Functional analysis of the EMT inducing transcription factor ZEB1 during malignant carcinoma progression

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<th>Full Form</th>
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<tbody>
<tr>
<td>Ago</td>
<td>Argonaute</td>
</tr>
<tr>
<td>alphaSMA</td>
<td>alpha smooth muscle actin</td>
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<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumine</td>
</tr>
<tr>
<td>DKK</td>
<td>Dickkopf</td>
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<tr>
<td>Dsg</td>
<td>Desmoglein</td>
</tr>
<tr>
<td>DSH</td>
<td>Dishevelled</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGR</td>
<td>Early growth response</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial mesenchymal Transition</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular regulated kinase</td>
</tr>
<tr>
<td>ESR1</td>
<td>Estrogen receptor 1</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 2</td>
</tr>
<tr>
<td>HES</td>
<td>Hairy and enhancer of split 1</td>
</tr>
<tr>
<td>Hey1</td>
<td>Hairy/ enhancer of split related 1</td>
</tr>
<tr>
<td>HGF/SF</td>
<td>Hepatocyte growth factor/ Scatter factor</td>
</tr>
<tr>
<td>HIF1alpha</td>
<td>Hypoxia inducible factor 1 alpha</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>ID</td>
<td>Inhibitor of differentiation</td>
</tr>
<tr>
<td>IF</td>
<td>Intermediary filaments</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IGFR</td>
<td>Insulin-like growth factor receptor</td>
</tr>
<tr>
<td>IKK</td>
<td>IKB Kinase</td>
</tr>
<tr>
<td>JAM</td>
<td>Junctional adhesion molecule</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun Kinase</td>
</tr>
<tr>
<td>LSF</td>
<td>Lymphoid enhancer factor</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal epithelial transition</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>NC</td>
<td>Neural Crest</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor k B</td>
</tr>
<tr>
<td>NICD</td>
<td>Notch intracellular domain</td>
</tr>
<tr>
<td>PAK1</td>
<td>P21 activated kinase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phospho buffered saline</td>
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<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<td>--------------</td>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<tr>
<td>PI3K</td>
<td>Phospho inositol 3 kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKP</td>
<td>Plakophilin</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>Smurf</td>
<td>Smad ubiquitination regulatory factor</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight junction</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>Xbra</td>
<td>Xenopus Brachyury</td>
</tr>
<tr>
<td>ZEB</td>
<td>Zinc finger E-box binding factor</td>
</tr>
<tr>
<td>Zn</td>
<td>Zinc finger E-box binding factor</td>
</tr>
<tr>
<td>ZO1</td>
<td>Zona occludens 1</td>
</tr>
<tr>
<td>ZONAB</td>
<td>ZO1 associated nucleic acid binding protein</td>
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1 LITERATURE STUDY
1.1 Epithelial mesenchymal transition and cancer: a historical perspective

The epithelium is one of the major types of tissue in metazoans forming sheets of closely attached cells that are characterized by an apico-basal polarization (Hay, 2005). One of its crucial functions is the separation of internal and external surfaces, which is made possible by the presence of several junctional complexes. These complexes allow the cells to form tight contacts with each other resulting in an impermeable layer (Thiery & Sleeman, 2006). Already from the earliest appearance of metazoan organisms, the epithelium shows a strict divergence with another major tissue type, the mesenchyme (Micalizzi et al., 2010; Shook & Keller, 2003). In contrast to their epithelial counterparts, mesenchymal cells do not form sheet-like structures as consequence of the absence of specialized junctional complexes. This additionally implies that an apico-basal polarization is not present. Nevertheless, contacts between neighbouring mesenchymal cells still occur via focal interaction points (Thiery & Sleeman, 2006). It has long been assumed these epithelial and mesenchymal cell types had a stable phenotype (Overton, 1977). However, experiments conducted by Greenberg and Hay showed that the epithelial phenotype is subject to a wide spectrum of plasticity (Greenburg & Hay, 1988). Indeed, when epithelial cell explants from the embryonic notochord and limb ectoderm were seeded within a collagen I matrix, a shift towards a mesenchymal morphology could be observed after 36 hours. This was not restricted to cells of embryonic origin since the same effect occurred when adult anterior lens epithelium was used. Next to undergoing a mesenchymal transformation, the cells invaded into the surrounding collagen matrix (Greenburg & Hay, 1982). Further experiments led to the discovery that this mesenchymal transformation was accompanied by the replacement of the cytokeratins by the intermediate filament protein vimentin. In addition, epithelial corneal cells did not undergo this transformation when seeded into a matrix based on basal membrane constituents (Greenburg & Hay, 1988). The existing plasticity between the epithelial and mesenchymal phenotype was further confirmed in a report studying the formation of the secondary palate. It was first thought that the disappearance of the medial edge epithelium in the developing palate was due to the induction of apoptosis. However, further investigation led to the conclusion that instead of undergoing cell death, the medial...
The epithelium is subject to a mesenchymal transformation whereby the basal membrane is degraded and the cells subsequently migrate into the existing neighbouring mesenchyme (Fitchett & Hay, 1989). This epithelial mesenchymal transition (EMT) is pivotal in the formation of a multitude of organ and tissue types during metazoan embryogenesis. Indeed, both during gastrulation and neural crest formation an EMT takes place. One of the first indications that neural crest cells had migratory capacities came from a study in the pickerel frog *Rana palustris* wherein it was found that the skeletal connective tissues of the upper face were of neural crest origin (Stone, 1929). These findings could be extended to higher vertebrates when Johnston studied the origin of the cranial mesenchymal tissues in mouse embryos (Johnston, 1966; Johnston et al, 1973). Since then, it has become clear that neural crest derivatives are responsible for much of the facial skeleton, the development of the peripheral nervous system and pigment cells (Sauka-Spengler & Bronner-Fraser, 2008b). Accordingly to neural crest development, the process of gastrulation also depends on cellular migration. Initial research on the event of gastrulation was already being performed during the 19th and early 20th century on amphibian embryos. During this period, the formation of the different germ layers and the polarization of the developing body-axes were accurately described (Beetschen, 2001). During the following decades, a more precise image of gastrulation and the fate of the different germ layers was formulated across different species of amphibians. It was not until research started in *Drosophila* that the molecular mechanisms laying behind the complex structural rearrangements of gastrulation started to emerge. As such, it was discovered in *Drosophila* that the transcription factors Twist and Snail are both activated in the mesodermal primordium. Moreover, embryos mutant for Twist and Snail fail to generate a functional mesoderm (Smith et al, 1992). Cloning of the homologues of Snail in *Xenopus laevis* and mouse not only revealed its necessity for a correct gastrulation, but also demonstrated its importance during migration of the neural crest (Smith et al, 1992). These findings were also confirmed in the chick embryo with the transcription factor Slug, another member of the Snail gene family (Nieto et al, 1994).

Together with the above observations it was found that the epithelial adhesion molecule E-cadherin was repressed during mesoderm formation (Butz & Larue, 1995). Furthermore, the view was arising that the development of malignant tumors was partly dependent on the ability of the cells to overcome cell-cell adhesions. Since E-cadherin is
a major adhesion protein in epithelial tissues, it was soon discovered that the transition from a benign tumor towards a cancer with malignant properties often was accompanied by the loss of E-cadherin (Berx & van Roy, 2009; Birchmeier & Behrens, 1994; Frixen et al, 1991; Mareel et al, 1992; Perl et al, 1998b; Takeichi, 1993). Moreover, overexpression of E-cadherin in invasive tumor cell lines led to a stabilization of the epithelial phenotype with a complementary reduction of the invasive phenotype (Vleminckx et al, 1991). The parallel between E-cadherin loss during mesoderm formation and the development of carcinomas was further established by Cano et al., showing that the transcription factor Snail was capable of directly repressing E-cadherin via binding to specific E-box sequences in the promoter which was going hand in hand with the induction of EMT. Additional expression analysis of cancer cell lines revealed that Snail was mainly expressed in epithelial cancer cell lines and that this misexpression was accompanied by the loss of E-cadherin (Cano et al, 2000b). In human breast carcinomas expression of Snail was identified in infiltrating ductal carcinomas and was inversely correlated with the differentiation grade of these tumors (Blanco et al, 2002). Since E-cadherin could be repressed by binding of specific transcription factors to the E-box sequences in its promoter, several other candidates were considered to perform a similar function as Snail. The mechanism of transcriptional silencing of the earlier identified ZEB1 was associated with its specific binding with E-box sequences in the delta-crystallin enhancer (Sekido et al, 1994). Accordingly, further analysis indeed confirmed that ZEB2 mediated repression occurred also via binding to an E-box sequence in the Xbra2 promoter (Remacle et al, 1999). This resulted in the assumption that genes with E-boxes present in their regulatory regions like E-cadherin might be target genes as well. It was shown that ZEB2 was able to bind to an oligonucleotide containing the E-boxes of the E-cadherin promoter. In parallel with the transcription factor Snail, both ZEB proteins play a pivotal role during gastrulation, migration of the neural crest cells and maturation of neural crest derivatives in several species (Lerchner et al, 2000; van Grunsven et al, 2006). The first connection that also ZEB2 was able to play a role in malignant tumor progression came from Comijn et al., where it was shown that binding of ZEB2 to the E-cadherin promoter directly led to transcriptional repression. In addition, conditional expression of ZEB2 in tumor cell lines led to an EMT and a subsequent gain in invasive capacities (Comijn et al, 2001; Vandewalle et al, 2005). Similar conclusions could be made for ZEB1 (Aigner et al,
The in vivo relevance of these findings was further stipulated by the insight that ZEB1 was expressed in highly aggressive uterine cancers and in invasive cells at the tumor border of advanced colorectal carcinoma (Spaderna et al, 2006; Spoelstra et al, 2006).

