complementarity of miRNAs and targeted transcripts. Here, we describe miTRAP (miRNA trapping by RNA *in vitro* affinity purification), an advanced protocol of previously reported MS2-tethering approaches. MiTRAP allows the rapid identification of miRNAs targeting an *in vitro* transcribed RNA in cell lysates. Selective co-purification of regulatory miRNAs was confirmed for the MYC- as well as ZEB2-3′UTR, two well-established miRNA targets *in vivo*. Combined with miRNA-sequencing, miTRAP identified in addition to miRNAs reported to control MYC expression, 18 novel candidates including not *in silico* predictable miRNAs. The evaluation of 10 novel candidate miRNAs confirmed 3′UTR-dependent regulation of MYC expression as well as putative non-canonical targeting sites for the not *in silico* predictable candidates. In conclusion, miTRAP provides a rapid, cost-effective and easy-to-handle protocol allowing the identification of regulatory miRNAs for RNAs of choice in a cellular context of interest. Most notably, miTRAP not only identifies *in silico* predictable but also unpredictable miRNAs regulating the expression of a specific target RNA.

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Additional results: Re-expression of *IGF2BP1* in anaplastic thyroid carcinomas promotes cancer progression

The identification of the TGFB-ZEB1-*miR-200-3p-miR-30-5p* axis, that most likely triggers malignant EMT in ATCs, suggested an interplay of transcriptional and post-transcriptional networks in the reprogramming of gene expression observed during thyroid carcinogenesis. In addition to miRNAs, RNA-binding proteins have been suggested to enhance or antagonize cancer progression by modulating gene expression post-transcriptionally [Kim *et al.*, 2009b]. One RNA-binding protein that was frequently identified in cancer is the *IGF2* mRNA-binding protein 1 (reviewed in [Bell *et al.*, 2013]). In non-transformed tissues, *IGF2BP1* expression is essentially lost in adulthood. However, during development and the regeneration of tissue IGF2BP1 was suggested to promote cell proliferation and migration (reviewed in [Bell *et al.*, 2013]). Thus, it appeared tempting to speculate that IGF2BP1 becomes *de novo* synthesized in ATCs and promotes or sustains EMT in thyroid cancer. The latter was recently supported by the observation that IGF2BP1 promotes the expression of *LEF1*, a pro-mesenchymal transcriptional regulator [Zirkel *et al.*, 2013].

Introduction

IGF2BP1 belongs to a highly conserved protein family that comprises the orthologues members VG1RBP (*Vg1*-mRNA binding protein, *Xenopus laevis)*, IGF2BPs (Insulin-like growth factor 1-binding proteins, *Homo sapiens*), CRD-BP (*c-myc* mRNA coding region determinant-binding protein, *Mus musculus*), KOC (KH-domain-containing protein overexpressed in cancer; *Homo sapiens*) and ZBP1 (Zipcode-binding protein 1, *Gallus gallus*) (Figure 8A) [Yisraeli, 2005]. All IGF2BPs comprise two N-terminal RNA-recognition motifs (RRMs) and four HNRNPK (heterogeneous nuclear ribonucleoprotein K) homology domains (KH domains) arranged as di-domains (KH1+2 and KH3+4) that mediate the RNA-binding capacity (Figure 8B) [Farina *et al.*, 2003; Nielsen *et al.*, 2003; Bell *et al.*, 2013; Wachter *et al.*, 2013]. A mutation of the GxxG-loop (x = any residue with a preference for basic residues) into GEEG (Glycine-Glutamate-Glutamate-Glycine) within each KH-domain of IGF2BP1 dramatically reduced RNA binding *in vitro* and subcellular localization of the protein [Nielsen *et al.*, 2003; Wachter *et al.*, 2013]. Under normal conditions IGF2BP1 localizes predominantly to cytoplasmic granule-like structures [Nielsen *et al.*, 2003; Wachter *et al.*, 2013], whereas under conditions of cellular stress it is recruited to stress granules [Stohr *et al.*, 2006]. Instead, IGF2BP1 KH1-4-GxxG-mutants

show a homogeneous cell distribution with a cytoplasmic as well as nuclear localization, and they do not associate with stress granules [Nielsen *et al.*, 2003; Stohr *et al.*, 2006; Wachter *et al.*, 2013]. These findings indicated that the function and subcellular distribution of IGF2BP1 is essentially determined by RNA binding and association with other RBPs [Wachter *et al.*, 2013]. The characterization of IGF2BP1-containing protein complexes by co-immunoprecipitation analyses revealed various associating RBPs like STAU1 (staufen double-stranded RNA-binding protein 1), FMRP (fragile X mental retardation protein) and ELAVL2 (embryonic lethal abnormal vision like RNA-binding protein 2), which co-localize with IGF2BP1 in cytoplasmic RNA-granules [Jonson *et al.*, 2007; Weidensdorfer *et al.*, 2009].

A

	IGF2BP3 / VG1RBP *Xenopus laevis*	IGF2BP1 / ZBP1 *Gallus gallus*	IGF2BP1 / CRD-BP *Mus musculus*	IGF2BP1 *Homo sapiens*	IGF2BP2 *Homo sapiens*	IGF2BP3 *Homo sapiens*
IGF2BP3 / VG1RBP *Xenopus laevis*	100	76	74	74	67	81
IGF2BP1 / ZBP1 *Gallus gallus*	76	100	95	94	67	74
IGF2BP1 / CRD-BP *Mus musculus*	74	95	100	99	65	73
IGF2BP1 *Homo sapiens*	74	94	99	100	66	74
IGF2BP2 *Homo sapiens*	67	67	65	66	100	65
IGF2BP3 *Homo sapiens*	81	74	73	74	65	100

B

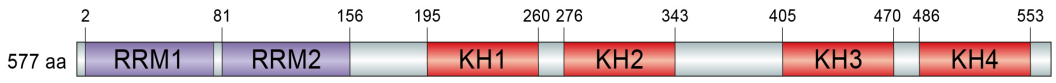

Figure 8 | Amino acid identity of IGF2BP1 paralogues and orthologues, and domain structure of IGF2BP1.
(A) Table shows percentage of amino acid identity of the most widely studied IGF2BP1 para- and orthologues from frog (*Xenopus leavis*), chicken (*Gallus gallus*), mouse (*Mus musculus*) and human (*Homo sapiens*) determined by protein-protein BLAST (blastp) alignment (http://blast.ncbi.nlm.nih.gov/Blast.cgi). IGF2BP1 shows highest identity with its orthologues from chicken and mouse. (B) IGF2BP1 consists of two RNA recognition motifs and four HNRNPK homology domains, which facilitate RNA binding and subcellular localization. Numbers above the scheme indicate amino acids of domains or linker regions, respectively.

Depending on the target transcript, IGF2BP1 regulates RNA turnover and/or translation leading to either an increase or decrease in gene expression (reviewed in [Bell *et al.*, 2013]). In response to environmental stress when bulk mRNA translation is blocked, IGF2BP1 was suggested to exclusively prevent target mRNA decay. This was also demonstrated for target mRNAs regulated at the level of translation in non-stressed cells, for instance the *ACTB* (*β-actin*) mRNA [Stohr *et al.*, 2006]. Moreover, to support cell migration as well as axon guidance IGF2BP1 also facilitates the transport of *ACTB* mRNA to the leading edge of fibroblasts [Shestakova *et al.*, 2001; Oleynikov and Singer,

2003] or the exploratory growth cones of developing neurons [Zhang *et al.*, 2001; Huttelmaier *et al.*, 2005].

The expression of *IGF2BP1* is characterized by a so-called 'onco-fetal' pattern. Although expressed in various embryonic tissues, the gene shows negligible expression levels in adult organisms, but was frequently described to be re-expressed in aggressive forms of cancer [Nielsen *et al.*, 1999; Hansen *et al.*, 2004; Hammer *et al.*, 2005; Bell *et al.*, 2013]. During mouse embryogenesis, *IGF2BP1-3* expression was induced at embryonic day (E) 10.5. Until E12.5, mRNA levels raised before declining again towards birth [Mueller-Pillasch *et al.*, 1999; Nielsen *et al.*, 1999; Hansen *et al.*, 2004; Dai *et al.*, 2011]. In contrast to *IGF2BP1*, *IGF2BP2* expression was detectable in all adult mouse tissues so far analyzed by RT-PCR as well as Western blot analysis. *IGF2BP3* expression is restricted to distinct organs like pancreas and testes [Hammer *et al.*, 2005; Dai *et al.*, 2011; Bell *et al.*, 2013].

Numerous studies identified the expression of *IGF2BP1* by either RT-PCR or IHC staining in neoplasia including carcinomas [Ross *et al.*, 2001; Ioannidis *et al.*, 2003; Ioannidis *et al.*, 2004; Dimitriadis *et al.*, 2007; Kobel *et al.*, 2007; Vainer *et al.*, 2008; Boyerinas *et al.*, 2012; Gutschner *et al.*, 2014], sarcomas [Ioannidis *et al.*, 2001], melanomas [Elcheva *et al.*, 2008], brain tumors, meningiomas [Ioannidis *et al.*, 2004], and various testicular tumors [Hammer *et al.*, 2005]. However, IHC might have been problematic due to the high sequence identity of IGF2BP1 paralogues (see Figure 8). In breast [Ioannidis *et al.*, 2003], ovary [Kobel *et al.*, 2007] and colorectal cancer [Dimitriadis *et al.*, 2007] the incidence of *IG2BP1* expression increased with tumor grading that quantitatively assesses the differentiation status from well- (lowest grade) to undifferentiated tumors (highest grade). Potentially due to cancer cell dedifferentiation, in both colorectal [Dimitriadis *et al.*, 2007] and ovarian [Kobel *et al.*, 2007] carcinomas *IGF2BP1* expression was shown to be associated with a higher metastasis potential, and a shorter recurrence-free and overall survival. Accordingly, IGF2PB1 was suggested to be a marker of cancer aggressiveness [Dimitriadis *et al.*, 2007].

The role of IGF2BP1 during development and tissue regeneration

To understand the role of *IGF2BP1* in tumorigenesis, it is necessary to review its proposed roles during development and tissue regeneration. Breeding data from *IGF2BP1* knockout mice revealed high prenatal mortality, and only 50% of homozygous animals were alive three days after birth suggesting an important function of *IGF2BP1* during development [Hansen *et al.*, 2004]. Moreover, Hansen *et al.* observed a growth

retardation that remained into adult life. This was reminiscent of the dwarfism phenotype described for *IGF2* (*insulin-like growth factor 2*) knockout mice [DeChiara *et al.*, 1990]. Accordingly, the authors suggested that the observed reduction of *IGF2* translation in E12.5 embryos upon *IGF2BP1* knockout could be one prerequisite for this phenotype [Hansen *et al.*, 2004]. This stimulatory effect of IGF2BP1 on *IGF2* translation was most recently confirmed in mouse embryonic fibroblasts [Dai *et al.*, 2013]. Consistent with the impact of *IGF2* on bone development [Minuto *et al.*, 2005], *IGF2BP1* knockout mice showed smaller bones as well as a loss of cartilage in extremities and the tail. Global gene expression profiling of E12.5 embryos and postnatal mice identified the downregulation of genes expressing extracellular matrix components like collagens, which are known to be necessary for cartilage formation as well as intestinal mucosa development, which was also impaired in *IGF2BP1* knockout mice [Hansen *et al.*, 2004].

Its role in intestinal mucosa homeostasis seems to be preserved into adulthood. During the response of mucosal damage, Manieri *et al.* identified re-expression of *IGF2BP1* in adult colonic mesenchymal stem cells (cMSCs) and suggested this to be necessary for the stabilization of the *PTGS2* (*prostaglandin-endoperoxide synthase 2*) mRNA. PTGS2 is required for the production of immunomodulatory prostaglandins [Manieri *et al.*, 2012], important for cMSC self-renewal [Walker *et al.*, 2010]. In agreement with its role in adult stem cell renewal, *IGF2BP1* expression was identified in embryonic stem cells of dorsal telencephalon, which develops into the cerebral cortex of the adult brain [Nishino *et al.*, 2013]. In *IGF2BP1* knockout animals, both embryonic and adult brains were significantly smaller due to a reduced number of neurons that differentiated from the reduced number of neural stem cells (Nishino et al., 2013).

Supporting its role in pre-differentiation processes, *IGF2BP1* expression was suggested to be irrelevant for the differentiation process of developing cortical neurons, because axonal and dendritic outgrowth, as wells as dendrite number and growth cone size of neurons cultured from *IGF2BP1* knockout embryos were similar to wild type neurons [Welshhans and Bassell, 2011]. However, IGF2BP1-mediated local translation of *ACTB* mRNA in neurons enhanced filopodia size at the axonal growth cone [Leung *et al.*, 2006; Yao *et al.*, 2006; Sasaki *et al.*, 2010; Welshhans and Bassell, 2011]. Growth cone filopodia are plasma membrane protrusions composed of bundled actin that direct growth cone migration, and function as sensing units for molecular cues, which direct axon guidance/pathfinding during development [Mattila and Lappalainen, 2008; Welshhans and Bassell, 2011].

During *Xenopus leavis* development, the IGF2BP1 orthologue VG1RBP was suggested to be required for the migration of cells forming the roof plate of the neural tube, and for neural crest cell migration [Yaniv *et al.*, 2003]. Taken together, IGF2BP1 plays an essential role during development by facilitating cell migration and proliferation. Hence, the loss of *IGF2BP1* or its orthologues led to hypoplastic [Hansen *et al.*, 2004; Nishino *et al.*, 2013] or abnormally formed organs [Yaniv *et al.*, 2003].

The role of IGF2BP1 in cancer

In addition to descriptive clinical studies, the molecular function of IGF2BP1 in tumorigenesis was investigated in cancer-derived cell lines and mouse xenograft models. Consistent with its role in development, pro-survival and pro-migratory functions were proposed [Wang *et al.*, 2004; Kobel *et al.*, 2007; Lapidus *et al.*, 2007; Boyerinas *et al.*, 2008; Elcheva *et al.*, 2008; Noubissi *et al.*, 2009; Noubissi *et al.*, 2010; Mongroo *et al.*, 2011; Stohr *et al.*, 2012; Gutschner *et al.*, 2014]. As prerequisite for its role in proliferation the potential of IGF2BP1 to stabilize the mRNAs of *BTRC (β-transducin repeat containing E3 ubiquitin protein ligase*) [Noubissi *et al.*, 2006], *GLI1 (GLI family zinc finger 1*) [Noubissi *et al.*, 2009], *MYC* [Kobel *et al.*, 2007; Gutschner *et al.*, 2014], and *KRAS* (*Kirsten rat sarcoma viral oncogene homologue*) [Mongroo *et al.*, 2011] was suggested. *KRAS* encodes a small GTP-binding protein, which is often activated by coding sequence mutations in cancer, leading to enhanced cell proliferation [Jancik *et al.*, 2010]. Increased levels of the transcription factor MYC also accelerate cancer cell proliferation [Kobel *et al.*, 2007; Meyer and Penn, 2008], whereas elevated levels of BTRC were suggested to protect cells from apoptosis [Elcheva *et al.*, 2008]. *BTRC* encodes a substrate-specific adaptor of an E3 ubiquitin ligase responsible for degradation of inhibitors of NF-κB, which suppresses apoptosis [Elcheva *et al.*, 2008]. *GLI1* encodes a transcriptional activator that attenuated the inhibitory effect of *IGF2BP1*-depletion on proliferation of colorectal cancer cells [Noubissi *et al.*, 2009]. A correlation between the expression of these factors and *IGF2BP1* in cancer was only given for *GLI1*, which showed in five of six tested *IGF2BP1* positive primary colorectal tumor samples increased mRNA amounts [Noubissi *et al.*, 2009].

Moreover, the IGF2BP1-mediated stabilization of *MDR1* (*multidrug resistance protein 1*) mRNA associated IGF2BP1 with the property of cancer cells to avoid chemotherapeutic treatment, a characteristic of cancer stem cells [Boyerinas *et al.*, 2012]. These findings, the aforementioned potential to facilitate the self-renewal of fetal neural stem cells (Nishino et al., 2013), as well as the positive impact on *CD44* expression [Vikesaa *et al.*, 2006], a gene highly expressed in CSCs from various cancer types [Jaggupilli and Elkord, 2012],

may indicate a role for IGF2BP1 in cancer stem cells. This hypothesis is supported by the IGF2BP1-dependent increased survival of a subpopulation of colorectal cancer cells that exhibited characteristics of CSCs [Hamilton *et al.*, 2013]. Further evidence for a role in tumor initiation was suggested by a transgenic mouse model, in which *IGF2BP1* was expressed from the whey acidic promoter (WAP) in mammary epithelial cells of lactating adult female mice [Tessier *et al.*, 2004]. Supporting a dose-dependent effect, 95% of high *IGF2BP1*-expressing and 60% of low *IGF2BP1*-expressing animals developed mammary tumors within 60 weeks. Some of these tumors even metastasized [Tessier *et al.*, 2004].

Indicated by the pro-migratory potential of VG1RBP and IGF2BP1 in neural crest cells and chicken embryonic fibroblasts, respectively [Farina *et al.*, 2003; Yaniv *et al.*, 2003], *IGF2BP1* gain- and loss-of-function studies in osteosarcoma-derived U2OS cells and ovarian carcinoma-derived ES-2 cells indicated a function of the protein in promoting the velocity as well as directionality of cancer cell migration [Stohr *et al.*, 2012]. These findings were supported by earlier studies in metastatic rat mammary carcinoma-derived MTLn3 cells that showed an increased polarity, persistence and directionality in cell motility upon ectopic *IGF2BP1* expression [Wang *et al.*, 2004; Lapidus *et al.*, 2007]. However, the stable polarization of this cell phenotype was suggested to reduce the ability to respond to chemotactic signals, e.g. an EGF gradient, necessary for cancer cell invasion [Lapidus *et al.*, 2007]. On the contrary, xenograft mouse models demonstrated the association between *IGF2BP1* expression and tumor cell dissemination into the blood [Hamilton *et al.*, 2013]. Moreover, the combined knockdown of *IGF2BP1* and *IGF2BP3* was suggested to reduce *in vitro* cell migration by decreasing invadopodia formation [Vikesaa *et al.*, 2006]. These actin-based membrane protrusion have been suggested to facilitate tumor cell invasion [Paz *et al.*, 2013].

Regulation of IGF2BP1 expression

As demonstrated in HEK293T cells [Noubissi *et al.*, 2006], in breast cancer-derived cells [Gu *et al.*, 2008], and fetal telencephalon [Nishino *et al.*, 2013] canonical Wnt signaling promotes *IGF2BP1* expression. Oncogenic stabilization of CTNNB1 in colorectal cancer [Noubissi *et al.*, 2006] and melanomas [Elcheva *et al.*, 2008] was suggested to be the driving force for *IGF2BP1* expression in cancer. In breast cancer cells, Gu *et al*. proposed a feedback regulation between CTNNB1 and *IGF2BP1*, which, when overexpressed, stabilized *CTNNB1* mRNA [Gu *et al.*, 2008].

The transcription factor MYC, which mRNA is stabilized by IGF2BP1, was also suggested to feed back by accelerating *IGF2BP1* expression [Noubissi *et al.*, 2010]. As demonstrated for CTNNB1, MYC directly interacted and activated the *IGF2BP1* promoter in HEK293T and HeLa cells, respectively, and *MYC* overexpression increased *IGF2BP1* protein and mRNA levels in both cell lines [Noubissi *et al.*, 2010]. Moreover, one could envision an indirect enhancement of *IGF2BP1* expression by the MYC-mediated repression of *let-7-5p f* expression. MYC was suggested to repress transcription [Chang *et al.*, 2008; Wang *et al.*, 2011] as well as maturation [Chang *et al.*, 2009] of several *let-7-5p* family members, which are strong repressors of *IGF2BP1* expression [Boyerinas *et al.*, 2008; Nishino *et al.*, 2013].

As an alternative mechanism to avoid miRNA-mediated repression, Mayr and Bartel proposed alternative polyadenylation of the *IGF2BP1*-3'UTR [Mayr and Bartel, 2009]. In various cancer-derived cell lines and in HEK293 cells, three *IGF2BP1*-3'UTR variants were observed, and reporters harboring the shortest variant were expressed at higher levels than reporters comprising the full length 3'UTR [Mayr and Bartel, 2009]. The impact of miRNA-mediated control of *IGF2BP1* expression was further supported by a most recent report that suggested the loss of global miRNA expression in an adult mesenchymal stem cell line as a trigger to induce *IGF2BP1-3* transcription [Gurtan *et al.*, 2013]. Comparing a *Dicer* knockout with a wild type cell line revealed a dramatic downmodulation of miRNA expression accompanied with increased levels of mRNAs encoding for factors highly expressed during mid-embryogenesis, where miRNA expressions have not yet globally activated [Gurtan *et al.*, 2013].

Results & Discussion

This project was conducted in collaboration with Alexander Mensch (MD thesis candidate).

IGF2BP1 is expressed in ATCs and marks epithelial dedifferentiation

Aiming at determining the expression status of *IGF2BP1* in thyroid cancer, total RNA of tumor tissue samples from patients suffering from FTCs, PTCs or ATCs was analyzed by means of qRT-PCR (Figure 9A). Tissue samples were different from those used by Braun *et al.* [Braun *et al.*, 2010] and provided by Dr. Kerstin Lorenz from the university hospital Halle (Saale). Based on the evaluation by the local pathologist, samples were categorized into tumor tissues or non-transformed thyroid tissues from FTC or PTC patients. Up to now the patient's disease progression is not available. Levels of *IGF2BP1* mRNA were

determined relative to *RPLP0* (*ribosomal protein large P0*) and *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) mRNAs by using the ΔCt-method. Consistent with the finding that *IGF2BP1* re-expression is linked to advanced carcinoma stages [Ioannidis *et al.*, 2003; Dimitriadis *et al.*, 2007; Kobel *et al.*, 2007], its expression was strongly elevated in tissues from ATC patients, but with the exception of one PTC-diagnosed patient, not detectable in tumors or non-transformed tissues from FTC and PTC patients (Figure 9A). However, although 14 ATC patients showed high *IGF2BP1* mRNA levels, seven expressed the mRNA at negligible levels (Figure 9B). Accordingly, ATC patients were assigned into an IGF2BP1-positive and an IGF2BP1-negative population.

To further evaluate *IGF2BP1* expression in ATC tissues and to correlate the expression with epithelial dedifferentiation, protein levels were determined by Western blot analysis (Figure 9C). CDH1 protein served as the primary epithelial marker, whereas ZEB1, ZEB2 and TGFBR1 were used as mesenchymal markers and to indicate EMT. In line with the qRT-PCR analyses, IGF2BP1 protein was exclusively detectable in the 14 ATC samples that showed a high IGF2BP1 mRNA expression (compare Figure 9B with C). In agreement to previous findings [Braun *et al.*, 2010], 15 of 21 ATC tissues (A1, A6, A8, A9, A11-A21) were characterized by a reduced or lost *CDH1* expression. Strikingly, in essentially the same samples an upregulation of *ZEB1*, *ZEB2* and *TGFBR1* expression was observed. Semi-quantitative Western blotting confirmed significantly reduced levels of CDH1 and significantly increased levels of ZEB1 in IGF2BP1-positive tissues compared to IGF2BP1-negative tissues (Figure 9D). Although the *ZEB2* and *TGFBR1* expressions were significantly elevated in ATC tissues compared with non-transformed tissues, there was no significant difference between IGF2BP1-positive and -negative tissues. To further evaluate the downregulation of *CDH1* expression and the upregulation of *ZEB1* expression in IGF2BP1-positive tissues, mRNA levels of *CDH1* and *ZEB1,* as well as of *VCL* (*vinculin*) as control were determined and normalized to *GAPDH* and *RPLP0* mRNA amounts. *CDH1* mRNA was barely detectable in IGF2BP1-positive ATC samples, whereas *ZEB1* mRNA levels were significantly increased in IGF2BP1-positive ATC tissues compared with IGF2BP1-negative ATC tissues (Figure 9E). Notably, with exception of one sample (A17), *VCL* mRNA levels remained unaffected between IGF2BP1-positive and IGF2BP1-negative ATC tissues.

The expression data from thyroid cancer tissues suggested an association of *IGF2BP1* re-expression with epithelial dedifferentiation of anaplastic thyroid carcinomas. To further support this notion, expression levels of the pro-epithelial miRNAs *141-3p* and *200c-3p* [Burk *et al.*, 2008; Gregory *et al.*, 2008a] in ATC as well as NT samples were

analyzed by means of qRT-PCR and using the snRNA U18 for normalization (Figure 9F). Consistent with the miRNA expression data determined by Braun *et al.*, tumor tissues derived from ATC patients showed a downmodulation of *miR-141-3p* and *miR-200c-3p* expression when compared with non-transformed tissues. In accord with the co-expression of *IGF2BP1* and *ZEB1*, the abundance of both miRNAs, although only significant for *miR-141-3p*, was more severely decreased in IGF2BP1-positive ATCs.

In addition to tumor-suppressive miRNAs, the expression of the pro-metastatic, pro-proliferative oncomiR *21-5p* [Yan *et al.*, 2011; Bornachea *et al.*, 2012] was determined. In breast [Yan *et al.*, 2008] and colorectal cancer [Xia *et al.*, 2013] *miR-21-5p* expression was associated with poor survival and advanced-staging. Consistent with the results from the comparative microarray analyses (Table 1 in [Braun *et al.*, 2010]), *miR-21-5p* was significantly upregulated in tumor tissues from ATC patients, with severely elevated levels in IGF2BP1-positive tissues ((Figure 9G).

In summary, the expression analyses of six non-transformed and 21 ATC tissue samples identified a correlation between *IGF2BP1* re-expression and epithelial dedifferentiation of ATCs, characterized by the downmodulation of *CDH1* [Scheumman *et al.*, 1995; Naito *et al.*, 2001; Wiseman *et al.*, 2007; Slowinska-Klencka *et al.*, 2012] and *miR-141-3p/200c-3p* expression [Park *et al.*, 2008]. Most interestingly, the expression of *ZEB1*, the reported transcriptional repressor of *CDH1* [Eger *et al.*, 2005] and *miR-141/200c-3p* [Burk *et al.*, 2008], correlated positively with *IGF2BP1* expression. Although also expressed in IGF2BP1-negative ATC tissues, *ZEB1* protein and mRNA levels were significantly elevated in IGF2BP1-positive tissues. A similar correlation was identified for the expression of oncomiR *21-5p*. This provided strong evidence that IGF2BP1 is a novel biomarker of ATCs with a potential role in malignant EMT.