Together with the discovery of the EMT inducing transcription factors came the notion that induction of EMT occurred in a non cell autonomous way. This implied the requirement of external stimuli in order for EMT to take place. These conclusions were mainly made on the basis of in vitro studies. One of the first manuscripts reporting induction of an EMT like event as a result of an external stimulus came from Blay and Brown in 1985 where the authors found that the growth factor EGF was able to direct the migration of rat intestinal epithelial cells (Blay & Brown, 1985). Similar outcomes were made for the growth factors FGF and TGF-β (Miettinen et al, 1994; Vallés et al, 1990).

Later on, the focus was set towards the signaling cascades that were necessary in order to induce EMT. A pivotal role for MAPK signalization had indeed been found to be required for EMT induction in specific in vitro cellular model systems (Boyer et al, 1997). Additionally, the WNT pathway was elucidated to be influencing the epithelial plasticity. This was based on the finding that nuclear β catenin was responsible for the mesenchymal transition in c-Fos overexpressing mammary epithelial cells (Eger et al, 2000; Fialka et al, 1996; Reichmann et al, 1992).

With the discovery of the first microRNAs in 2001 in the nematode C. elegans, the foundation of a novel and general mechanism of expression regulation was made (Lau et al, 2001; Lee et al, 2008). In a study of 2008 it was indeed established that also microRNAs were able to regulate epithelial plasticity and hence the process of EMT. In particular, microRNAs of the miR200 family act as stabilizing factors of the epithelial phenotype by directly repressing both ZEB transcription factors (Gregory et al, 2008; Korpali et al, 2008; Park et al, 2008). However, the opposite relation between the ZEBs and the miR200 family also holds true. Indeed, the induced EMT in several cancer cell lines was accompanied by repression of the microRNAs of the miR200 family. By directly targeting the E-boxes in the miR200 promoter, it was demonstrated that ZEB1 is able to induce and maintain a feedforward loop that destabilizes the epithelial phenotype (Burk et al, 2008a). Accordingly, expression of the miR200 family is absent during mesoderm formation in zebrafish (Wienholds et al, 2005). Several reports also linked the
absence of the mIR200 family to advanced cancer (Shinozaki et al, 2010; Wiklund et al, 2010).

The role EMT inducing transcription factors play in malignant cancer and their regulatory mechanisms is thus becoming well established. Recently however, a new paradigm is emerging that ascribes a new function to EMT in cancer progression. This is based on the view that macroscopic metastatic foci originate from a small group of extravasated cells. In order to reach a significant metastatic mass, these cells must possess strong replicative capacities. Since these cells might have undergone an EMT prior to reaching the bloodstream, a potential link with the formation of cancer stem cells exists. Indeed, recently it has been shown that the induction of EMT is able to generate cells that have stem cell like properties. The transcription factors Snail and Twist have as such been described as mediators of this phenotype (Mani et al, 2008). In particular, it seems that EMT is able to generate mesenchymal like stem cells (Battula et al, 2010). Both the ability of EMT inducers to modulate microRNAs that stabilize the epithelial phenotype and capacity to generate stem cell like cells are converged in a report by Wellner et al. where the authors describe that ZEB1 is able to bring forth tumor initiating capacities by repressing stemness inhibiting microRNAs of the miR200 family. This implies that the ZEB1-mIR200 axis is a critical component in the progress of malignant tumor formation and can be considered to be a valuable target for treatment (Wellner et al, 2009b).

From the first description of the plasticity of the epithelial phenotype in 1982 to the present day view on EMT a large amount of progress has been made. General regulatory mechanisms that give EMT its spacio-temporal characteristics during embryogenesis have been elucidated. During the past 20 years it has become clear that aberrant activation of the EMT program is involved in several pathologies, including fibrosis and cancer. The consequences of this pathologic EMT not only reveal themselves in an enhanced invasive capacity of the tumor cells, but is in addition reflected in a gain of stem cell like properties and an increased resistance against chemotherapy. Understanding the nature of EMT opens up the possibility to identify novel markers for carcinoma and for the development of specific treatments.
1.2 Foundations of the epithelial phenotype

1.2.1 Introduction

In order to fully understand the implications of EMT brings forth, it is necessary to have a clear view on both the epithelial and the mesenchymal phenotype. Epithelial organization is one of the defining properties of the metazoa (Shook & Keller, 2003). During development and throughout adult life stages, multicellular organisms are equipped with epithelial sheets on their outer surfaces. Indeed, epitheliazation can already be observed after the third or fourth cleavage after compaction (Fleming et al, 2000). The barrier function of epithelial cells is also necessary for the internal organization of organisms in order to create a regulated environment. As such the development of highly complex organs performing specific tasks becomes possible (Micalizzi et al, 2010). In general, the structure of epithelial tissue can be subdivided in a simple, stratified or glandular type. For both the simple and glandular type, one layer of cells generally separates the luminal side from the stroma. Exceptions to this are for instance the sebaceous glands, which are holocrine glands. Here, the cells from the basal layer are gradually displaced to the center of the gland and release their content in the duct by disintegration of the cell membrane (Schneider & Paus). Thus, the apical side of the (basal) cells consists of a non adhesive, free surface, while the lower side comes into contact with the basal membrane. In the case of stratified epithelium, the cells form highly structured layers which again are superposed onto the basal membrane. These different layers strongly adhere to each other in order to maintain structural integrity. (Hay, 2005) The basal membrane is a thin sheet of extracellular matrix that supports the epithelium, provides a barrier separating the epithelium from the stroma, blocks the passage of macromolecules and delivers growth and differentiation signals to the contacting cells (Sherwood, 2006).

The nature of epithelial cell-cell adhesion only started to be elucidated at the molecular level with the advent of electron microscopy. One of the first reports studying the intricate adhesion complexes of epithelial cells came from Farquhar in 1963. By making use of electron microscopy the authors described for the first time the existence of the major
adhesive structures found in epithelial cells: the *zonula occludens* (tight junctions), *zonula adhaerens* (intermediary junctions) and the *macula adhaerens* (desmosomes) (Farquhar & Palade, 1963). In brief, tight junctions are characterized by fusion of adjacent cell membranes, circumscribing the cell as a belt at the apical side. In addition, tight junctions are considered to be rate limiting barriers for the diffusion of specific solutes (Furuse, 2010). Also adherens junctions are able to form a belt like structure surrounding the cell and are situated less apical when compared to the tight junctions. Dense cytoplasmic plaques are associated at cell-cell contacts that are mediated by adherens junctions. The main components of these plaques have been identified to be actin filaments. In contrast to tight junctions, the plasma membranes of two adjacent cells at the sites of adherens junctions do not contact each other. A 200 Å intercellular space is maintained, suggesting that the adherens junctions are less capable of forming a barrier against diffusing solutes. Instead, it is assumed that adherens junctions are necessary to maintain structural integrity of epithelial tissues (Harris & Tepass, 2010). The third main junctional complex are the desmosomes and are located more basical relative to the adherens junctions. Contacts between desmosomes leave an intercellular space and are characterized by the presence of dense plaques in the cytoplasm at sites of desmosomal contact. These plaques are conversion sites for bundles of intermediary filaments (IF) and it is therefore postulated that also desmosomes are mediators of mechanical rigidity (Franke, 2009). An schematical overview of these distinct junctional complexes is given in Figure 1 at p 23.