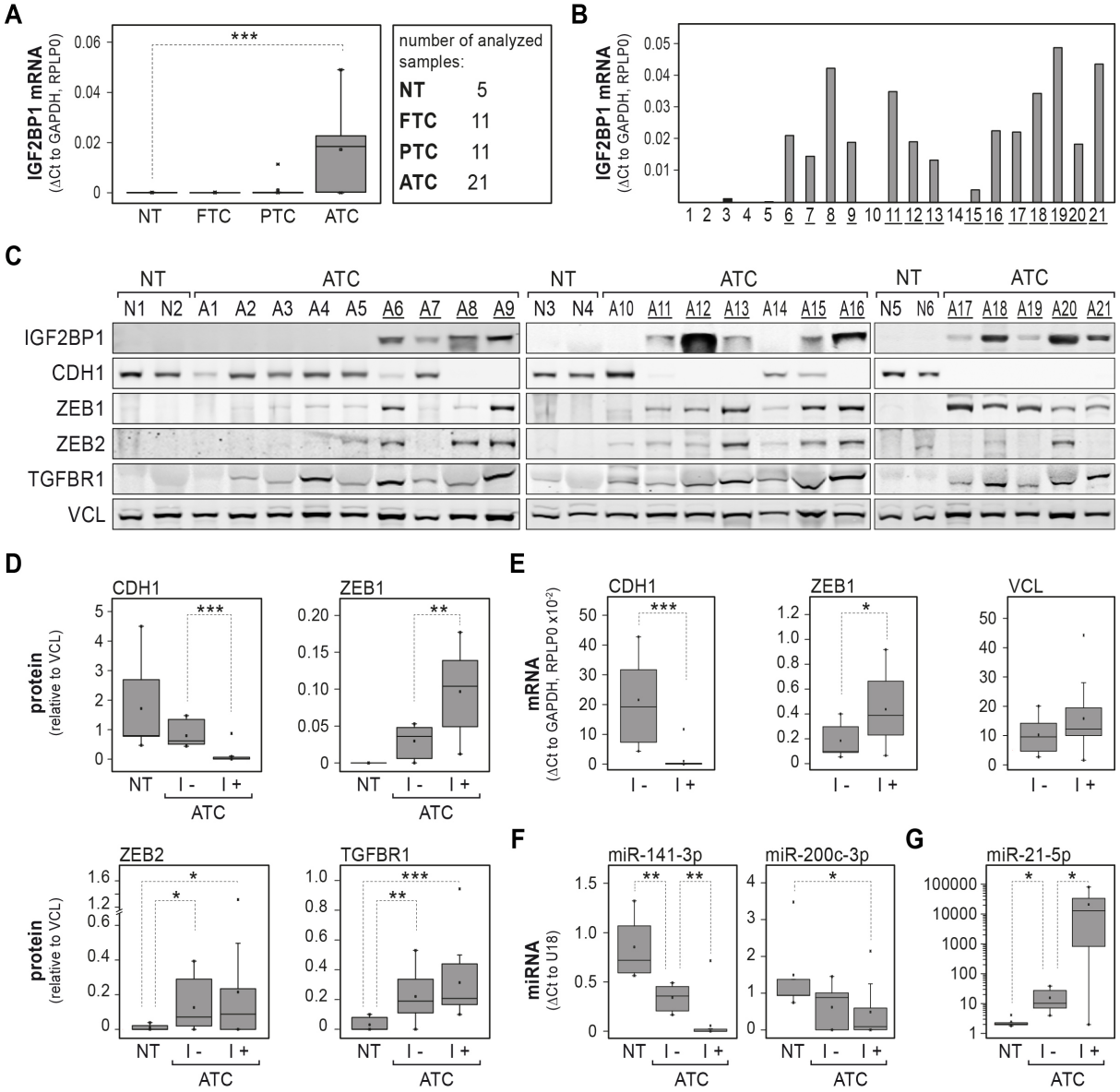

Figure 9 | *IGF2BP1* was re-expressed in anaplastic thyroid carcinomas with mesenchymal properties.
(A) Total RNA of tumor tissue samples from patients suffering from FTCs, PTCs or ATCs was analyzed for I*GF2BP1*, *GAPDH* and *RPLP0* mRNAs by qRT-PCR. RNA was extracted by means of phenol-chloroform extraction. Reverse transcription was performed by using random primers. Tumor adjacent non-transformed (NT) tissue originates from FTC or PTC patients. The number of tissue samples is depicted at the right panel. mRNA abundance of *IGF2BP1* was determined relative to *GAPDH* and *RPLP0* mRNA levels by cross-normalization using the ΔCt-method. Mean values of *IGF2BP1* mRNA abundance with error bars are presented as box plots. Student's t-testing determined statistical significant increase of *IGF2BP1* mRNA abundance in ATC samples in comparison to NT samples (***, $P < 0.0005$). (B) Bar diagram depicts *GAPDH*- and *RPLP0*-crossnormalized *IGF2BP1* mRNA abundance from individual ATC tissue samples. According to the *IGF2BP1* mRNA levels, ATC patients were assigned to an *IGF2BP1*-positive (underlined) and *IGF2BP1*-negative population. (C) Protein abundance of six NT tissues (N1-6) and 21 ATC tissues (A1-21) was determined by Western blot analyses with the indicated antibodies. Protein from tissue samples was extracted by using RIPA buffer and total protein concentration of extracts was determined by D_c protein assay (Bio-Rad, München, Germany) to allow equal loading. VCL served as loading control. Blots were analyzed by infrared scanning using the Odyssey scanner (LI-COR Biosciences). IGF2BP1-positive ATC tissue samples are labeled by underlined numbers. Western blot analyses for IGF2BP1, CDH1 and ZEB1 represent a reproduction of Western blot analyses performed by Alexander Mensch. (D) Fold change of CDH1, ZEB1, ZEB2 and TGFBR1 protein abundance was determined by normalization to VCL protein abundance. All blots were incubated with the same concentration of antibody and detection range. Mean values of relative protein abundance with error bars are presented as box plots for non-transformed tissue (NT) samples, IGF2BP1-negative ATC samples (I-) and IGF2BP1-positive ATC samples (I+). Student's t-testing determined statistical significant decrease of CDH1 protein abundances, and statistical significant increase of ZEB1 protein abundances in IGF2BP1-positive samples in comparison to IGF2BP1-negative samples, as well as statistical significant increase of ZEB2 and TGFBR1 protein abundances in ATC tissues in comparison to NT tissues (***, $P < 0.0005$; **, $P < 0.005$; * $P < 0.05$). (E) mRNA of ATC tissue samples depicted in (C) was analyzed for *CDH1, ZEB1, VCL, GAPDH* and *RPLP0* by means of qRT-PCR. *CDH1, ZEB1* and *VCL* mRNA abundance was determined relative to *GAPDH* and *RPLP0* mRNA levels by cross-normalization using the ΔCt-method. Mean values of relative mRNA abundance with error bars are presented as box plots for IGF2BP1-negative and IGF2BP1-positive ATC samples (see B, C). Note, y-axis scaling represents ΔCt value x 10^{-2}. Student's t-testing determined statistical significant decrease of *CDH1* mRNA, and statistical significant increase of *ZEB1* mRNA levels in IGF2BP1-positive samples in comparison to IGF2BP1-negative samples (***, $P < 0.0005$; *, $P < 0.05$).
Continued on next page

Figure 9 Continued
(F, G) Abundance of (F) *miR-141-3p*, *miR-200c-3p*, and (G) *miR-21-5p* was analyzed by means of TaqMan-based qRT-PCR (Life Technologies, Carlsbad, CA, USA) and normalized to snRNA U18 levels by using the ΔCt-method. Mean values of miRNA abundance with error bars are presented as box plots. Note logarithmic scale for *miR-21-5p* plot. Student's t-testing determined statistical significant decrease of *miR-141-3p* and *miR-200c-3p* abundances, as well as statistical significant increase of *miR-21-5p* abundances (**, $P < 0.005$; *, $P < 0.05$). (Antibodies and oligonucleotides are depicted in APPENDIX.)

IGF2BP1 is co-expressed with mesenchymal markers in carcinoma-derived cell lines

The expression analyses in ATC tissues supported the view that *IGF2BP1* expression marks epithelial dedifferentiation. To evaluate this assumption and to correlate the expression of *IGF2BP1* with mesenchymal markers, 12 carcinoma-derived cell lines were analyzed for *IGF2BP1*, *CDH1*, and mesenchymal marker expression (Figure 10A). Consistent with the inverted expression pattern of *IGF2BP1* and *CDH1* in ATC tissues, *IGF2BP1* was expressed at negligible levels in the *CDH1*-expressing cell lines derived from a primary pancreas carcinoma (BxPC3, Sigma-Aldrich, St. Louis, USA), primary colorectal carcinomas (HCT 116; HRT-18; HT-29, ATCC, Manassas, USA) and a primary mammary carcinoma (MCF7, Sigma-Aldrich, St. Louis, USA). In contrast, high levels of IGF2BP1 protein were observed in the *ZEB1*-, *ZEB2*- as well as *VIM*-expressing cell lines derived from a poorly differentiated clear-cell ovarian carcinoma (ES-2, ATCC, Manassas, USA), a lymph node metastasis of follicular thyroid carcinoma (FTC-133; Sigma-Aldrich, St. Louis, USA), a poorly differentiated papillary thyroid carcinoma (B-CPAP, DSMZ, Braunschweig, Germany), primary anaplastic thyroid carcinomas (8505C, Sigma-Aldrich, St. Louis, USA; C-643, CLS, Eppelheim, Germany), a cervical carcinoma (HeLa, ATCC, Manassas, USA) and a poorly differentiated pancreas carcinoma (PANC-1, ATCC, Manassas, USA). To further evaluate the epithelial characteristics of the carcinoma-derived cell lines, the expression of the non-coding epithelial markers *miR-141-3p* and *miR-200c-3p* was analyzed in addition to the expression of *CDH1* (Figure 10B). In agreement with the repressive function of the transcription factors ZEB1 and ZEB2 [Burk *et al.*, 2008], both miRNAs were barely detectable in mesenchymal-like cell lines, but highly expressed in *CDH1*-expressing cells. Surprisingly, the expression of *SNAI1*, *SNAI2* and *LEF1*, reported mesenchymal markers [Zeisberg and Neilson, 2009] and transcriptional repressors of *CDH1* [Cano *et al.*, 2000; Bolos *et al.*, 2003; Jesse *et al.*, 2010] correlated only to some extend with the expression pattern of *ZEB1*/*ZEB2* and *VIM*.

Most interestingly, the expression of the IGF2BP1 paralogues *IGF2BP2* and *IGF2BP3* was not restricted to mesenchymal-like cells (Figure 10A). This accounts also for *CTNNB1* and *MYC*, which encode a transcriptional co-activator and a transcription

factor, respectively, suggested to activate *IGF2BP1* transcription [Noubissi *et al.*, 2006; Noubissi *et al.*, 2010].

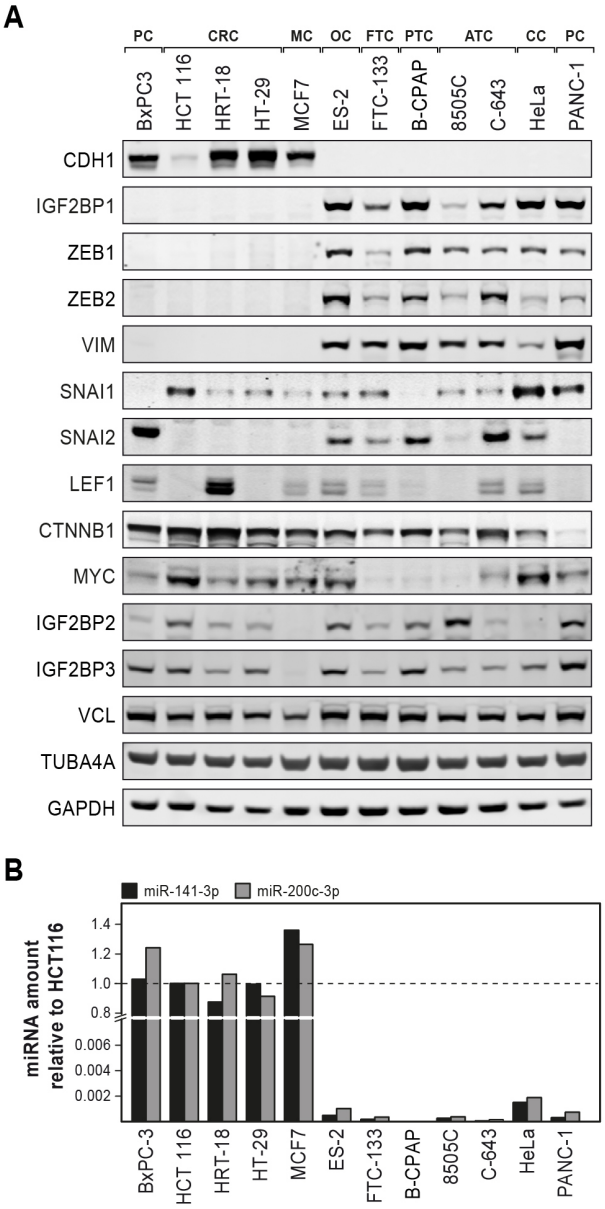

Figure 10 | IGF2BP1 was detectable in carcinoma-derived cell lines that express mesenchymal markers.
(A) Indicated carcinoma-derived cell lines were cultured for 48 hours and harvested at 90% confluence before analyzing the abundance of indicated proteins by Western blotting. Cells were lysed by RIPA buffer and total protein concentration of lysates was determined by D_c protein assay (Bio-Rad, München, Germany) to allow equal loading. VCL, α-tubulin 4A (TUBA4A) and GAPDH served as loading controls. *CDH1*-expressing pancreas carcinoma (PC)-derived BxPC-3, colorectal carcinoma (CRC)-derived HCT116, HRT-18 and HT-29, and mammary carcinoma (MC)-derived MCF7 expressed negligible levels of IGF2BP1, whereas the following cell lines expressed high levels of IGF2BP1: ovarian carcinoma (OC)-derived ES-2, follicular thyroid carcinoma (FTC)-derived FTC-133, papillary thyroid carcinoma (PTC)-derived BCPAP, anaplastic thyroid carcinoma (ATC)-derived 8505C and C-643, cervical carcinoma (CC)-derived HeLa, and PC-derived PANC-1 cells. (Alexander Mensch performed Western blot analyses for CDH1, IGF2BP1 and ZEB1; antibodies are depicted in APPENDIX.) (B) RNA of indicated cell lines was analyzed for *miR-141-3p, miR-200c-3p, miR-16-5p* and snRNA *U18* by means of TaqMan-based qRT-PCR (Life Technologies, Carlsbad, CA, USA). Abundance of *miR-141-3p* and *miR-200c-3p* was determined relative to HCT116 cells, and *U18* and *miR-16-5p* levels for normalization by using the ΔΔCt-method.

Depletion of IGF2BP1 impairs mesenchymal-like cell properties of ATC-derived cells

The mesenchymal expression pattern of *IGF2BP1* in ATC tissues and carcinoma-derived cell lines suggested a role for IGF2BP1 in promoting mesenchymal-like properties of anaplastic thyroid carcinoma cells. Previous studies indicated a function of IGF2BP1 and its orthologues in the migration of neural crest cells [Yaniv *et al.*, 2003], chicken embryonic fibroblasts [Farina *et al.*, 2003], and cancer-derived cells [Wang *et al.*, 2004; Lapidus *et al.*, 2007; Stohr *et al.*, 2012; Zirkel *et al.*, 2013]. To test the pro-migratory role of

IGF2BP1 in ATC-derived cells, Alexander Mensch compared the migratory potential of 8505C cells treated with two different siRNAs directed against *IGF2BP1*, respectively, with 8505C cells treated with a control siRNA. Cell motility was determined by wound closure assays. Indicated by the decreased potential to close the wound, the knockdown of *IGF2BP1* significantly impaired cell migration (Figure 11A). Knockdown efficiencies were verified by Western blot analyses (data not shown).

In addition to an increased motility, cancer cells with mesenchymal-like cell properties posses the ability to digest extracellular matrix components and invade adjacent tissues or blood vessels. Vikesaa *et al*. hypothesized an impact of IGF2BP1 on cell invasiveness, because the combined knockdown of *IGF2BP1* and *IGF2BP3* reduced invadopodia formation and the expression of cell adhesion molecules [Vikesaa *et al.*, 2006]. To test the role of IGF2BP1 in directing cell invasion, *in vitro* invasiveness of 8505C cells was analyzed by using a transmigration assay (InnoCyte™ Cell Invasion Assay, Merck, Darmstadt, Germany). *IGF2BP1* knockdown and control cells were seeded in serum-free media into cell culture inserts (upper chamber) with an 8 μm pore size polycarbonate membrane coated with a layer of basement membrane matrix (BMM). After 48 hours of incubation, the number of cells that invaded the BMM into the lower chamber of the tissue culture plate was determined by staining viable cells with calcein AM (acetomethoxy derivate of calcein). The lower chamber contained media with 10% fetal bovine serum as a chemoattractant. In comparison to control cells, from which about 11% invaded the BMM, invasion of *IGF2BP1*-depleted cells was reduced up to three-fold (Figure 11B). Because IGF2BP1 is a well-known pro-proliferative factor (reviewed in [Bell *et al.*, 2013]), the number of viable cells was determined over time by using the CellTiter-Blue Cell Viability Assay (Promega, Madison, WI, USA) parallel to the cell invasion assays. In agreement with studies performed in ES-2 cells [Kobel *et al.*, 2007], the *IGF2BP1* knockdown population ceased to divide (Figure 11C). Hence, the strongly reduced invasive potential of *IGF2BP1* knockdown cells (Figure 11B) may result from impaired invasion as well as decreased proliferation. To further test the impact of IGF2BP1 on cancer cell invasion, and to separate this effect from cell proliferation, microscopy-assisted single cell analyses remain to be performed in future studies. Moreover, the formation of invadopodia, which mediate invasion, may be analyzed upon *IGF2BP1* knockdown in ATC-derived cells.

Consistent with the reduced migratory and invasive potential, *IGF2BP1*-depleted 8505C cells altered their morphology from a more spindle-shaped to a flattened, round-shaped morphology reminiscent of a MET (Figure 11D, left panel). Moreover, the cells

grew in closer contact to each other. Immunostaining of the adherens junction component CTNNB1 revealed pronounced interactions of *IGF2BP1*-depleted cells (Figure 11D, right panel). However CDH1 was not detectable by immunostaining or Western blot analyses, and an increased expression of *CDH1* upon *IGF2BP1* knockdown was also not observed at the mRNA level (data not shown). Surprisingly, however, the knockdown of *IGF2BP1* reduced the expression of *ZEB1* (Figure 11E). On the contrary, the expression of both *ZEB2* isoforms was not reduced in *IGF2BP1* knockdown cells; instead it was even slightly increased. Immunostaining for MYC served as a positive control for the *IGF2BP1* knockdown-mediated changes in gene expression [Kobel *et al.*, 2007].

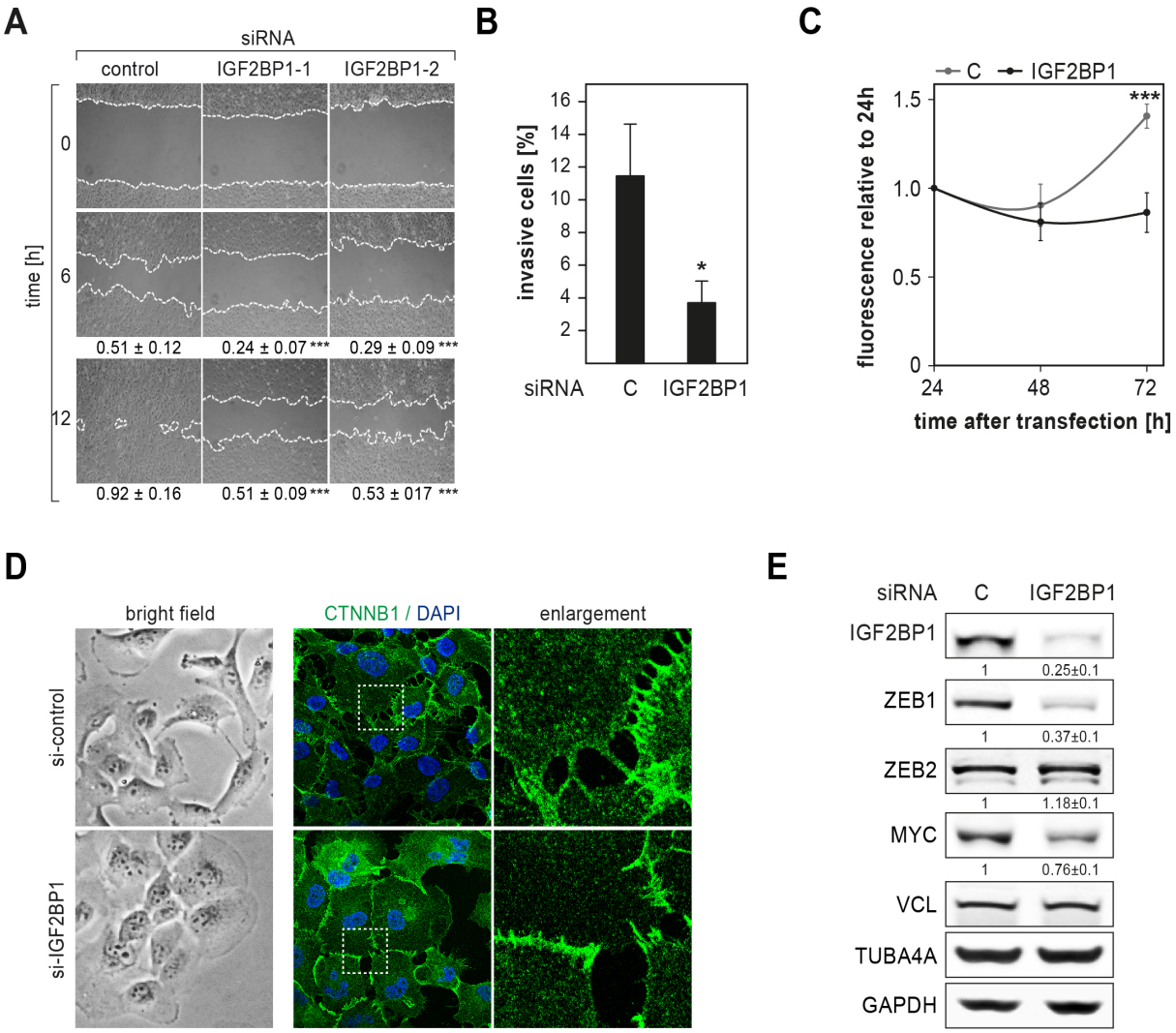

Figure 11 | *IGF2BP1* knockdown impairs mesenchymal cell properties of ATC-derived 8505C cells.
(A) 300,000 8505C cells were transfected with 75 pmol of indicated siRNAs by using Lipofectamin RNAiMax (Life Technologies, Carlsbad, CA, USA). The control siRNA encoded *cel-miR-239b-5p*. 48 hours after transfection, 200.000 cells were seeded in 24-well tissue culture plates. After 12 hours of incubation, cell populations were scratched before wound closure was determined every six hours over
Continued on next page

Figure 11 Continued
12 hours by means of microscopy (scratch, 250 μm). Cell migration was assessed by quantitative means using an automated segmentation algorithm [Glaß *et al*., 2012]. SD was determined over three independent analyses. Statistical significance was validated by Student's t-testing (***, $P < 0.0005$). The *IGF2BP1* knockdown was monitored by Western blot analyses of protein extracts from the residual cells (data not shown). (Experiments depicted in (A) were conducted by Alexander Mensch.) (B-D) 600,000 8505C cells were transfected with a mix of IGF2BP1-1 and IGF2BP1-2 siRNAs (total 150 pmol), or 150 pmol control siRNA by using Lipofectamin RNAiMax (Life Technologies, Carlsbad, CA, USA). 20 hours after transfections cells were counted and seeded for (B) invasion assay (300,000 cells in invasion chamber), (C) viability assay (3x 10,000 cells in 96-well plates in duplicated), (D, E) microscopy analyses and Western blot analyses (200.000 cells in one well of a 6-well plate, which harbored one cover-slip for immunostaining). (B) *In vitro* cell invasion was analyzed 48 hours after seeding in invasion chamber by using the InnoCyte™ Cell Invasion Assay Kit (Merck, Darmstadt, Germany). SD was determined over three independent analyses. Statistical significance was validated by Student's t-testing (*, $P < 0.05$). (C) Cell viability was determined by using the CellTiter-Blue Viability Assay Kit (Promega, Darmstadt, Germany) at indicated time points after *IGF2BP1*-directed siRNA transfection. The assay is based on the fluorescence dye resazurin, which is reduced by viable cells to resofurin upon incubation for four hours. Therefore the 20 hours time point is indicated as 24 hours. The fluorescence intensity of resofurin at the 48 and 72 hours time point was normalized to the 24 hours time point. (D) Cell morphology of control and IGF2BP1 depleted cells was monitored by light microscopy. Cell shape and cell-cell interactions were further analyzed by immunostainig for CTNNB1. Enlargements of box regions (left panel) are shown in the right panels (enlargement). Note altered cell morphology as well as reduced gap size between individual cells. (E) Cells seeded for Western blot analyses were harvested and lysed in RIPA buffer. Total protein concentration of lysates was determined by D_c protein assay (Bio-Rad, München, Germany) to allow equal loading. VCL, TUBA4A and GAPDH served as loading controls. Fold change of IGF2BP1, ZEB1, ZEB2 and MYC protein abundance on *IGF2BP1* knockdown from three independent experiments were determined relative to control cells by normalization to GAPDH protein abundance, as indicated below the panel. (Antibodies, siRNA and oligonucleotides are depicted in APPENDIX.)

Taken together, these results suggest that the re-expression of *IGF2BP1* in ATCs may contribute to the high invasive and proliferative potential of this aggressive malignancy. The reduced motility and invasion of 8505C cells upon *IGF2BP1* depletion indicated a pro-mesenchymal role of IGF2BP1 and supported previous findings suggesting IGF2BP1 as a key regulator of cell migration and invasion (reviewed in [Stohr and Huttelmaier, 2012]). The modulation of *ACTB* expression and of microfilament dynamics by IGF2BP1 were suggested to promote velocity of cell migration and also cell-matrix adhesion, a prerequisite for cell migration [Stohr and Huttelmaier, 2012]. IGF2BP1 was shown to inhibit *ACTB* mRNA translation and in addition it was proposed to modulate G-actin sequestering by interfering with the translation of the *MAPK4* mRNA [Huttelmaier *et al*., 2005; Stohr *et al*., 2012]. On the other hand, the stimulatory effect on tumor cell invasion was suggested to be facilitated by the IGF2BP1-mediated stabilization of *CD44* mRNA, which enhances the formation of invadopodia *in vitro* [Vikesaa *et al*., 2006]. To link these features with ATC tumorigenesis, it remains to be analyzed if, how and to what extend IGF2BP1 modulates these processes in ATC-derived cells. Moreover, the expression of respective IGF2BP1 target genes might be determined in ATC tissue samples.

Consistent with observations from ovarian [Kobel *et al*., 2007] and hepatocellular carcinoma-derived cells [Gutschner *et al*., 2014], depletion of *IGF2BP1* inhibited the proliferation of 8505C cells. Interestingly, the ceased proliferation was accompanied by morphological changes, characterized by the gain of a large and flattened cell shape. Although this phenotype may be reminiscent of an epithelial morphology, it may also be reminiscent of a senescent morphology. Senescent cells, which lost their proliferative capacity, can become flat and large [Kuilman *et al*., 2010]. Accordingly, it will be interesting to investigate whether IGF2BP1 antagonizes senescence and thereby

contributes to cancer cell proliferation. A prominent, and easy-to-analyze senescent biomarker is senescence-associated β-galactosidase, which increases its activity under senescent conditions [Kuilman *et al.*, 2010]. In addition, it remains to be analyzed whether the reduced cell number in the IGF2BP1 depleted population results from the induction of apoptosis, as recently suggested for hepatocellular carcinoma-derived cells [Gutschner *et al.*, 2014]. A link to all these functions was given by the reduced expression of *MYC* and *ZEB1* in 8505C upon *IGF2BP1* knockdown. Both transcription factors have been associated with pro-proliferative as well as pro-metastatic processes (reviewed in [Browne *et al.*, 2010; Wolfer and Ramaswamy, 2011; Dang, 2013]). However, in contrast to *MYC*, which represents a well-characterized IGF2BP1 target [Doyle *et al.*, 1998; Lemm and Ross, 2002; Bell *et al.*, 2013], *ZEB1* presented a potential novel IGF2BP1 target mRNA.

Depletion of IGF2BP1 reduces ZEB1 mRNA stability

The reduced expression of *ZEB1* in *IGF2BP1*-depleted 8505C cells suggested *ZEB1* mRNA as a novel IGF2BP1 target transcript. To further test this notion, *ZEB1* expression upon *IGF2BP1* depletion was, in addition to 8505C cells, also analyzed in ATC-derived C-643 cells. In both cell lines, the *IGF2BP1* knockdown directed by two different siRNAs led to a significant decrease of steady state ZEB1 protein as well as *ZEB1* mRNA levels (Figure 12A, B). According to the Gene database of the National Center for Biotechnology Information (NCBI) *ZEB1* encodes six protein-coding transcripts that result from alternative splicing and alternative translation initiation sites. The qRT-PCR primers used in this study allowed detection of all isoforms. The antibody used for Western blot analyses was supposed to recognize all isoforms as well, however due to low differences in the molecular weight, distinct isoforms may not be recognizable. The decreased steady levels of *ZEB1* mRNA suggested that IGF2BP1 promoted *ZEB1* expression by stabilizing *ZEB1* mRNA, as previously demonstrated for *MYC* [Weidensdorfer *et al.*, 2009], *PTEN* [Stohr *et al.*, 2012], and *LEF1* [Zirkel *et al.*, 2013]. To analyze the role of IGF2BP1 in preventing *ZEB1* mRNA degradation, Alexander Mensch monitored *ZEB1* transcript turnover upon *IGF2BP1* knockdown in 8505C cells by using Actinomycin D (ActD). ActD was proposed to inhibit transcription by preventing elongation of growing RNA chains [Sobell, 1985]. These analyses revealed a significant destabilization of *ZEB1* mRNAs in response to *IGF2BP1* depletion (Figure 12C). In contrast, levels of *VCL* mRNA remained insignificantly affected upon reduction of

IGF2BP1 expression. Both *ZEB1* and *VCL* mRNA levels were internally normalized to *RPLP0* transcript levels.