A final major type of junctional complex are the gap junctions. These are intercellular channels that permit the exchange of small molecules between neighbouring cells. Examples are cAMP, ADP, ATP and adenosine. Gap junctions are comprised of the proteins belonging to the connexin family, which form hexamers (Saez et al., 2003).
1.2.2 Specialized junctional complexes provide the mechanical rigidity for epithelial tissues

1.2.2.1 Tight junctions

In epithelial cells, tight junctions are stable, multimeric protein structures comprising at least 40 different proteins (Yamazaki et al, 2008). Two types of transmembrane proteins have been described so far: four pass transmembrane proteins such as claudins and occludins on one hand and single span proteins including the junctional adhesion molecules (JAMs) on the other hand (Balda & Matter, 2008). Apart from these constituents, recent proteomics studies suggest that additional uncharacterized transmembrane proteins are present in tight junctions (Yamazaki et al, 2008). Both occludins and claudins were identified by the group of Tsukita in 1993 and 1998, respectively (Furuse et al, 1998; Furuse et al, 1993). To date 24 different claudin members have been identified that encode proteins with molecular masses of 20-27 kDa (Koval, 2006; Tsukita et al, 2001). For the claudins, the N- and C-terminal domains are oriented to the cytoplasmic side and contain two extracellular loops in between. All claudins have a C-terminal PDZ domain allowing them to interact with cytoplasmic tight junction proteins such as ZO-1,-2,-3 (Zona Occludens), MUPPI (Multiple PDZ domain Protein 1) and PATJ (Pals1 Associated Tight junction Protein). Interaction with ZO-1 and ZO-2 link the claudins to the actin cytoskeleton and as such stabilizes the junction (Umeda et al, 2006). The first extracellular loop of the claudins has a length of ~52 amino acids and contains the highly conserved [Gly-Leu-Trp-xx-Cys-(8–10aa)-Cys] motif. The second loop contains 16-33 residues and is folded in a helix-turn-helix conformation, which participates in claudin-claudin interactions (Piontek et al, 2008). Claudins can interact with each other in a homo- or heterophilic manner and have the ability to form a multimeric complex with each other in the lateral plane of the membrane (Koval, 2006). Together with the fact that several members can be co-expressed in the same cell, the freedom of forming both homo- and heterophilic interactions allows the formation of tissue specific tight junctions. As such, it becomes possible to regulate the specificity of the barrier function towards different solutes (Anderson & Van Itallie, 2009). For
example, it has been shown that the modulation of claudin 2 and 3 have a significant impact on the permeability for mono- and divalent cations (Milatz et al, 2010). Moreover, overexpression of claudin -4, -5, -8, -11 and -14 decreases the permeability in several cell lines (Van Itallie et al, 2001) (Ben-Yosef et al, 2003; Wen et al, 2004).

The second transmembrane protein that can be found in tight junctions is occludin. Just like the claudins, occludin spans the cellular membrane four times, although no sequence similarity is present (Furuse et al, 1993). Unlike the claudins, occludin does not seem to be a main structural component of the tight junctions. However, occludin is an invariant component found in all tight junctions of epithelial and endothelial origin (Van Itallie et al, 2010). By transfecting different mutants of occludin in MDCK cells, it was observed that the permeability of the epithelial layer was strongly affected. It is thus becoming clear that occludin is necessary for the modulation of the transepithelial barrier function (Balda et al, 2000). Via their cytoplasmic domains, both occludin and the claudins are able to directly interact with the PDZ domain containing proteins ZO-1, -2 and -3. This binding allows the recruitment of a myriad of different structural proteins, signaling proteins and the actin cytoskeleton to the underlying surface of the tight junctions (Furuse, 2010). The other prominent function of the ZO-proteins in tight junction is the regulation of claudin polymerization at the membrane. Indeed, depletion of both ZO-1 and ZO-2 in mammary epithelial cell lines prevents the formation of functional tight junctions. In addition, recent evidence points to the direction that the ZO-proteins are necessary for the correct apico-basal polarity in epithelial cell lines. Depletion of ZO-1 and -2 results in an abnormal distribution of the polarity marker Par3 (Umeda et al, 2006).

1.2.2.2 Adherens junctions

Just like tight junctions, adherens junctions form a belt surrounding epithelial cells. The cytoplasmic side of adherens junctions is characterized by dense plaques that have been identified to be actin fibres (Hirokawa et al, 1982; Miyaguchi, 2000). The transmembrane proteins that make contact with their counterparts in neighboring cells are the cadherin proteins. Cadherins were discovered in the early 1980s as a result of research in the field of cell adhesion during embryonic development (Franke, 2009). One of the first cloned cadherins was epithelial cadherin (E-cadherin) in 1987 by the group of Takeichi.
Transfection of E-cadherin into fibroblast results in a significant increase in Ca$^{2+}$ dependent adhesion and goes together with clustering of the cells, indicating E-cadherin plays a major role in cell adhesion (Nagafuchi et al, 1987). The mature E-cadherin is a transmembrane single pass protein. Its cytoplasmic domain consists of 150 amino acids, while the ectodomain has a length of 550 residues. The ectodomain is made up of five tandemly repeated domains and is responsible for homophilic cell adhesion (Nose et al, 1988; van Roy & Berx, 2008). The first ectodomain (EC1) contains the typical HAV sequence that is necessary for E-cadherin mediated cell adhesion and is conserved in all four classical cadherins (Blaschuk et al, 1990). Each ectodomain consists of 110 amino acids containing three conserved sequences, namely, DRE, DXNDNXPXF, and DXD. Mutational analysis revealed that these motifs are detrimental for Ca$^{2+}$ dependent cell adhesion (Ozawa et al, 1990). The functional unit of E-cadherin is a cis dimer formed by the interaction of the extracellular domains of two cadherins on the same cell surface. Cell-cell adhesion is accomplished by the formation of trans adhesive complexes between the EC1 domains of cis dimers on opposing cell surfaces (Shapiro et al, 1995). Stronger adhesion is accomplished by trans binding of additional EC domains. Adhesion is further enhanced by the fact that E-cadherin dimers cluster together and as such cooperate to form stronger adhesive structures (Chen et al, 2005).

The intracellular domain of E-cadherin is highly conserved among the classical cadherins and is responsible for the interaction with key regulators of the adherens junctions. Via its armadillo domain, β-catenin associates with the cytoplasmic domain of E-cadherin. This interaction already starts in the endoplasmatic reticulum (ER) and is believed to be crucial for the correct transport of the E-cadherin protein to the cell surface (Chen et al, 1999). With its N-terminal domain β-catenin binds to α-catenin and the latter protein interacts with the actin cytoskeleton. As such E-cadherin is linked to the cytoskeleton, stabilizing and strengthening the adherens junction. Conflicting data exists whether the only role of β-catenin in this complex is its adapter function. Indeed, a cadherin-α-catenin fusion is able to replace the function of the β-catenin homologue during D. melanogaster oogenesis (Pacquelet & Rørth, 2005). However, the same fusion protein cannot replace the β-catenin function during dorsal closure of the fly embryo, indicating that during different stages of the embryonic development in D. melanogaster, β-catenin has additional functions besides linking E-cadherin to α-catenin (Gorfinkiel & Arias, 2007).
In addition, it has been shown that phosphorylation of β-catenin has a profound influence on cadherin-catenin phosphorylation and endocytosis of cadherins (Delva & Kowalczyk, 2009; Lilien & Balsamo, 2005; Rhee et al, 2007).

Another member of the catenin family that associates with the cytoplasmic domain of E-cadherin is p120 catenin. Interaction of p120 catenin with the highly conserved YDEEGGGE motif in the juxtamembrane domain of E-cadherin is believed to stabilize E-cadherin at the plasma membrane during the formation of adherens junctions (Thoreson et al, 2000). Besides a direct stabilization of the adherens junctions p120 catenin prevents the internalization and subsequent degradation of the cadherins (Davis et al, 2003). Furthermore, it has been shown that p120 catenin is able to regulate cell motility via its association with the Rho family of GTPases. Overexpression of p120 catenin in fibroblasts and MDCK cells enhances migration as a consequence of the activation of Rac and Cdc42 (Noren et al, 2000). Strikingly, disruption of the nuclear localization signal (NLS) of p120 catenin abolishes the induction of the migratory capacities that would otherwise be induced (Kim et al, 2004).

### 1.2.2.3 Desmosomes

Desmosomes, another type of intracellular adhesion complexes, are electron dense plaques that are highly enriched in tissues that are prone to experiencing mechanical stress such as the myocardium, the bladder, the gastrointestinal mucosa and skin (Holthöfer et al, 2007). Traditionally, desmosomes have been regarded as relatively static complexes that reinforce the cellular adhesion, in particular in tissues that have to withstand high mechanical stress. Loss of desmosomal functionality can lead to a plethora of pathological phenotypes, including epidermal fragility and cardiomyopathy (Dusek and Attardi, 2011). With the advent of electron microscopy it was already known by the end of the 1970s that desmosomes associated with intermediate filaments (IF) at the cytoplasmic side of the junctions. The insertion of the IF occurs at electron dense plaques that are mainly consisting of the proteins desmoplakin I and II (Franke, 2009). While the C-terminal end of the desmoplakins connects the desmosomes with the IF cytoskeleton, the N-terminal end makes contact with the linker protein plakoglobin. In addition, direct
interaction with members of the plakophilin family and the IF network have been described. The desmoplakins have been shown to interact with the keratin, desmin and vimentin type of IF networks (Green and Simpson, 2007). The strength of this interaction is modulated by the phosphorylation of a serine residue at position 2849. Mutation of this amino acid leads to an impaired desmoplakin assembly into the tight junctions (Godsel et al., 2005). To date, at least three plakophilins (PKPs) have been described. While PKP1 is mainly expressed in stratified epithelium, both PKP2 and 3 can be found back in simple and stratified epithelial layers throughout the body. Moreover, PKP2 is also expressed in cardiac myocytes and in the lymph node germinal center (Bass-Zubek et al., 2009). In general, PKPs are considered as structural scaffolding proteins that significantly increase the plaque density leading to additional mechanical strength of the desmosomal junction. Plakoglobin and plakophilins on their turn interact with the transmembrane proteins desmoglein and desmocollin that are responsible for the formation of the actual intercellular contacts (Holthöfer et al, 2007). Both desmoglein and desmocollin are members of the Ca\textsuperscript{2+} dependent cadherin superfamily. At the extracellular side, both proteins contain four cadherin homology repeats and a juxtamembrane extracellular anchor. At the cytoplasmic side, an intracellular cadherin like sequence (ICS) can be found that binds plakoglobin. Furthermore, the cytoplasmic part of the desmogleins contain additional domains such as a proline rich linker and a glycine rich terminal domain (Delva et al, 2009). In human, four isoforms of desmoglein and three isoforms of desmocollin exist. Desmoglein 2 and desmocollin 2 are the predominant isoforms in simple epithelial tissues. In contrast, for stratified epithelium and in particular in the epidermis, higher concentrations of desmocollin 2, 3 and desmoglein 3 can be found in the basal cells, with their expression gradually diminishing towards the luminal side. Vice versa, desmoglein 1 and desmocollin 1 are preferentially expressed in the upper layers, while being marginally expressed in the basal cells. It is postulated that this isoform specific patterning of specific desmosomal cadherins contributes to tissue morphogenesis and differentiation (Green and Simpson, 2007). In addition, the desmosomes of the suprabasal cornified layers contain a glycoprotein corneadesmosin, which contains homophilic adhesive properties (Jonca et al., 2002). This highly specific expression profile of the different desmosomal components in stratified epithelium might form the basis of the contradictory results that have been obtained regarding correlation studies of
desmosomal expression and cancer development. Indeed, for cancers of the skin, head and neck, both upregulation and downregulation of desmosomal components has been brought into association with progressive status (Dusek and Attardi, 2011). A more clear view has been established by making use of the Rip1Tag2 transgenic model for pancreatic cancer, whereby it was shown that local invasion is preceded by loss of desmosomal components (Perl et al., 1998). It is postulated that desmosomes are often lost during the initial steps of local cell invasion and that subsequent loss of adherens junction functionality further supports the invasion into neighboring tissues (Dusek and Attardi, 2011).