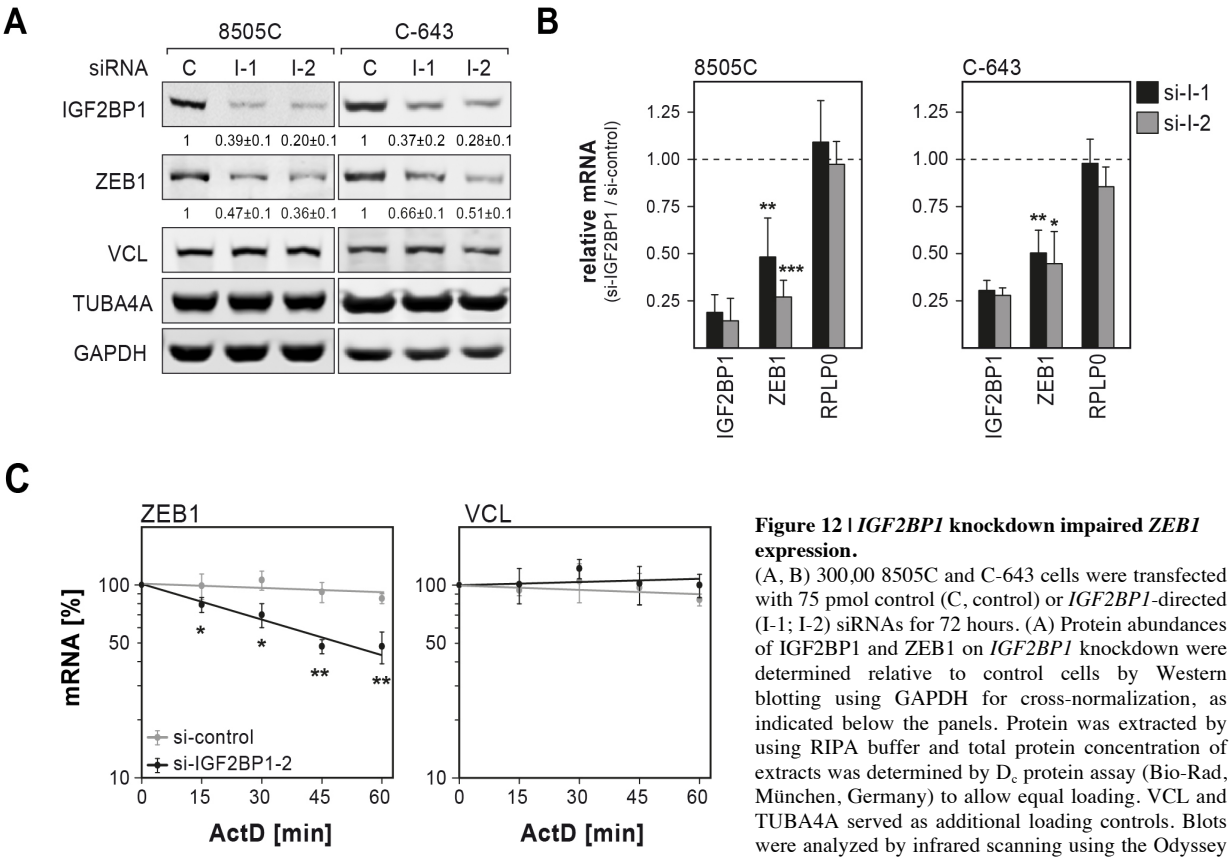

Figure 12 | *IGF2BP1* knockdown impaired *ZEB1* expression.
(A, B) 300,00 8505C and C-643 cells were transfected with 75 pmol control (C, control) or *IGF2BP1*-directed (I-1; I-2) siRNAs for 72 hours. (A) Protein abundances of IGF2BP1 and ZEB1 on *IGF2BP1* knockdown were determined relative to control cells by Western blotting using GAPDH for cross-normalization, as indicated below the panels. Protein was extracted by using RIPA buffer and total protein concentration of extracts was determined by D_c protein assay (Bio-Rad, München, Germany) to allow equal loading. VCL and TUBA4A served as additional loading controls. Blots were analyzed by infrared scanning using the Odyssey scanner (LI-COR Biosciences). (The Western blot shown for 8505C cells displays a reproduction performed by Alexander Mensch.) (B) *IGF2BP1*, *ZEB1* and *RPLP0* mRNA levels were analyzed by means of qRT-PCR. Total RNA was extracted by means of phenol-chloroform extraction. Reverse transcription was performed by using random priming. Changes in *IGF2BP1*, *ZEB1* and *RPLP0* mRNA abundance on IGF2BP1 knockdown were determined relative to control cells by the ΔΔCt-method using *GAPDH* for normalization. Error bars indicate SD of at least three independent analyses. Student's t-testing determined statistical significant decrease of *ZEB1* mRNA abundance in *IGF2BP1* knockdown cells (*, $P < 0.05$; **, $P < 0{,}005$; ***, $P < 0.0005$). (C) mRNA decay was monitored in 8505C cells transfected with indicated siRNAs for 72 hours by blocking mRNA synthesis using 5μM Actinomycin D (ActD) for indicated times. mRNA levels were determined by means of qRT-PCR and normalization to *RPLP0* by the ΔΔCt-method. *VCL* served as a control. mRNA decay is depicted in semi-logarithmic scale. Statistical significant decrease of *ZEB1* mRNA abundance upon *IGF2BP1* knockdown determined over three independent experiments was analyzed by Student's t-testing (*, $P < 0.05$; **, $P < 0{,}005$). Decay experiments were performed by Alexander Mensch. (Antibodies, siRNA and oligonucleotides are depicted in APPENDIX.)

The role of IGF2BP1 in interfering with *ZEB1* mRNA turnover suggested a direct association of IGF2BP1 with the *ZEB1* mRNA. To test this hypothesis, Alexander Mensch analyzed mRNAs co-immunoprecipitated with IGF2BP1 protein from HeLa cell lysate by means of qRT-PCR. As observed in ATC-derived cells, the depletion of *IGF2BP1* in HeLa cells reduced ZEB1 protein as well as *ZEB1* mRNA abundance (Figure 12A, B). Indicated by their increased abundance within the IGF2BP1-co-IP fraction compared to the bead control (BC), *ZEB1* as well as *LEF1* mRNAs were associated with IGF2BP1 protein from HeLa cell lysates (Figure 13C). *LEF1* was identified as an IGF2BP1 target mRNA by Nadine Bley during the time of this study, and served as a positive control for the IP analyses [Stohr *et al.*, 2012; Zirkel *et al.*, 2013]. In contrast to *ZEB1* and *LEF1*,

GAPDH and *RPLP0* mRNAs showed a similar abundance within the IGF2BP1 co-IP and BC fractions.

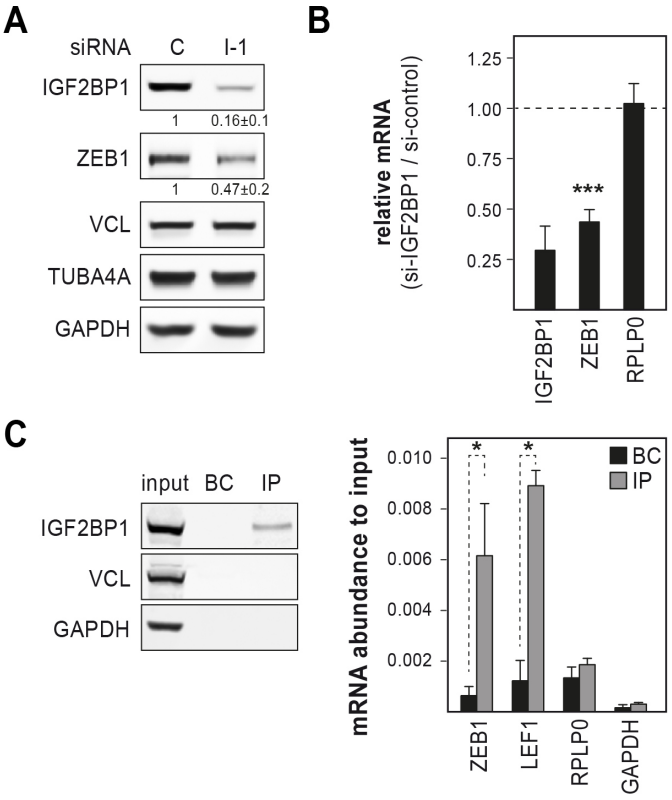

Figure 13 | *ZEB1* mRNA was specifically co-purified with IGF2BP1 from HeLa cell lysate.
(A, B) 300,000 HeLa cells were transfected with 75 pmol control (C, si-control) or *IGF2BP1*-directed (I-1, si-IGF2BP1) siRNAs for 72 hours. (A) Protein abundances of IGF2BP1 and ZEB1 on *IGF2BP1* knockdown were determined as described in Figure 12. (B) *IGF2BP1*, *ZEB1* and *RPLP0* mRNA levels were analyzed as described in Figure 12. Student's t-testing determined statistical significant decrease of *ZEB1* mRNA abundance in *IGF2BP1* knockdown cells (***, $P < 0.0005$). (C) *In vitro* association of indicated mRNAs with IGF2BP1 protein was analyzed by RNA co-immunoprecipitation (co-IP). Endogenous IGF2BP1 was immunopurified by a polyclonal antibody (IGF2BP1 (2), see APPENDIX)) as indicated by Western blot analyses (left panel). Co-purification of indicated mRNAs was analyzed by using qRT-PCR. IgG-agarose served as a control for unspecific mRNA binding (BC, bead control). The enrichment of mRNAs by IGF2BP1-IP was determined relative to the input fraction by using the ΔCt-method. Student's t-testing determined the statistical significant enrichment of *ZEB1* and *LEF1* mRNAs within the IGF2BP1-co-IP fraction compared to BC fraction (*, $P < 0.05$). Error bars indicate SD of at least three independent analyses. (All described experiments were performed by Alexander Mensch; antibodies, siRNAs and oligonucleotides are depicted in APPENDIX.)

Taken together, these results suggest *ZEB1* as a novel IGF2BP1 target mRNA. Similar to the IGF2BP1-directed control of e.g. *MYC*, *PTEN* or *LEF1* expression [Weidensdorfer *et al.*, 2009; Stohr *et al.*, 2012; Zirkel *et al.*, 2013], the time-dependent decrease of *ZEB1* mRNA abundance upon *IGF2BP1* depletion and the block of transcription by actinomycin D indicated that IGF2BP1 prevented *ZEB1* mRNA degradation. Under steady-state conditions, the knockdown of *IGF2BP1* reduced *ZEB1* protein and mRNA abundance. Consistent with these findings, *ZEB1* mRNA and protein levels were significantly increased in IGF2BP1-expressing tumors from patients suffering from ATCs (see Figure 9C, D). This provides a strong argument that IGF2BP1 may promote *ZEB1* expression in ATCs. However, to further verify this promoting effect, it remains to be demonstrated that *ZEB1* expression increases upon *IGF2BP1* gain-of-function.

Strikingly, the here presented findings support the notion that IGF2BP1 enhances or sustains pro-mesenchymal cell properties. Previous studies indicated this by demonstrating the pro-migratory effect of IGF2BP1 by reorganizing the actin cytoskeleton [Stohr and Huttelmaier, 2012], by promoting *LEF1* and, potentially as a result of this, *SNAI2* expression [Zirkel *et al.*, 2013]. IGF2BP1 was suggested to associate

with *LEF1* transcripts and to prevent their degradation. The transcription factor LEF1 was proposed to transcriptionally activate *SNAI2* expression, and to promote cell migration and invasion [Huang *et al.*, 2012; Zirkel *et al.*, 2013]. Accordingly, one may hypothesize that IGF2BP1 controls additional genes involved in pro-mesenchymal cell behaviors. Therefore, it would be highly interesting to monitor global gene expression upon *IGF2BP1* knockdown in mesenchymal-like carcinoma-derived cells of e.g. ATC origin. Until now, global gene expression under *IGF2BP1* knockdown conditions was only analyzed in HEK293 [Hafner *et al.*, 2010] and U2OS cells [Stohr and Huttelmaier, 2012].

ZEB1 sustains IGF2BP1 expression

IGF2BP1 expression was suggested to be controlled by CTNNB1 [Noubissi *et al.*, 2006; Gu *et al.*, 2008], MYC [Noubissi *et al.*, 2010] or the *let-7-5p* family [Gu *et al.*, 2008]. However, these regulators did not show a mesenchymal-like expression pattern like *IGF2BP1* (see Figure 10A; Figure 2A in [Park *et al.*, 2008]; Figure 6A in [Zirkel *et al.*, 2013]). In contrast, *ZEB1* showed a strikingly similar expression pattern as *IGF2BP1* in ATC tissues as well as in carcinoma-derived cell lines (see Figure 9C, and Figure 10A; Figure 6A in [Zirkel *et al.*, 2013]). Hence, the co-expression of *IGF2BP1* and *ZEB1* suggested not only a promoting effect of IGF2BP1 on *ZEB1* expression, but *vice versa* also a stimulatory effect of ZEB1 on the synthesis of IGF2BP1. Most studies revealed that ZEB1 functions as a transcriptional repressor (reviewed in [Gheldof *et al.*, 2012]). However, it was suggested that dependent on the expression of specific co-factors, ZEB1 could also function as a transcriptional activator [Gheldof *et al.*, 2012]. Moreover, by suppressing miRNAs like *miR-203a* or members of the *miR-200-3p* family, ZEB1 indirectly promotes the expression of genes like *BMI1* (*BMI1 polycomb ring finger oncogene*) [Wellner *et al.*, 2009].

To evaluate a promoting function of ZEB1 on *IGF2BP1* expression, IGF2BP1 protein and *IGF2BP1* mRNA abundances were analyzed upon *ZEB1* knockdown in 8505C and C-643 cells by using two distinct siRNAs (Figure 14A, B). Consistent with the co-expression in ATC tissues and carcinoma-derived cell lines, ZEB1 sustained *IGF2BP1* expression in ATC-derived cells with a pronounced effect at the protein level (compare Figure 14A with B). Although IGF2BP1 protein levels were reduced by approximately 40-50% in both cell lines; *IGF2BP1* mRNA levels were exclusively reduced in C-643 cells by approximately 25-40%, depending on the siRNA. These findings suggested a cell context-dependent regulation of *IGF2BP1* expression by ZEB1. This was reflected by the

different extend of *CDH1* re-expression upon *ZEB1* knockdown. In 8505C cells, *CDH1* mRNA as well as CDH1 protein levels were strongly increased, whereas *CDH1* expression was only modestly accelerated in C-643 cells upon *ZEB1* depletion. Accordingly, CDH1 protein was not detectable by Western blot analyses. In these cells additional transcription factors or cofactors may synergize with ZEB1 to silence the *CDH1* promoter.

To further evaluate the involvement of ZEB1 in sustaining *IGF2BP1* expression, Alexander Mensch performed a *ZEB1* knockdown in HeLa cells. Consistent with the findings from C-643 cells, IGF2BP1 protein and *IGF2BP1* mRNA amounts were reduced in *ZEB1*-depleted cells (Figure 14C, D). Again, the effect on IGF2BP1 protein abundance was stronger than on *IGF2BP1* mRNA abundance. The increased expression of *CDH1* was sufficiently high to be detected by Western blot analyses. Taken together, these findings suggest a positive feedback regulation between IGF2BP1 and ZEB1. However, the only modest change in *IGF2BP1* mRNA versus IGF2BP1 protein levels observed upon manipulating *ZEB1* expression argued against a transcriptional activation of *IGF2BP1* by ZEB1. In contrast, ZEB1 may repress post-transcriptional or post-translational regulators that attenuate *IGF2BP1* expression. In view of the strong inhibition of some miRNAs by ZEB1 [Burk *et al.*, 2008; Wellner *et al.*, 2009; Ahn *et al.*, 2012], it was hypothesized that the observed effects were at least partly due to the suppression of *IGF2BP1*-inhibiting miRNAs (Figure 14E).

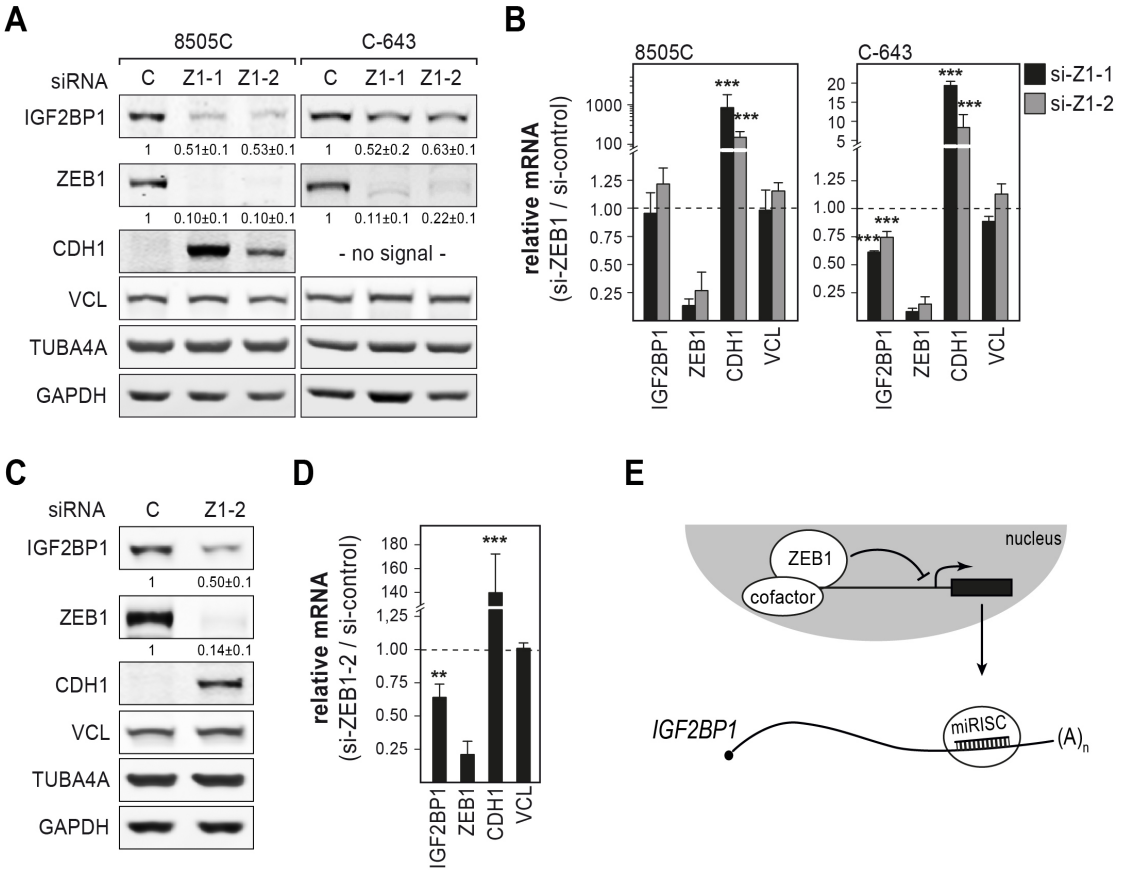

Figure 14 | *ZEB1* knockdown impaired *IGF2BP1* expression.
(A, B) 300,000 8505C, C-643 and (C, D) HeLa cells were transfected with 75 pmol control (C, *cel-miR-239b-5p*) or *ZEB1*-directed (Z-1; Z-2) siRNAs for 72 hours. (A, C) Protein abundances of IGF2BP1 and ZEB1 on *ZEB1* knockdown were determined relative to control cells by Western blotting using VCL for cross-normalization, as indicated below the panels. Protein was extracted by using RIPA buffer and total protein concentration of extracts was determined by D_c protein assay (Bio-Rad, München, Germany) to allow equal loading. GAPDH and TUBA4A served as additional loading controls. Blots were analyzed by infrared scanning using the Odyssey scanner (LI-COR Biosciences). Representative Western blots of at least three independent analyses are shown. (B, D) *IGF2BP1*, *ZEB1*, *CDH1* and *VCL* mRNA levels were analyzed as described in Figure 12. Student's t-testing determined statistical significant decrease of *IGF2BP1* mRNA abundance and increase of *CDH1* mRNA abundance in *ZEB1* knockdown cells (*, $P < 0.5$; **, $P < 0.005$; ***, $P < 0.0005$). (The Western blot shown for 8505C cells displays a reproduction performed by Alexander Mensch; mRNA results for 8505C cells include reproductions performed by Alexander Mensch; experiments with HeLa cells were performed by Alexander Mensch; antibodies, siRNAs and oligonucleotides are depicted in APPENDIX.) Note, that y-axes in B differ in two orders of magnitude. (E) Model for ZEB1-mediated regulation of *IGF2BP1* expression: The mechanism by which ZEB1 sustains *IGF2BP1* expression in ATC-derived and HeLa may involve the repression of IGF2BP1-targeting miRNAs by interfering with their transcription.

To test the involvement of post-transcriptional regulators that mediate translational repression of *IGF2BP1* mRNA upon *ZEB1* knockdown, Alexander Mensch analyzed mRNA co-sedimentation with polysomes from HeLa cell extracts. Cycloheximide-treated cell extracts from HeLa cells transfected with a *ZEB1*-directed siRNA or a control siRNA were separated by centrifugation through a 15 – 45 % sucrose gradient. The absorption profile monitored at 260 nm allowed the determination of polysomes (fraction 11 - 22), and monosomes or ribonucleoproteins (RNPs) (fraction 1 – 10) (Figure 15A). Gradient fractions were analyzed for *IGF2BP1, VCL, ACTB* and *GAPDH* mRNA content by means of qRT-PCR. These analyses identified a shift of *IGF2BP1* mRNA from polysomal to monosomal fractions in *ZEB1* knockdown cell lysates (Figure 15B). In contrast to control lysates, from which 84±1% of *IGF2BP1* mRNA associated with

polysomes, only 57±1% of *IGF2BP1* mRNA from *ZEB1*-depleted lysates co-sedimented with polysomes. Notably, the polysomal abundance of three control mRNAs including *VCL*, *ACTB* and *GAPDH* was barely affected by the depletion of *ZEB1*. These results suggested that the pronounced reduction of IGF2BP1 protein abundance upon *ZEB1* knockdown resulted at least partially from a reduced translation efficiency of *IGF2BP1* mRNA. However, additional regulation at the post-translational level cannot be excluded at this point and thus remains to be investigated by future studies.

Hypothesizing the involvement of *IGF2BP1* expression-antagonizing miRNAs, the expression of reporters harboring a control, a shortened and the full-length *IGF2BP1*-3'UTR was analyzed upon *ZEB1* knockdown. The more than 10-fold shortened *IGF2BP1*-3'UTR was suggested to be essentially resistant to miRNA-mediated repression [Mayr and Bartel, 2009]. The activity of reporters comprising the vector-derived control 3'UTR or the shortened *IGF2BP1*-3'UTR remained unaffected by the *ZEB1* knockdown (Figure 15C). On the contrary, the activity of the *IGF2BP1*-3'UTR-comprising reporter was significantly reduced by *ZEB1* depletion (Figure 15C). Although these reporter studies strongly argued for an involvement of post-transcriptional regulators like miRNAs, the reduction of *IGF2BP1*-3'UTR-reporter activity was less severe than the reduction of endogenous IGF2BP1 protein (compare Figure 14C with Figure 15C). Accordingly, additional *cis*-acting elements of the endogenous *IGF2BP1* mRNA may play a role in the post-transcriptional control or, as mentioned before, post-translational regulation of IGF2BP1 protein abundance. Future studies also remain to evaluate whether the reduction of reporter expression is predominantly due to the reduction of reporter mRNA levels.

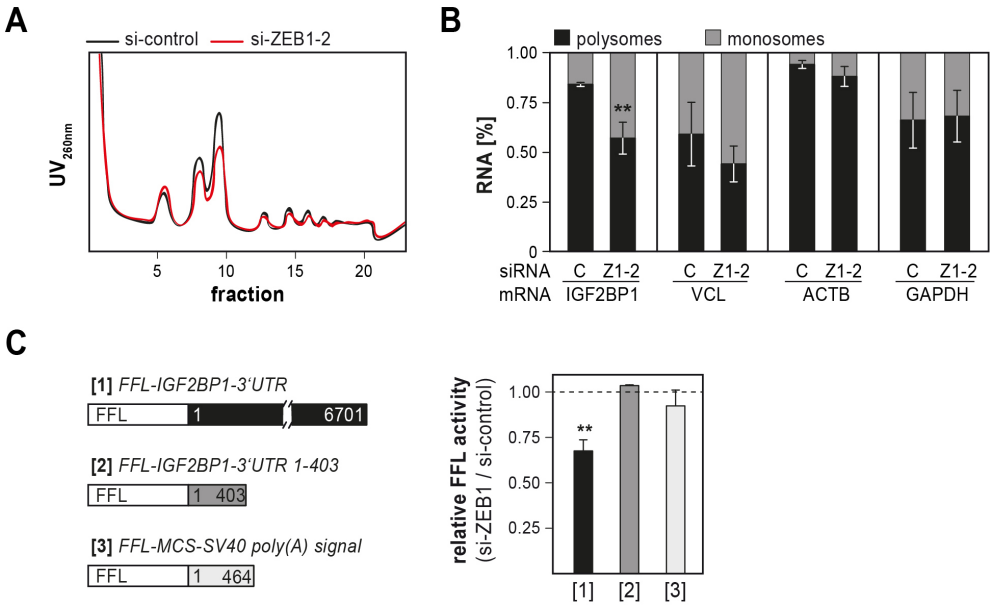

Figure 15 | *ZEB1* knockdown reduced polysomal association of *IGF2BP1* mRNA.
(A, B) 800,000 HeLa cells were transfection with 200pmol control (si-control, C) or *ZEB1*-directed (si-ZEB1-2, Z) siRNAs. After 72 hours, cells were incubated for 1 hour with 1:1000 cylcoheximide solution (100mg/ml, #66-81-9, Sigma Aldrich, St. Louis, USA) and lysed in 1.2 ml gradient buffer (10mM HEPES pH 7.2, 150 mM KCl, 5 mM $MgCl_2$) supplemented with 0.5% NP-40, protease inhibitor cocktail (1:250, Sigma-Aldrich, St. Louis, USA), RNase inhibitor (RNasin Plus, #N2615, 1:1000, #P8340, Promega, Madison, WI, USA), and 1:1000 cycloheximide. One ml of cell extract was loaded onto a 10 ml 15 - 45 % sucrose gradient and centrifuged for 2 hours at 40,000 rpm and 4°C. Fractions of 0.5 mL were collected from the top to the bottom of the gradient. Left panel displays representative 260 nm absorbance profiles from extracts of cells treated with control siRNA (si-control, black line) or ZEB1-2 siRNA (si-ZEB1-2, red line) plotted (A) against the fraction number. (B) From the 22 fractions, two successive fractions were pooled and RNA was isolated by means of phenol-chloroform extraction from the resulting 11 fractions. The amount of mRNA per fraction relative to the amount of all fractions was determined by means of qRT-PCR. The bar diagram depicts the summation the relative amounts of indicated mRNAs in percent from monosomal (fraction 1-10, black bars) and polysomal fractions (fraction 11-22, grey bars). Student's t-testing determined statistical significant increase of *IGF2BP1* mRNA amounts in monosomal fractions from *ZEB1* knockdown cell extracts (Z, siZEB1-2) compared to control knockdown (C, si-control) cell extracts (**, $P < 0.005$). Error bars indicate SD of at least three independent analyses. Gradient analyses were performed by Alexander Mensch. (C) Left panel: Scheme of used Firefly luciferase (FFL) reporters comprising the Firefly open reading frame fused to the full lengths *IGF2BP1*-3'UTR [1], the shortened *IGF2BP1*-3'UTR [2] comprising the 3'UTR 5'-sequence until the first alternative poly(A)-signal, or the pmiR-GLO vector encoded multiple cloning site fused to the SV-40 poly(A)-signal [3]. Right panel: HeLa cells were transfected with control (si-control) or a mix of *ZEB1*-directed siRNAs (si-ZEB1-1, si-ZEB1-2) for 48 hours before transfection of pmiR-GLO vectors encoding FFL reporters as well as Renilla luciferase. Changes in activity of Firefly luciferase reporters [1-3] upon *ZEB1* knockdown (si-ZEB1) were determined relative to controls (si-control) by normalization to Renilla luciferase activities. Statistical significance was validated by Student's t-testing (**, $P < 0.005$). Error bars indicate SD of at least three independent analyses. (siRNAs and oligonucleotides are depicted in APPENDIX.)