1.2.3 Distinct components of the junctional complexes contribute to cell signaling events

Apart from playing a mechanistic role by forming stable intercellular contacts, it is becoming increasingly clear that the different junctional complexes are also implicated in cellular signaling. As such, the overall stability or functionality of a specific junctional complex can provide additional information to the cell resulting in a corresponding response. As will be exemplified below, this furthermore implies that several post transcriptional modifications and/or somatic mutations in distinct components of the adherence complexes, which at first sight do not seem to play a direct role in cell adhesion, are able to contribute to alterations in cell signaling.

1.2.3.1 Cell signaling at the level of the tight junctions

As mentioned before, ZO-1 is considered to be a prominent member of the tight junction proteins which is strictly necessary for the correct functionality of these junctional complexes. However, recent evidence is emerging that members of the ZO family are also implicated in cell signaling. For instance, apart from binding to the claudins, distinct
physical interactions between ZO-1 and several members of the Par family have been reported. Via these Par proteins, a complex is made with Cdc42, Tiam1 and protein kinase c (PKC), which have been shown to be necessary for modulating the polarity of the cell. Removing PKC from the tight junctions furthermore results in functional impairment of the junctional complex as a whole (Lin et al., 2000; Joberty et al., 2000). Strikingly, in vitro experiments have shown that ZO-1 is able to interact with the transcription factor ZONAB at the tight junctions (ZO-1-associated nucleic acid-binding protein). The ZO-1-ZONAB-complex is located at the cell membrane, whereas ZONAB can also be found in the nucleus. Overexpression of ZO-1 in MDCK cells seeded at low cell density resulted in the upregulation of the ErbB-2 promoter activity, while overexpression of ZONAB lead to repression of the promoter. It is thus suggested that ZONAB is a repressor of ErbB-2 and that overexpression of ZO-1 leads to sequestration of ZONAB to the cell membrane, preventing to exert its function in the nucleus (Balda & Matter, 2000). A similar effect can be observed for ZO-2. Translocation to the nucleus at low cell densities of ZO-2 allows the interaction with the transcription factors Jun, Fos, AP-1 and C/EBP to regulate proliferation (Betanzos et al, 2004). This working model is thought to be further implicated in the regulation of cell proliferation. Indeed, in subconfluent conditions, the abundance of tight junction complexes at the membrane is relatively low. In contrast, in a confluent layer, the density of tight junctions is much higher, resulting in the sequestration of key signaling molecules to the membrane. For example, under these conditions, ZO-1 has been shown to bind PTEN and ZONAB at the cell membrane. As such, the tumor suppressing capabilities of PTEN are stimulated by bringing it into close contact with its membrane associated lipid substrates. For ZONAB, it has been shown that a physical interaction with cyclin dependent kinase 4 (CDK4) occurs. Location of ZONAB to the cellular membrane thus leads to sequestration of CDK4 to the membrane, preventing the latter to exert its function in the nucleus during G1-S transition of the cell cycle (Matter and Balda, 2003). Additional tumor suppressing effects have been observed in Raf-1 transformed cells whereby overexpression of occludin was sufficient to inhibit the transformed phenotype and disrupted the anchorage independent growth is soft agar (Li and Mrsny, 2000).

The involvement of tight junctions in cellular signaling suggests that disruption of the complex might contribute to pathological onset. Indeed, a connection between
disfunctional tight junctions and cancer has been established. For example, low expression of ZO-1 in several carcinomas is associated with invasive status and poor prognosis (Hoover et al, 1998; Kleeff et al, 2001). Moreover, forced expression of occludin in breast cancer cells has been shown to decrease cancer cell migration and invasion both in vitro and in vivo (Osanai et al, 2006). Similar conclusions can be made for different members of the claudin family. For instance, promoter hypermethylation and subsequent downregulation of claudin 11 has been shown to contribute to increased invasion of gastric cancer cells (Agarwal et al, 2009). Furthermore, breast cancer tumors with low claudin-3, -4 and -7 and high mesenchymal markers are associated with poor overall survival (Hennessy et al, 2009). In addition, claudin-1 has proven to be a good predictor for cancer recurrence and the overall malignant status in breast cancer (Morohashi et al, 2007). New evidence suggests the involvement of disfunctional tight junctions in the process of EMT. Indeed, overexpression of mutated ZO-1 proteins that are only present in the nucleus results in an EMT in MDCK and corneal epithelial cells (Ryeom et al, 2000). Together with this shift towards a mesenchymal phenotype, accumulation of nuclear β-catenin can be observed (Reichert et al, 2000). The link between ZO-1 and β-catenin is also observed when wild type ZO-1 is transfected into breast cancer cells. Ectopic ZO-1 expression results in activation of the TCF/LEF pathway and in parallel with activation of the membrane type 1 matrix metalloprotease (MT1-MMP). Interestingly, when only overexpressing the N-terminal PDZ domain of ZO-1, a similar effect could be observed (Polette et al, 2005). How exactly ZO-1 is able to activate TCF/LEF mediated transcription is not known. It has however been demonstrated that ZO-1 is able to form a complex with α-catenin and β-catenin and could as such be able to modulate the TCF/LEF pathway. Whether or not the disfunctionality of tight junctions lies at the basis of cancer development or is merely a consequence of malignant progression is less clear.

1.2.3.2 Cell signaling at the level of the adherence junctions

The functionality of the adherens junctions is imperative in preventing tumor cells from disseminating from the primary tumor mass. Indeed, a multitude of studies report that
E-cadherin function is frequently inactivated during the development of human cancer, including those of breast, colon, prostate, stomach, liver, oesophagus, skin, kidney, and lung (Birchmeier & Behrens, 1994; Bracke et al, 1996). More specifically, the suppression of E-cadherin function has been shown to induce invasive capacities in epithelial cells in vitro. In extension to this, re-expressing E-cadherin into highly invasive cells resulted in the loss of their migratory and invasive abilities. Moreover, ectopic E-cadherin overexpression in invasive cancer cell lines resulted in a partial redifferentiation of the cancerous cells in xenograft experiments (Vleminckx et al, 1991). This view was further established in a study of the transgenic Rip1Tag2 model where it was shown that E-cadherin expression was responsible for maintaining a differentiated state of the developing tumors (Perl et al, 1998a). The fact that E-cadherin overexpression could induce redifferentiation suggests that, besides playing a mechanical role, E-cadherin functionality also has an influence on cellular signaling. Indeed, several reports revealed an interplay between the E-cadherin/ catenin complex and various protein kinases and phosphatases. Initial research showed that cadherin based contacts are enriched with phosphotyrosine residus. Further investigation led to the identification of a myriad of protein kinases and phosphatases and revealed that interaction with the E-cadherin/ catenin complex brought these mediators of cellular signaling into an activated status (McLachlan & Yap, 2007). The list of associating tyrosine kinases encompasses both cytoplasmic and receptor bound tyrosine kinases. Examples of the former are members of the Src family and the kinases FER and Abl (Behrens et al, 1993; Fox & Peifer, 2007; Rosato et al, 1998). Known receptor bound kinases that associate with the E-cadherin/ catenin complex are members of the epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor (FGFR). An exhaustive list of the known kinases and phosphatases that interact with adherens junctions can be found in the review of McLachlan and Yap (McLachlan & Yap, 2007).