IGF2BP1 is targeted by known ZEB1-repressed miRNAs

Aiming at identifying *IGF2BP1*-regulatory miRNAs from epithelial cells, Bianca Busch conducted in the context of her PhD thesis two independent miTRAP experiments by using MCF7 cell lysate and the *IGF2BP1*-3'UTR as a bait RNA. MCF7 cells are characterized by a typical epithelial-like cell morphology (not shown), the expression of epithelial markers, and the absence of *ZEB1* as well as *IGF2BP1* expression (see Figure 10). Accordingly, the presence of miRNAs negatively regulated by ZEB1 was expected. The comprehensive analysis of miTRAP eluates by next generation sequencing identified in addition to *IGF2BP1*-regulatory miRNAs of the *let-7-5p* family, 25 miRNAs specifically enriched in the IGF2BP1 miTRAP eluates (Figure 16A). Most interestingly, these candidates comprised 14 miRNAs predicted to target the *IGF2BP1*-3'UTR, moreover, two of these were reported to be repressed by ZEB1 (*miR-203a* [Wellner *et al.*, 2009; Moes *et*

al., 2012]; *miR-34a-5p* [Siemens *et al.*, 2011; Ahn *et al.*, 2012]). These findings suggested that ZEB1 sustains IGF2BP1 expression by repressing *miR-34a-5p* and *miR-203a*. Accordingly, the impact of these miRNAs on IGF2BP1 expression was of particular interest. To evaluate their regulatory potency to control endogenous *IGF2BP1* expression, *miR-34a-5p* and *miR-203a* mimics were transfected in C-643 cells. Indicated by the reduced IGF2BP1 protein and *IGF2BP1* mRNA abundance, both miRNAs decreased *IGF2BP1* expression (Figure 16B, C). Ectopic expression of *let-7d-5p* served as a positive control. Potentially due to a high number of *let-7-5p f* targeting sites, *IGF2BP1* expression was more severely decreased by *let-7d-5p* than by *miR-34a-5p* or *miR-203a*. Nonetheless, these findings suggest that miRNAs transcriptionally repressed by ZEB1 have the potential to control *IGF2BP1* expression. Although a direct interaction with the *IGF2BP1*-3'UTR was demonstrated by miTRAP experiments *in vitro*, ongoing research by Bianca Busch focuses on the verification of the direct interaction of miTRAP-identified miRNAs with the *IGF2BP1*-3'UTR *in vivo*. To further validate the involvement of miRNAs in the positive feedback regulation of ZEB1 and IGF2BP1, future studies remain to analyze the association of endogenous *IGF2BP1* mRNA to protein components of miRISC upon *ZEB1* depletion. Additionally, it remains to be identified which miRNAs alter their expression upon *ZEB1* knockdown in ATC-derived cells, and to what extend a ZEB1-affected miRNA population overlaps with an *IGF2BP1*-targeting miRNA population. To expand the hypothesis to anaplastic thyroid carcinomas, it will also be interesting to compare miRNA expression patterns between IGF2BP1-positive and -negative tumor tissues.

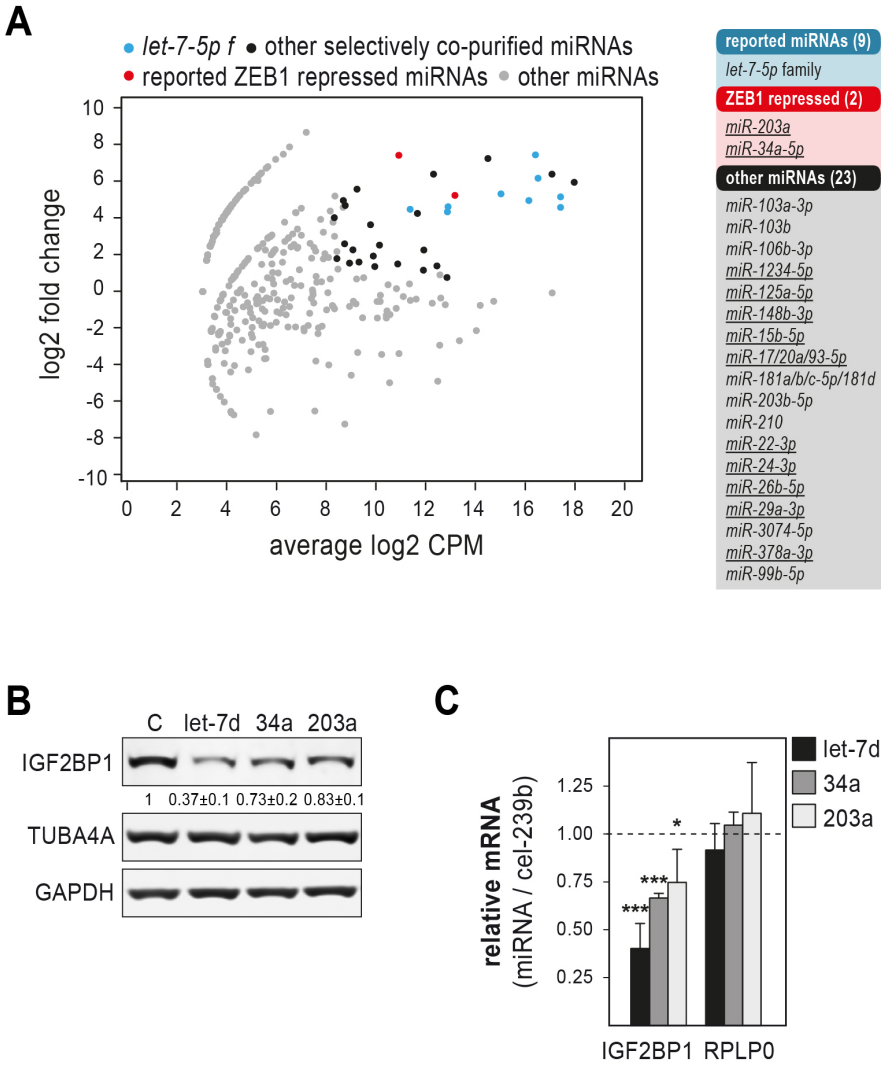

Figure 16 | *IGF2BP1* mRNA was targeted by ZEB1-repressed miRNAs.
(A) Two independent miTRAP experiments using the *IGF2BP1*-3'UTR as bait RNA and MCF7 cell lysate were performed by Bianca Busch. Left panel: Scatter plot depicts the log2 fold change (FC) between the IGF2BP1 and MS2 control libraries over the averaged TMM-normalized log2 CPM (counts per million) of miRNAs. A Poisson exact test [Robinson and Smyth, 2008] determined all nine *let-7-5p* family members (blue), two known ZEB1-repressed miRNAs (red) as well as 23 other potential *IGF2BP1*-regulatory miRNAs (black) as significantly enriched in the IGF2BP1 libraries compared to the MS2 control libraries. Right panel: Table summarizes all miRNAs selectively co-purified with the *IGF2BP1*-3'UTR bait RNA. Color coding refers to color coding of scatter plot in the left panel. Members from the same miRNA seed family were clustered. MiRNAs *in silico* predicted as potential *IGF2BP1*-regulatory miRNAs by miRANDA or TargetScan are underlined. (B, C) 300,000 C-643 cells were transfected with 75 pmol of *let-7d-5p (let-7d)*, *miR-34a-5p (34a)* or *miR-203a (203a)* mimics for 72 hours. Control cells (C, control) were transfected with *cel-miR-239b-5p*. (B) Protein abundance of IGF2BP1 on miRNA overexpression was determined as described in Figure 12. (B) *IGF2BP1* and *RPLP0* mRNA levels were analyzed as described in Figure 12. Student's t-testing determined statistical significant decrease of *IGF2BP1* mRNA abundance in miRNA-overexpressing cells (*, $P < 0.5$; ***, $P < 0.0005$). (Antibodies and oligonucleotide sequences are depicted in APPENDIX.)

Conclusions & Outlook

Anaplastic thyroid carcinoma patients suffer from rapidly growing tumors and lymphonodal as well as distant metastasis. These characteristics of late-stage cancers (ATCs are classified as stage-IV disease) are reminiscent to processes like tissue growth and cell segregation during embryonic development. Moreover, they have been suggested to rely on similar molecular mechanisms [Kelleher *et al.*, 2006; Micalizzi *et al.*, 2010]. Along these lines, global gene expression analyses revealed a positive correlation between tumor stage and the presence of key regulators of embryonic stem cell identity

[Ben-Porath *et al.*, 2008]. Consistent with these findings, IGF2BP1, a protein with crucial roles in development, was *de novo* synthesized in ATCs, but not in well-differentiated subclasses of thyroid cancer. Initial experiments in ATC-derived cells demonstrated that *IGF2BP1* depletion impaired migratory, invasive as well as proliferative traits. Together with observations from osteosarcoma-, ovarian carcinoma-, melanoma- and hepatocellular carcinoma-derived cells [Weidensdorfer *et al.*, 2009; Stohr *et al.*, 2012; Zirkel *et al.*, 2013; Gutschner *et al.*, 2014], these findings indicate that post-transcriptional gene regulation by IGF2BP1 enhances or sustains aggressive tumor cell behavior including mesenchymal-like cell properties. Consistent with these findings, *IGF2BP1* re-expression in ATCs correlated to dedifferentiation of the epithelium.

Moreover, the obtained results suggest that IGF2BP1 promotes the expression of *ZEB1*, which encodes a potent EMT-inducing transcription factor (reviewed in [Gheldof *et al.*, 2012]) (Figure 17). These findings support the previously gained notion that IGF2BP1 promotes mesenchymal-like expression patterns [Zirkel *et al.*, 2013]. By preventing *LEF1* mRNA degradation, IGF2BP1 not only enhances *LEF1* expression, but also *SNAI2* and *FN1* transcription [Zirkel *et al.*, 2013] (Figure 17). *FN1* encodes the extracellular matrix component fibronectin, a *bona fide* mesenchymal marker.

Regarding the mechanism of how IGF2BP1 stabilizes *ZEB1* mRNA, several scenarios can be envisioned: 1) IGF2BP1 prevents an endonucleolytic attack as proposed for *MYC* or *MDR1* mRNA [Lemm and Ross, 2002; Sparanese and Lee, 2007]. 2) IGF2BP1 prevents miRNA targeting as proposed for *BTRC* mRNA [Elcheva *et al.*, 2009]. 3) IGF2BP1 prevents targeting of destabilizing RNA-binding proteins. To unravel the mechanism, IGF2BP1-binding affinities of *cis*-acting elements of *ZEB1* will be determined in future studies, and tested for their regulatory potency upon *IGF2BP1* depletion. Furthermore, other *trans*-acting factors like miRNAs or RNA-binding proteins will be determined, to analyze whether IGF2BP1 competes with respective binding sites. Finally, the regulatory potency of these factors as well as their association with *ZEB1* mRNA depending on the cellular IGF2BP1 status will be investigated.

Most interestingly, experiments from three different carcinoma-derived cell lines suggested that *IGF2BP1* expression in turn was reduced by the depletion of *ZEB1* (Figure 17). This and the reduced association of *IGF2BP1* mRNA with polysomes proposed a positive feedback regulation between *IGF2BP1* and *ZEB1*. However, the promoting effect of ZEB1 on *IGF2BP1* expression is potentially mediated by post-transcriptional and post-translational mechanisms, and therefore it remains elusive how the induction of *IGF2BP1* transcription in ATCs is triggered. Though, one could speculate that the *IGF2BP1*

promoter is activated through the release of epigenetic silencing. It was proposed that cancerogenesis is accompanied by global genomic hypomethylation, although tumor suppressor genes are transcriptional silenced by hypermethylation [Esteller, 2003].

In regard of the positive feedback regulation between IGF2BP1 and ZEB1, it will be interesting to analyze if and to what extend both proteins are involved in the cancer-promoting functions of the respective other protein, like migration, invasion and proliferation (reviewed in [Gheldof *et al.*, 2012; Bell *et al.*, 2013]). In addition to cancer, it remains to be addressed whether this positive feedback regulation plays as role during embryogenesis. Previous studies suggested an essentially similar tissue expression pattern of both genes in mouse and frog embryogenesis [Takagi *et al.*, 1998; Yaniv *et al.*, 2003; Hansen *et al.*, 2004; van Grunsven *et al.*, 2006].

Taken together, the oncofetal RNA-binding protein IGF2BP1 represents a novel post-transcriptional regulator in thyroid carcinogenesis and a potential novel biomarker for ATC diagnosis. The latter is specifically relevant as IGF2PP1 was not detectable in non-transformed thyroid tissue and well-differentiated subclasses of thyroid cancer. Whether IGF2BP1 also displays a prognostic marker for ATC progression remains to be evaluated by analyzing ATC patient prognoses in regard of *IGF2BP1* expression status.

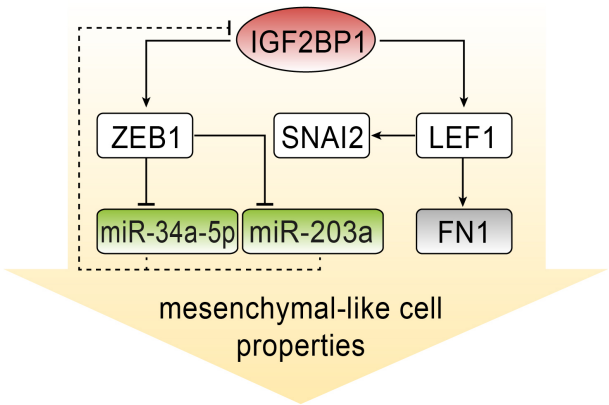

Figure 17 | IGF2BP1 promotes mesenchymal-like cell properties by sustaining the expression of EMT-driving transcriptional regulators.
Hypothetical scheme of IGF2BP1 function in promoting mesenchymal-like expression patterns. IGF2BP1 prevents degradation of *LEF1* [Zirkel *et al.*, 2013] and *ZEB1* mRNAs. ZEB1 in turn represses the *IGF2BP1* expression interfering miRNAs 34a-5p and 203a. The direct interaction of these miRNAs with *IGF2BP1* transcripts remains to be verified *in vivo* (dashed lines). LEF1 transcriptionally activates *SNAI2* and *FN1* expression.