A clear link between E-cadherin and EGFR signaling has been demonstrated in several reports. For instance, the formation of intercellular adherens junctions and hence the clustering of cadherins is a crucial requirement for the EGFR dependent activation of the Rac GTPase in keratinocytes (Betson et al, 2002). In addition, the physical association between E-cadherin and the EGFR has been shown to result in a diminished ligand dependent activation of the receptor. This suggests that loss of functional E-cadherin
might contribute to the frequently observed ligand dependent activation of receptor tyrosine kinases during cancer progression (Qian et al, 2004). In contrast, the E-cadherin mediated activation of the EGFR and subsequent activation of the MAPK signaling pathway has also been observed. Strikingly, this stimulation takes place in the absence of the EGF ligand, indicating that the formation of an E-cadherin/ EGFR complex is sufficient to induce signaling from the outside to the inside of the cell (Pece & Gutkind, 2000). Apart from having an influence on the MAPK pathway, E-cadherin is also involved in AKT signaling. Indeed, upon the establishment of functional homophilic E-cadherin contacts in MDCK cells, the PI3 kinase is recruited to the adherens junctions and subsequently phosphorylated. This in turn leads to the activation of the AKT protein, which results in the modulation of cell growth and survival (Pece et al, 1999).

Besides E-cadherin, several other protein members of the adherens junctions are implicated in cellular signaling. For instance, reduced levels of α-catenin or p120 catenin in basal cell carcinoma lead to activation of the NFκB pathway, resulting in subsequent inflammation (Kobielak & Fuchs, 2006; Perez-Moreno et al, 2006). Interestingly, the loss of α-catenin disturbs contact inhibition in keratinocytes as a consequence of constitutive activation of the MAPK pathway (Vasioukhin et al, 2001). When taking into account the MAPK modulating capacities of E-cadherin, these findings suggest the existence of several regulatory mechanisms for extracellular mitogenic stimuli at the site of the adherens junctions. Other cellular signaling cascades that may be regulated by α-catenin is the Hedgehog pathway. Tissue-specific deletion of α-catenin results in activation of the Hedgehog pathway, resulting in shortening of the cell cycle and subsequent hyperplasia (Lien et al, 2006).

Additional evidence that the adherence junctions are implicated in cell signaling is provided when looking to the p120 catenin, of which it has been found to interact with the transcription factor Kaiso in the nucleus (van Roy and McCrea, 2005). The role of p120 catenin in this nuclear complex is to repress Kaiso mediated transcription at sensitive promoters. Moreover, the p120 catenin-Kaiso complex is able to modulate similar target genes as the β-catenin-TCF complex. One major difference however is that free cytoplasmic p120 catenin is relatively stable in comparison with free β-catenin in the absence of Wnt signaling (van Roy & McCrea, 2005).
Perhaps the most studied adherens junction protein involved in cellular signaling is β-catenin. Developmental studies during the 1980s and 1990s showed that overexpression of E-cadherin in *Xenopus* embryos could inhibit dorsal axis formation, a process that is highly dependent on Wnt signaling (Heasman et al, 1994). Furthermore, β-catenin mutant embryos in *Drosophila* show disturbed segment polarity, which is also controlled by Wnt signaling (Cox & Peifer, 1998). These data support a model whereby β-catenin is involved in cellular adhesion on one hand and has the potential to act as a transcription factor on the other hand. The finding that relocation of β-catenin from the nucleus to the plasmamembrane by overexpressing N-cadherin led to inhibition of LEF transcriptional activity, proved the involvement of β-catenin in the canonical Wnt pathway (Heuberger & Birchmeier, 2010). Based on these findings, the question arises whether accumulation of free cytosolic β-catenin due to major disturbances in the stability of the adherens junctions de facto leads to nuclear translocation and subsequent transcriptional activity. It has been shown that siRNA mediated knockdown of E-cadherin in colon cancer cells only leads to β-catenin transcriptional activity when a Wnt signal is present. In keratinocytes that lack active Wnt signaling, β-catenin gets degraded by the proteasome (Kuphal & Behrens, 2006). Indeed, in the absence of Wnt, free cytoplasmic β-catenin is phosphorylated by a protein complex comprising GSK3β, APC and axin. The phosphorylated β-catenin is as such marked for ubiquitination, leading to proteasomal degradation. When a Wnt signal is present, the activity of the GSK3β complex is inhibited, preventing the degradation of β-catenin (Heuberger & Birchmeier, 2010).

Strikingly, Wnt signaling is known to modulate target genes that cause a switch from an epithelial appearance towards a mesenchymal phenotype. For example, it has been shown that E-cadherin is negatively regulated by Wnt signaling via direct binding of the LEF-1/β-catenin complex to the E-cadherin promoter (Huber et al, 1996). Moreover, other Wnt target genes are implicated in the regulation of the stability of the adherens junctions. Examples are the induction of matrix metalloproteinases (MMPs) stromelysin and matrilysin (Brabletz et al, 1999; Prieve & Moon, 2003). Expression of these MMPs cleave E-cadherin at the cell surface releasing an 80kDa ectodomain fragment that acts in a paracrine manner to inhibit homotypic cell adhesion. The result is an increase in invasive potential and reduction of E-cadherin mediated cell adhesion (Noe et al, 2001). In addition, the activation of the Wnt pathway can also enhance invasive and migratory
capacity of tumor cells by modulating expression of fascin, EphB/Ephrin and S100A4 (Stein et al, 2006; Vignjevic et al, 2007).

Having these profound effects on epithelial plasticity, it was soon discovered that Wnt signaling was able to modulate several transcription factors that are capable of inducing a complete EMT. For example, in the presence of Wnt1, the LEF/β-catenin complex is able to induce TWIST1 expression, leading to a dedifferentiated phenotype in mammary epithelial cells (Howe et al, 2003). In addition, the Slug promoter contains a LEF binding site and is shown to be directly activated by the LEF/β-catenin complex. This leads to decreased E-cadherin expression, further increasing the cytoplasmic pool of free β-catenin (Conacci-Sorrell et al, 2003). For the transcription factor Snail, a direct activation by Wnt signaling has not been shown. However, the stability of the Snail protein is determined by its phosphorylation status, which is determined by the kinase GSK3β. Since Wnt activation inhibits the kinase activity of GSK3β, this implies in an increased stability of Snail. Moreover, Snail is known to interact with β-catenin and is able to enhance Wnt target gene expression. As such, a positive feed back loop exists between Snail functionality and the Wnt pathway (Stemmer et al, 2008; Yook et al, 2005)

1.2.3.3 Cell signaling at the level of the desmosomes

The myriad of different effects that are observed in transgenic mice with altered expression of desmosomal components suggests that also desmosomes are implicated in cellular signaling next to providing mechanical rigidity to epithelial tissue. For instance, overexpression of desmoglein 3 in the epidermis gives rise to hyperproliferation and aberrant differentiation, resembling the phenotypes of overexpression of growth factors and cytokines in the epidermis (Merritt et al, 2002). In extension, abnormal expression of desmoglein 2 in the epidermis causes susceptibility to chemically induced carcinogenesis (Brennan et al, 2007a). On the other hand, ablation of desmoglein 3 gave rise to impaired cell-cell interactions in the oral mucosa, characterized by separation of the keratinocytes (Koch et al, 1997). The specific mechanisms that are responsible for these signaling
events have recently started to be elucidated. Indeed, it has been recently shown that plakoglobin is able to activate WNT signaling (Chidgey & Dawson, 2007). Plakoglobin is a homologue of β-catenin and is able to bind to the APC complex and to LEF/TCF factors (Kodama et al, 1999). In addition, overexpression of plakoglobin in desmosome negative cell lines results in its accumulation in the nucleus and subsequently leads to increased levels of nuclear β-catenin. Moreover, a highly potent activation domain has been identified in plakoglobin (Simcha et al, 1998). As such, plakoglobin has been shown to be a strong transcriptional activator of the pro survival gene Bcl-2 and c-myc, leading to transformation of plakoglobin overexpressing cells (Hakimelahi et al, 2000; Kolligs et al, 2000). Concerning the activation of the LEF/TCF pathway, similar results have been obtained in the skin of mice that overexpress desmocollin 3 under control of the keratin 1 promoter (Hardman et al, 2005). Furthermore, in colorectal cancer expression of desmocollin 2 is often reduced, thereby suggesting its role in development and/or progression of tumorigenic lesions. Further investigations led to the conclusion that loss of desmocollin 2 is a driving event in cell proliferation via activation of the β-catenin TCF/LEF pathway (Kolegraff et al., 2010). This might be explained by the fact the several members of the catenin family, including β-catenin and p120 catenin, have been found to associate at the desmosomes. Disruption of the desmosomal integrity might thus result in a free pool of β-catenin (McCrea et al, 2009). In contrast, shuttling of plakoglobin due to loss of desmoplakins at the membrane of cardiomyocytes, has been shown to result in a decrease of canonical WNT signaling and in a concomitant reduction of nuclear β-catenin levels. While this resulted in a marked downregulation of the β-catenin target genes c-myc and cyclin D1, an increase in adipogenic gene transcription was observed (Garcia-Gras et al, 2006). In addition to having a modulatory effect on the β-catenin TCF/LEF pathway, other signaling cascades have been found to be regulated by desmosomal components. For example, an enhanced activation of the Akt, MEK, Stat3 and NFκB pathway in the skin of mice which overexpressed desmoglein 2 suprabasally in the epidermis has been discovered. As a consequence, hyperproliferation and an apoptosis resistant phenotype could be observed (Brennan et al, 2007b). The influence of desmosomal components on cell signaling was further established when studying the role of desmoglein 1 during keratinocyte differentiation. Hereby it was found that reduced expression levels of desmoglein 1 lead to impaired expression of several markers for
keratinocyte differentiation and that this was the consequence of aberrant activation of the EGF receptor. Interestingly, by making use of specific deletion constructs, it was found that the extracellular domain of desmoglein 1 was not necessary for the repression of EGF receptor activation, further implying the adhesion independent nature of this effect (Getsios et al, 2009).