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6 APPENDIX

List of figures

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Abbreviations

A	adenine
AAT	serpin peptidase inhibitor, clade A, member 1
ACTB	β-actin
ActD	Actinomycin D
AGO	argonoute
AJ	adherens juction
AKT2	v-akt murine thymoma viral oncogene homologue 2
APC	adenomatous polyposis coli
ATC	anaplastic thyroid carcinoma
bHLH	basic helix-loop-helix
BL	Basal lamina
BMI1	BMI1 polycomb ring finger oncogene
BMM	basement membrane
bp	base pair
BRAF	v-raf murine sarcoma viral oncogene homologue B
BTRC	β-transducin repeat containing E3 ubiquitin protein ligase
C	cytosine
CAF1	CCR4-NOT transcription complex, subunit 8
CCR4	CCR4-NOT transcription complex, subunit 6
CDH1	E-cadherin
CDC25A	cell division cycle 25A
CDK6	cyclin-dependent kinase 6
CDKI	cyclin-dependent kinase inhibitor
CDKN1A	cyclin-dependent kinase inhibitor 1A
CDS	coding sequence
cel	*Ceanorhabditis elegans*
CK1	casein kinase 1
CLASH	crosslinking, ligation and sequencing of hybrids
CLIP	crosslinking immunoprecipitation
cMSC	colonic mesenchymal stem cells
co-IP	co-immunoprecipitation
CP	coat protein
CPM	counts per million
CRD-BP	c-myc mRNA coding region determinant-binding protein
CSC	cancer stem cell
CTNNA1	α-catenin

CTNNB1	β-catenin
DCP	Decapping protein
DDX6	DEAD box helicase 6
DNA	desoxyribonucleic acid
dre	*Danio rerio*
Dvl	dishevelled
E	embryonic day
EDC4	enhancer of mRNA decapping 4
e.g.	exempli gratia
E2F1	E2F transcription factor 1
E47	transcription factor 3
EC	extracellular cadherin domain
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
eIF	eukaryotic translation initiation factor
ELAVL1	embryonic lethal abnormal vision like RNA-binding protein 1
ELAVL2	embryonic lethal abnormal vision like RNA-binding protein 2
EMT	epithelial-to-mesenchymal transition
ERK	MAPK extracellular regulated kinase
f	family
FC	fold change
FFL	Firefly luciferase
FGF	fibroblast growth factor
FMRP	fragile X mental retardation protein
FN1	fibronectin 1
FNAB	fine-needle aspiration biopsy
FTC	follicular thyroid carcinoma
G	guanine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GEEG	Glycine-Glutamate-Glutamate-Glycine
GF	growth factors
GLI1	GLI family zinc finger 1
GRB2	growth factor receptor-bound protein 2
GSK3	glycogen synthase kinase 3
GxxG	Glycine-any residue-any residue-Glycine
Hand2	heart and neural crest derivatives expressed 2

HDAC	histone deacetylase
HITS-CLIP	High-throughput sequencing of RNA isolated by crosslinking immunoprecipitation
HMGA2	high mobility group AT-hook 2
HNRNPK	heterogeneous nuclear ribonucleoprotein K
HOXB8	homeobox B8
hsa	*Homo sapiens*
IGF2	Insulin-like growth factor 2
IGF2BP	*IGF2* mRNA-binding protein 1
IGF2BP2	*IGF2* mRNA-binding protein 2
IGF2BP3	*IGF2* mRNA-binding protein 3
IHC	immunohistochemistry
ILK	integrin-linked kinase
IM	interstitial matrix
IP	immunoprecipitation
IRES	internal ribosomal entry site
KH	HNRNP homology
KLF8	Kruppel-like factor 8
KOC	KH-domain-containing protein overexpressed in cancer
KRAS	Kirsten rat sarcoma viral oncogene homologue
LEF1	lymphoid enhancer binding factor 1
LRP	low-density lipoprotein receptor related protein
MAPK	mitogen-activated kinase
MDCK	Madin Darby canine kidney
MDR1	multidrug resistance protein 1
MEK	MAPK/ERK kinase
MET	mesenchymal-to-epithelial transition
miRISC	miRNA-induced silencing complex
miRNA	microRNA
mirtron	intron that encodes pre-miRNA
MMP	matrix metalloprotease or matrix-metallopeptidase
mmu	*Mus musculus*
mRNA	messenger RNA
MTC	medullary thyroid carcinoma
MTS	microRNA targeting site
MYC	v-myc avian myelocytomatosis viral oncogene homologue
NCBI	National Center for Biotechnology Information

NOT	CCR4-NOT transcription complex, subunit 1
nt	nucleotide
NT	non-transformed tissue
OSBPL8	oxysterol binding protein-like 8
p.	page
PABPC	poly(A)-binding protein, cytoplasmic
PAN2	PAN2 poly(A) specific ribonuclease subunit
PAN3	PAN3 poly(A) specific ribonuclease subunit
PAR-CLIP	Photoactivatable-ribonucleoside-enhanced crosslinking and IP
PDTC	poorly differentiated thyroid carcinoma
PI3K	phosphatidylinositol-3 kinase
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
poly(A)	polyadenylated
pre-miRNA	premature miRNA
pri-miRNA	primary precursor of mature miRNA
PTC	papillary thyroid carcinoma
PTEN	phosphatase and tensin homologue
PTGS2	prostaglandin-endoperoxide synthase 2
qRT-PCR	quantitative real-time polymerase chain reaction
RAF	oncogene homologue of rodent raf (rapidly accelerated fibrosarcoma or rat fibrosarcoma)
RAS	rat sarcoma viral oncogene homologue
RBP	RNA-binding protein
RNA	Ribonucleic acid
RNP	ribonucleoprotein
RPLP0	ribosomal protein large P0
rpm	rounds per minute
RPTOR	regulatory associated protein of MTOR, complex 1
RTK	receptor tyrosine kinase
SHCA	Src homology 2 domain containing
SILAC	stable isotope labeling by/with amino acids in cell culture
SIRT1	sirtuin 1
SMAD	similar to the gene products of the D. melanogaster gene 'mothers against decapentaplegic' (Mad)
SNAI1	Drosophila homologue Snail 1
SNAI2	Drosophila homologue Snail 2

snoRNA	small nucleolar RNA
SOS	son of sevenless
STAT3	signal transducer and activator of transcription 3
T	thymidine
T3	triiodothyronine
T4	thyroxine
TCF4	T-cell specific transcription factor 4
TF	transcription factor
TGFB	transforming growth factor β
TGFBR	TGFB receptor
TIC	tumor-initiating cell
TMM	trimmed mean of M-values
TNRC6	trinucleotide repeat containing 6
TP53	tumor proten p53
TRBP	TAR (HIV-1) RNA binding protein 2
TUBA4A	α-tubulin 4a
TWIST	twist family bHLH transcription factor
UTR	untranslated region
VCL	vinculin
VG1RBP	*Vg1*-mRNA binding protein
VIM	vimentin
WAP	whey acidic promoter
XRN1	5'-3' exoribonuclease 1
ZBP1	Zipcode-binding protein 1
ZEB1	zinc-finger E-box-binding homeobox protein 1
ZEB2	zinc-finger E-box-binding homeobox protein 2

List of antibodies, siRNAs and oligonucleotides used in ADDITIONAL RESULTS

Antibodies

primary antibody	produced in	company	ID
anti-CDH1	rabbit	Abcam	ab40772
anti-CTNNB1	rabbit	Cell Signaling	#9562
anti-GAPDH	rabbit	biomol	A300-641A
anti-IGF2BP1	mouse	TU Braunschweig	6A9
anti-IGF2BP1 (2)	rabbit	Cell Signaling	#2852
anti-IGF2BP2	mouse	TU Braunschweig	6A12
anti-IGF2BP3	mouse	TU Braunschweig	6G8
anti-LEF1	rabbit	Cell signaling	2286S
anti-MYC	rabbit	Millipore	06-340
anti-SNAI1	mouse	Cell signaling	#3895
anti-SNAI2	rabbit	Cell signaling	#9585
anti-TGFBR1	rabbit	Santa Cruz	sc-402
anti-TUBA4A	mouse	Sigma Aldrich	T9026
anti-VCL	mouse	Sigma Aldrich	V9131
anti-VIM	mouse	BD Transductions	550513
anti-ZEB1	rabbit	Santa Cruz	sc-25388
anti-ZEB2	rabbit	Bethyl	A302-474A

secondary antibody	produced in	company
IRDye 680RD anti-IgG-mouse-infrared-dye	donkey	LI-COR Biosciences
IRDye 680RD anti-IgG-rabbit-infrared-dye	donkey	LI-COR Biosciences
IRDye 800CW anti-IgG-mouse-infrared-dye	donkey	LI-COR Biosciences
IRDye 800CW anti-IgG-rabbit-infrared-dye	donkey	LI-COR Biosciences
dylight488™-conjugated anti-mouse-IgG F(ab)2	donkey	Jackson ImmunoResearch

siRNAs

control siRNA (cel-miR-239b-5p)	UUGUACUACACAAAAGUACUG
si-IGF2BP1-1	UGAAUGGCCACCAGUUGGA
si-IGF2BP1-2	CCGGGAGCAGACCAGGCAA
ZEB1-1	GCAUCCAAAGAACAAGAAA
ZEB1-2	AGAUGAUGAAUGCGAGUCA

Oligonucleotides

ZEB1 s	TTCAAACCCATAGTGGTTGCT
ZEB1 as	TGGGAGACACCAAACCAACTG
VCL s	TTACAGTGGCAGAGGTGGTG
VCL as	TCACGGTGTTCATCGAGTTC
RPLP0 s	CCTCATATCCGGGGGAATGTG
RPLP0 as	GCAGCAGCTGGCACCTTATTG
LEF1 s	ACAGATCACCCCACCTCTTG
LEF1 as	TGAGGCTTCACGTGCATTAG
IGF2BP1 s	TAGTACCAAGAGACCAGACCC
IGF2BP1 as	GATTTCTGCCCGTTGTTGTC
GAPDH s	CGCTCTCTGCTCCTCCTGTT
GAPDH as	CCATGGTGTCTGAGCGATGT
CDH1 s	GCCGAGAGCTACACGTTCAC
CDH1 as	GTCGAGGGAAAAATAGGCTG
ACTB s	AGAAAATCTGGCACCACACC
ACTB as	AGAGGCGTACAGGGATAGCA

Darlegung des Eigenanteils

1. Braun J., Hoang-Vu C., Dralle H., and Hüttelmaier S. (2010).
Downregulation of microRNAs directs the EMT and invasive potential of anaplastic thyroid carcinomas., *Oncogene* 29: 4237-4244.
Eigenanteil: Planung, Durchführung und Auswertung aller Experimente. H.C. und D.H. isolierten das Tumorgewebe von Schilddrüsenkrebspatienten. Anfertigung der Abbildungen. Anfertigung von Teilen des Manuskripts.

2. Braun J. and Hüttelmaier S. (2011).
Pathogenic mechanisms of deregulated microRNA expression in thyroid carcinomas of follicular origin., *Thyroid Research* 4: S1.
Eigenanteil: Anfertigung der Abbildungen und von Teilen des Manuskripts.

3. Braun J., Misiak D., Busch B., Krohn K., and Hüttelmaier S. (2014).
Rapid identification of regulatory microRNAs by miTRAP (miRNA trapping by RNA *in vitro* affinity purification)., *Nucleic Acids Res.* 42:e66.
Eigenanteil: Planung, Durchführung und Auswertung aller Experimente. Abbildung 4B stellt eine Zusammenfassung meiner eigenen Experimente und der Experimenten von Bianca Busch dar. Das miRNA-sequencing wurde von Knut Krohn durchgeführt. Die Sequenzen wurden von Knut Krohn annotiert. Die miRNA-sequencing Daten wurden gemeinsam mit Danny Misiak bioinformatisch ausgewertet. Anfertigung der Abbildungen. Anfertigung von Teilen des Manuskripts.

Erklärung

Hiermit erkläre ich, dass ich meine Dissertationsschrift selbständig und ohne fremde Hilfe verfasst habe. Ich habe keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt. Die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen habe ich als solche kenntlich gemacht.
Mit der vorliegenden Arbeit bewerbe ich mich erstmals um die Erlangung des Doktorgrades.

Halle (Saale), Mai 2014

Juliane Braun

Danksagung

Prof. Stefan Hüttelmaier danke ich für sein Engagement, sein Vertrauen in meine Arbeit, seine Diskussionsbereitschaft und konstruktive Kritik, die Integration in das GRK 1591 als wissenschaftliche Koordinatorin, die Herstellung von Kontakten zu anderen Wissenschaftlern und die Förderung von Kongressteilnahmen.

Allen Mitarbeitern der Arbeitsgruppe von Prof. Hüttelmaier danke ich für das fantastische Arbeitsklima. Insbesondere möchte ich mich bei Anne Baude, Marlen Mrotzek, Claudia Reinke, Bianca Busch, Mechtild Wahle, Nadine Bley, Alexander Mensch, Nikolaos Pazaitis, Danny Misiak, Fred Junghans, Hendrik Täuber und Marcel Köhn für wissenschaftliche und mentale Unterstützung, sowie das Bereitstellen diverser Reagenzien bedanken. Bernadette Harwardt danke ich für technische Assistenz.

Meinen besonderen Dank möchte ich Alexander Mensch aussprechen, welcher mit außerordentlichem Engagement zum Gelingen des ‚IGF2BP1-ZEB1' Projekts beitrug.

Dr. Knut Krohn möchte ich danken für seine Bereitschaft miTRAP Proben mittels miRNA-Seq zu analysieren, und die vielen Tipps bei der bioinformatischen Auswertung von ‚sequencing' Daten. Außerdem danke ich Danny Misiak, der mich bei der Normalisierung der miRNA-Seq Daten unterstützt und mir eine Einführung in R gegeben hat.

Dr. Kerstin Lorenz, Dr. Hoang-Vu, Dr. Frank Bartel und Prof. Dr. Dralle danke ich für die Bereitstellung der Tumorproben von Schilddrüsenkrebspatienten.

Bedanken möchte ich mich zudem bei allen Mitgliedern des GRK 1591 für die großartige Zusammenarbeit, die Diskussions- und Hilfsbereitschaft, sowie die Unterstützung bei der Organisation des ‚International Meetings' und der GRK 1591 Evaluierung. Besonderer Dank gilt Juliane Brock, Selma Gago-Zachert, Juliane Buschman, und Michael Götze für die ständige Bereitschaft zur Unterstützung der GRK 1591 Koordination, was zum Vorankommen meiner Arbeit beigetragen hat. Juliane Brock und Alexander Mensch möchte ich außerdem für das Korrekturlesen meiner Arbeit danken.

Mein besonderer Dank gilt Matthias, meiner Familie und meinen Freunden für ihre ständige mentale Unterstützung während meiner Arbeit und die Bereicherung meines Lebens.

Curriculum Vitae

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Halle (Saale), Mai 2014,

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List of publications

1. **Braun J.**, Hoang-Vu C., Dralle H., and Hüttelmaier S. (2010).
Downregulation of microRNAs directs the EMT and invasive potential of anaplastic thyroid carcinomas., *Oncogene* 29: 4237-4244.

2. **Braun J.** and Hüttelmaier S. (2011).
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3. **Braun J**., Misiak D., Busch B., Krohn K., and Hüttelmaier S. (2014).
Rapid identification of regulatory microRNAs by miTRAP (miRNA trapping by RNA *in vitro* affinity purification)., *Nucleic Acids Res.* 42:e66.

Award

Merck-von-Basedow Preis 2010 of the Deutsche Gesellschaft für Endokrinologie (shared with Stefan Hüttelmaier)

Halle (Saale), Mai 2014

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