Additionally, members of the plakophilin (PKP) family of proteins have the ability to locate to the nucleus. For example, PKP2 is able to associate with components of the PolIII transcriptional complex and the transcription factor TFIIIB (Mertens et al, 2001). The localization of PKP2 is modulated by phosphorylation at specific sites by the Cdc25C-associated kinase 1, whereby PKP2 phosphorylation leads to accumulation at the membrane (Muller et al, 2003). In addition, an alternative splice form of PKP1, PKP1b, is exclusively located in the nucleus, even in cells with fully functional desmosomes (Schmidt et al, 1997).
Figure 1: *Overview of the main adhesive junctional complexes found in epithelial cells.*

The tight junctions are found at the most apical side of the cells and are linked to the actin cytoskeleton for overall stability. Located less apically are the adherence junctions, which are also linked to the actin cytoskeleton via α- and β-catenin. Apart from its adhesive function, β-catenin can also translocate to the nucleus and act as a transcription factor. A similar working model has been proposed for p120 catenin. Finally, the desmosomes are located most basally and form stable interactions with several types of intermediate filaments. In addition, several constituents of desmosomal junctions, such as PKP and plakoglobin have been found to modulate transcription in the nucleus.
1.3 The transcription factors of the ZEB family are crucial mediators of EMT in physiological and pathological conditions

1.3.1 Introduction

The human ZEB family of transcription factors consists of two members, ZEB1 (deltaEF1) and ZEB2 (SIP1). These proteins are highly homologous and are characterized by two clusters of zinc fingers separated by a homeodomain. They are crucial for migration of the neural crest cells and formation of the derivative structures during subsequent embryonic development. Detachment of single cells and invasion into the surrounding tissue are necessary for these processes, which are also considered to be driving forces behind malignant tumour progression (Vandewalle et al., 2009).

Ectopic expression of ZEB1/2 in epithelial cancer cells is associated with poor prognosis (Peña et al., 2005; Sayan et al., 2009; Spaderna et al., 2006). In analogy with their function during embryonic development, both proteins can direct the epithelial status of tumorigenic cells towards a more mesenchymal phenotype. This process, which is known as epithelial-mesenchymal transition (EMT), has been extensively studied during recent years (Berx et al., 2007; Peinado et al., 2007; Yang and Weinberg, 2008). EMT is characterised by downregulation of cellular adhesion complexes, loss of apicobasal polarity of epithelial cells, and dramatic changes in the organisation of the cytoskeleton caused by remodelling of cortical actin into fibres. The constitution of intermediary filaments is also altered, mainly by replacement of cytokeratins by vimentin. Cells that undergo EMT can change the constitution and remodelling of the basal membrane to allow them to invade into the neighbouring tissue. For example, ZEB1 is responsible for downregulation of basal membrane constituents at the invasive front of colorectal carcinomas (Spaderna et al., 2006). In addition, ZEB2 has been implicated in the progression of carcinoma. The latter ZEB2 effect was first illustrated by Comijn et al., who demonstrated that ZEB2 can induce EMT and enhance invasion in vitro (Comijn et al., 2001). Further investigations identified several junctional proteins as ZEB2 targets, and numerous reports have linked ZEB2 expression with advanced carcinoma stages (Elloul et al., 2005; Rosivatz et al., 2002; Vandewalle et al., 2005).
The functional similarity of the ZEB transcription factors is also evidenced by their involvement in a regulatory loop with the miR200 microRNA family. Members of the miR200 family are involved in stabilizing the epithelial phenotype, and both ZEBs can transcriptionally inhibit the expression of miR200 to allow EMT to take place. Reciprocally, members of the miR200 family can inhibit protein expression of both ZEB factors. The existence of this loop permits rapid switching towards an epithelial or a mesenchymal phenotype, depending on the stimulus (Brabletz and Brabletz, 2010).

It is thus becoming evident that members of the ZEB family play a major role in malignant cancer progression. The functions of ZEB expression during embryonic development and cancer and in the different signalling cascades that lead to ZEB modulation have been reviewed (Vandewalle et al., 2009). However, the molecular mechanisms by which ZEB proteins direct the downstream signalling events are less clear. This review aims to give a better insight into how the nuclear ZEB proteins function as transcription factors. By using an evolutionary approach, we gain detailed insight into the organisation and function of the different domains of the ZEB proteins. We also present a comprehensive overview of the different cofactors and post transcriptional modifications that are essential for the diverse range of nuclear effects induced by ZEB factors.

### 1.3.2 Discovery of the ZEB transcription factors

Since its discovery in 1991, the transcription factor ZEB1 has been the subject of many studies covering a broad range of topics. ZEB1 was initially identified as an important transcriptional regulator of the human interleukin 2 (IL-2) gene and the chicken delta-crystalline gene (Funahashi et al., 1993; Williams et al., 1991). Williams and colleagues reported that expression of IL-2 in T-lymphocytes triggered by antigen recognition is repressed by a protein that binds to a specific site in the IL-2 promoter. They named this protein negative inhibitor of IL-2a (NIL-2a). After cloning its cDNA, they found that it contained several Zn finger motifs. Independently, Funahashi et al. were studying the regulation of the chicken delta-crystalline gene during lens development. They found that
an enhancer sequence in the third intron was responsible for lens-specific expression of this gene (Funahashi et al., 1993). In several non lens cell lines, this enhancer sequence was bound by a repressing factor. Cloning of this factor revealed that it contained two different clusters of DNA binding zinc fingers separated by a homeodomain. The factor was named delta crystallin enhancer factor (deltaEF1). Other nuclear proteins that were independently found to be transcriptional modulators and later identified as ZEB1 are AREB6 (a modulator of the N, K- ATPase alpha1 regulatory element or ARE), ZEB (an inhibitor of the immunoglobulin heavy chain in B-cells) and BZP (a serum responsive transcriptional repressor in hamster, designated Beta-cell Zn finger Protein) (Franklin et al., 1994; Genetta et al., 1994; Watanabe et al., 1993). Remarkably, these initial studies showed that ZEB1 is involved in many cellular processes, indicating that complex and subtle levels of regulation influence the functionality of this transcription factor.

ZEB2 was discovered when trying to identify transcription factors that can bind the MH2 domain of Smad1 in *Xenopus*. Since the MH2 domain can by itself induce ventral cell types in the *Xenopus* embryo but cannot bind DNA, it was suspected of interacting with other transcription factors. Indeed, by using the MH2 domain as bait in a yeast two hybrid screening, several candidates were found to physically interact with this domain. One of these smad interacting proteins, SIP1 (Smad interacting protein 1) or ZEB2, was identified as a transcriptional repressor and was found to be a close homologue of ZEB1. This close homology between ZEB1 and ZEB2 is also reflected in their high binding affinity for E-box DNA sequences. (Verschueren et al., 1999). Further investigations showed that a bipartite E-box motif is necessary for full repression of the Xbra2 promoter by both ZEB proteins. Interestingly, the fact that this bipartite motif was also found to enable high affinity binding of ZEB1 to DNA, further emphasising their functional similarity in DNA binding (Remacle et al., 1999).

1.3.3 Correlating the functions of the ZEB proteins with their evolution

The appearance of vertebrates during metazoan evolution was accompanied by a dramatic shift towards a more complex body plan. Unlike their ancestors, vertebrates are
characterized by the development of a neural crest, ectodermal placodes and a cartilagenous or bony endoskeleton. Moreover, one of the closest living invertebrate relative of the vertebrates, the cephalochordate amphioxus, is largely devoid of mesenchyme (Holland et al., 2004).

It is remarkable that the emergence of vertebrates occurred simultaneously with the duplication of the ancestral ZEB gene (Figure 2). Indeed, the first appearance of a ZEB ancestor can be traced back to the starlet sea anemone *Nematostella vectensis*. The similarity of the putative ancestral ZEB protein (accession number XP_001637322) to human ZEB1 is restricted to the zinc fingers (Figure 2). The ancestral protein is predicted to have contained three consecutive zinc fingers. Strikingly, alignment shows that the first zinc finger of the *Nematostella* protein corresponds with the second N-terminal finger of human ZEB1 and that these two fingers have 30% sequence similarity. The other finger motifs of the *Nematostella* ancestor correspond to the second and third C-terminal zinc finger of human ZEB1 with 53% sequence similarity. This strongly indicates that the general structure of the ZEB proteins initially started as three consecutive zinc fingers in *Nematostella*. These fingers probably separated during evolution and generated two distinct clusters in the vertebrate ZEBs. In addition, the three consecutive zinc fingers in *Nematostella* ZEB are preceded by a sequence that shares 47% sequence similarity with the first N-terminal zinc finger of vertebrate ZEBs. However, the final histidine residue that is present in the C2H2 zinc finger type is missing. Nevertheless, this suggests that the first N-terminal zinc finger in vertebrate ZEB arose by evolutionary drift and not by intron/exon exchange.

The zinc finger motif was first discovered when trying to elucidate how transcription factor IIIA binds to RNA and DNA in *Xenopus laevis* oocytes (Miller et al., 1985). Since then, it has become clear that zinc fingers are found not only in the DNA binding helix-turn-helix motif, but also in a variety of transcription factors, and in about 3% of human genes (Evans and Hollenberg, 1988; Klug). There is also evidence that zinc fingers are also important mediators of protein-protein interaction (Brayer and Segal, 2008). However, for the ZEB proteins, and particularly ZEB1, the C-terminal zinc fingers have been observed to interact only with the upstream homeodomain (Sekido et al., 1994). There is no clear indication that these fingers interact with other proteins.
Ancestral ZEB proteins can be traced back to the starlet sea anemone Nematostella vectensis, of which only distinct parts correspond to the N- and C-terminal zinc fingers of human ZEB1. Duplication of the ancestral ZEB occurred in the vertebrata and was accompanied by major modifications that suggest stricter regulation of both ZEB1 and ZEB2. From the vertebrata on, the CtBP interacting domain (CID), Smad binding domain (SBD), homeodomain (HD), a predicted nuclear localization signal (NLS) and several sumoylation sites (sumo) are present in the ZEB proteins. The Smad binding domains (SBD) of ZEB1 and ZEB2 are substantially different and are therefore depicted in different colors. Similarly, the homeodomains of S. purpuratus, C. elegans and A. gambiae are depicted in gray because they are less similar to the homeodomains of the vertebrates.

Duplication of ZEB seems to have been simultaneous with the incorporation of additional regulatory sequences, such as sumoylation sites, the CtBP interaction domain (CID) and the Smad binding domain (SBD). This indicates that a more complex regulation of both ZEB proteins came into existence. However, sumoylation and Smad signalling had been well established when duplication occurred (Srilunchang et al., 2010; Wu and Hill, 2009). Quite likely, the homeodomain was incorporated before duplication. One of the first ZEB
ancestors to have a homeodomain is Zag1 of *C. elegans*. Strikingly, the homeodomain of Zag1 corresponds fully with the third exon and is separated from the exon containing the closest N-terminal zinc finger (Figure 3). This gene structure does not seem to be present in higher organisms, in which the coding sequence of the last N-terminal zinc fingers is always on the same exon as the homeodomain (Figure 3). These observations suggest that the origin of the homeodomain in *C. elegans* might be an external exon that was incorporated by intron/exon recombination. The originating homeodomain can be predicted by comparing the Zag1 homeodomain of *C. elegans* with those found in the organisms *Ciona intestinalis* and *Nematostella vectensis*, which both lack a homeodomain in their ZEB ancestors (Figure 4A). This analysis indicates that the homeodomain could have originated from several protein candidates, but it is most similar to the homeodomain of the Pou family of proteins in both *N. vectensis* and *C. intestinalis*.

Homeodomains are moderately conserved sequences of 60 amino acids and are important for both protein-DNA and protein-protein interaction (Holland et al., 2007; Mallo et al.). Many homeodomain-containing proteins have been discovered. Several families of such proteins play crucial roles during embryonic patterning and cell differentiation (Wolberger, 1996). Examples are members of the Hox, Pou and Lim family (Holland et al., 2007; Mallo et al., 2010). The domain structure is comprised of three alpha helices that can in monomeric form bind DNA (Wolberger, 1996). However, the homeodomain of ZEB1 and ZEB2 do not seem to be important for DNA binding. The canonical WFxNxR sequence, which is responsible for interaction with DNA, is altered to WFEKMQ in ZEB1 and to WFEQRK in ZEB2 of vertebrates. Most likely, these alterations perturb or destroy the DNA binding capacities of the ZEB1/2 homeodomains (Smith and Darling, 2003). It has been postulated that the homeodomain is responsible mainly for protein-protein interaction (Vandewalle et al., 2009).
Figure 3: *Organisation of the ZEB genes throughout evolution.* Intron-exon structure of the human ZEB1 and ZEB2 genes and C. elegans Zag1. In human, and by extension in all vertebrates, the fourth N-terminal zinc finger is located on the same exon as the
homeodomain. This organisation is not found in *C. elegans*, in which the second N-terminal zinc finger is on a different exon. This suggests that incorporation of the homeodomain during duplication of the ancestral ZEB gene was not due to incorporation of an external exon containing the homeodomain. In addition, several binding sites for miR200a and b are depicted in the 3’ UTR of both ZEB1 and ZEB2.

Indeed, although the homeodomains of several transcription factors bind to the same DNA sequence, distinct functions are generated by the specific interactions between the homeodomain and other cofactors. This strongly indicates a crucial role for the homeodomain in protein-protein interaction (Pomerantz et al., 1995). For example, the functional specificity of the Hox genes *Antp* and *Scr* in *Drosophila* are almost entirely determined by their homeodomains and adjacent sequences. Exchanging the homeodomain between these two proteins switches their functionality (Gibson et al., 1990). In addition, the homeodomain of the *Drosophila* gene *Dfd* is responsible for its transcriptional inhibitory function independently of its DNA binding capacity (Li et al., 1999). It has furthermore been demonstrated that the homeodomain in combination with an adjacent zinc finger motif is responsible for the *in vitro* binding of ZEB1 to the transcription factor Oct1 (Smith and Darling, 2003). In analogy with ZEB1, several human homeodomain-containing factors can form a complex with the transcriptional regulator Serum Response Factor (SRF) (Grueneberg et al., 1995; Nishimura et al., 2006). This interaction enhances the activation of the serum response element (SRE) and also facilitates the recruitment of the ternary complex factors (TCFs) to SRF (Grueneberg et al., 1995). Though the specific SRF interacting domains of ZEB1 and ZEB2 have not been identified yet, these data suggest that the ZEB homeodomain might be responsible for the interaction with SRF. Remarkably, the homeodomains of human ZEB1 and ZEB2 share only 37% sequence similarity, which might indicate that these proteins have other distinctive features. Indeed, though their expression patterns overlap, their functions seem to differ in some ways. In the *Xenopus laevis* embryo, ZEB1 is induced in the postgastrulation stage in mesodermal tissue, whereas ZEB2 is present from the early gastrula stage in dorsal epithelial cells. Moreover, only ZEB2 seems to be able to induce neuronal markers in ectodermal cells (van Grunsven et al., 2006). More differences are observed when examining the Smad binding domains (SBD) of both proteins. Aligning
the SBDs of ZEB1 and ZEB2 from different species reveals a striking divergence (Figure 4B). The SBD of ZEB1 and ZEB2 individually share high sequence similarity across species, but the similarity between the SBD of human ZEB1 and ZEB2 is only 30%. These findings indicate that the SBDs of ZEB1 and ZEB2 have been under different evolutionary pressures since the duplication event, and that the SBDs of the two proteins probably have different functions.

Figure 4: Evolutionary analysis of the ZEB SBD and the homeodomain  (A) Analysis of the similarity of the ZAG1 homeodomain with the most similar homeodomain-containing proteins from N. vectensis (Nv) and C. intestinalis (Ci). Ce: C. elegans, Hs: H. sapiens, Mm: M. musculus  (B) Smad binding domain (SBD) of ZEB1 and ZEB2. The SBD of ZEB1 for human, mouse and Xenopus laevis share high homology. This also holds true for ZEB2, but when the SBD is compared in the two ZEB proteins, much less similarity is observed. This suggests different evolutionary pressure on the SBDs of ZEB2 and ZEB1.
Although the structures of the ZEB1 and ZEB2 genes are highly similar, the 5' untranslated region (5' UTR) of mouse ZEB2 is more complex than the ZEB1 5' UTR (Figure 3). Interestingly, strong expression of ZEB2 protein can be dependent on a large intron in the 5'UTR that contains an internal ribosomal entry site. Snail-induced EMT prevents the processing of the ZEB2 intron by upregulating a natural antisense transcript that blocks the splicing of this intron. As such, an Internal Ribosomal Entry Site (IRES) is preserved in the primary transcript, resulting in increased expression of ZEB2 protein (Beltran et al., 2008). In addition, several splice variants of ZEB2 are expressed from different transcription start sites in the 5' UTR. The presence of these alternative transcripts varies among several types of tissue and cell lines. For example, the longer variants with exons lying in the 5' UTR are overrepresented in liver in comparison to lung (Nelles et al., 2003). Whether these splice forms affect the function of ZEB2 remains elusive, but the presence of alternative transcripts might further explain the differences in expression and function between ZEB1 and ZEB2. These differences are also evident during embryonic development. In the mouse embryo, expression of both ZEB1 and ZEB2 starts at E8.5 in the neuroepithelium. While ZEB2 can be detected in the headfolds, neural tube and the prospective neural plate, expression of ZEB1 is restricted to the headfolds (Takagi et al., 1998; Van de Putte et al., 2003). Later, ZEB2 is present at the neural crest, presomitic mesoderm and lens. Furthermore, ZEB2 is strongly expressed during neocortical development in the mouse embryo postmitotic neurons, where it regulates the fate of neuronal progenitor cells (Seuntjens et al., 2009). In addition, ZEB1, together with LSD1, is responsible for repression of growth hormone in lactotrophic cells in the anterior pituitary during brain development (Wang et al., 2007). ZEB1 is also mainly expressed in neural crest derivatives, such as the branchial arches, the cranial ganglia and several craniofacial structures. ZEB1 can also be found in skeletal precursors and limb bud mesenchyme (Takagi et al., 1998). The importance of both ZEB1 and ZEB2 in the above-mentioned tissues is also seen in knockout mice: in ZEB2 null animals, embryonic arrest occurs at E8.5 and defects in neural tube closure and neural crest migration are evident (Van de Putte et al., 2003). On the other hand, ZEB1 null mice have normal neural crest delamination but display major skeletal defects and die perinatally (Takagi et al., 1998). These observations suggest that duplication of the ancestral ZEB in
vertebrates gave rise to two similar proteins, each of which play a distinct role in the developing embryo.

### 1.3.4 The functionality of the ZEB proteins is linked to the cellular context

Most studies have shown that ZEB1 functions as a transcriptional repressor. It negatively regulates E-cadherin, MucI, Pkp3 and several polarity markers (Aigner et al., 2007; Eger et al., 2005; Guaita et al., 2002). However, it has become clear that the cellular context also plays an important role in determining whether ZEB1 acts as a transcriptional repressor or activator. For example, ZEB1 can activate transcription from the ATPase1 promoter in MDCK cells, but in rat fibroblasts it represses it, indicating that several additional cofactors might play a pivotal role in specifying the function of ZEB1 (Watanabe et al., 1993). This is further reflected in the different levels of repression of the E-cadherin promoter by ZEB1 in different cell lines. ZEB1 transfected in MCF-7 and SW480 cell lines can repress the E-cadherin promoter by 50%, but in SW13 and C33a cells the reduction was only 25%. The absence of the ATPase subunit BRG1 in the latter two cell lines has been shown to be responsible for these differential effects (Sanchez-Tillo et al., 2010).

Additional evidence that the functionality of ZEB1 is dependent on the availability of specific cofactors has been found while studying the dynamic regulation of the IL-2 promoter in Jurkat cells. Binding of the C-terminal zinc fingers of wild type ZEB1 to the IL-2 promoter represses transcription (Figure 5A). However, mutation of specific sites in the N-terminal zinc finger cluster prevents the interaction between this finger and the homeodomain and results in derepression of the IL-2 promoter. Intramolecular contact between the zinc finger and the homeodomain might enable specific cofactors to bind to ZEB1 and thereby determine whether activation or repression will take place (Ikeda and Kawakami, 1995). Binding of the C-terminal zinc fingers to the E-box target sequence does not automatically result in transcriptional activation. For example, in rat neuroblastoma and HeLa cells, interaction of the C-terminal fingers to the E-box sequence in the hsp70 promoter results in activation. Inserting the GTTTC/G sequence in combination with the E-box enhances the binding of the N-terminal fingers and leads to
repression of the promoter. Again, this strongly suggests that conformational changes of ZEB1 are responsible for the binding of other activating or repressing factors (Ikeda and Kawakami, 1995).

Figure 5: The function of ZEB transcription factors. (A) Regulation of the interleukin 2 (IL-2) promoter by ZEB1 in Jurkat cells. Binding of the ZEB1 C-terminal zinc fingers to the E-box sequence CACCTGT represses transcription from the IL-2 promoter. This repression is dependent on the interaction of the homeodomain (HD) with the N-terminal zinc fingers, since mutation of these zinc fingers results in transcriptional activation.
Sekido et al. proposed that due to this intramolecular interaction, the ZEB1 conformation perturbs potential transcriptional activators. Adapted from (Sekido et al., 1994). (B) Alternative binding model for both ZEB1 and ZEB2 in which two sequential E-box motifs are required. Neither the orientation nor the spacing of the two motifs is essential for stable DNA binding. (C) Overview of different factors and/or cofactors interacting with the ZEB transcription factors. The binding region where the BRG1 complex, p300, p/CAF, LMO2, Smad3, CtBP and NC2 interact with ZEB1 have been determined. The exact binding locations SRF and the PC2 complex have not been elucidated yet. However, the observation that SRF can bind different transcription factors via their homeodomain suggests that this might also be the case for ZEB1. In addition, PC2 can bind the CtBP corepressor, indicating that this also takes place in a protein complex with ZEB1. Dashed arrows depict the sumoylation capacities of PC2. The dashed line connecting p300, p/CAF and Smad3 points to the fact that Smad3 can interact with ZEB1 only when p300 is present in the complex. Furthermore, interaction with Oct1 is only possible in the presence of the homeodomain in combination with the N-terminal zinc finger.

The working model in which only one E-box site is bound by the ZEB proteins is complemented by the concept that interaction of both ZEB proteins with a specific target promoter is only possible in the presence of two consecutive E-box motifs (Figure 5B) (Remacle et al., 1999). When one of these motifs is mutated, neither of the ZEBs bind and the Xbra2 promoter is derepressed. The orientation and the relative spacing of the E-boxes do not seem to be critical for the DNA-ZEB interaction. It has been postulated that each separate zinc finger cluster binds an E-box sequence in a stable interaction. How one of the two binding modes is selected is not clear, but it might depend on the cell type and/or promoter. This is illustrated by the absence of skeletal defects in mutant mice lacking the C-terminal Zn fingers of ZEB1 (and so ZEB1 can only bind one E-box motif), in contrast to mice lacking ZEB1 expression (Higashi et al., 1997; Takagi et al., 1998).

The importance of the cellular or physiological context is further emphasised by the results of investigations of ZEB2 expression in murine embryos and adults. For instance, two ZEB2 transcripts of 5.3 and 8.3 kb are differentially expressed in adult mouse tissues. The 5.3-kb transcript was located in several organs, including the heart, brain, spleen,
lung, skeletal muscle and kidney, while the 8.3-kb variant was detected only in brain and lung (Liu et al., 2006). The involvement of ZEB2 in different ontogenic processes, such as migration of the neural crest cells and neurogenesis in the developing brain, point to its ability to play different roles in different contexts (Vandewalle et al., 2009).

1.3.5 The ZEB transcription factors are part of a multimeric protein complex in which CtBP plays a pivotal role

The influence of the cellular context on the functionality of the ZEBs implies that an intricate interaction with specific cofactors determines whether ZEB1 functions as an activator or as a repressor. Indeed, several proteins interact with ZEB1 (Figure 5C). For example, Sun et al. discovered that the N-terminal part of ZEB1 is responsible for interaction with the hematopoietic regulator LIM only domain 2 (LMO2). Formation of this complex is necessary for repression of the ZEB1 target genes matrix metalloproteinase 1 (MMP-1) and Vascular Endothelial Growth Factor (VEGF) (Sun et al., 2010). In addition, a short sequence in front of the C-terminal zinc fingers (amino acids 754–902) is responsible for the functional and direct interaction of ZEB1 with the transcriptional repressor Negative Cofactor 2 (NC2). NC2 is a functional inhibitor of the TATA binding protein (Mermelstein et al., 1996). Mutation of N769Q, an N-glycosylation site, can abolish the binding of NC2 and thus terminate the repressive function of the complex (Ikeda et al., 1998). Directly upstream of the NC2 binding region there is a PLDLSL motif (amino acids 712–717) that can bind the corepressor proteins C-terminal Binding Protein 1 and 2 (CtBP1, 2) (Furusawa et al., 1999; Papadopoulou et al., 2010; Postigo and Dean, 1999; Shi et al., 2003). Mutation of this site to PLASSL disrupts the binding between ZEB1 and CtBP1/2 and leads to derepression. This indicates that two other related motifs (745 PLNLSC and 712 PLDLSL) near the PLDLSL sequence do not play a major role in the interaction of CtBP with ZEB1 (Furusawa et al., 1999). However, in contrast with this report, Postigo et al. claimed that all three CtBP binding motifs are involved in ZEB1 mediated transcriptional repression. Only mutating all three sites
simultaneously could prevent the binding of CtBP to ZEB1. Mutation of the three sites also led to full derepression of the SV40 promoter (Postigo and Dean, 1999).

CtBP was discovered as an interaction partner of the C-terminal part of the adenoviral protein E1a (Boyd et al., 1993; Chinnadurai, 2002). Moreover, E1a has been described in the context of tumor initiation and progression (Frisch, 1991; Gallimore and Turnell, 2001). Grooteclaes and Frisch discovered that E1a can induce an epithelial morphology in melanoma cells through its interaction with CtBP. Further experimental work led to the conclusion that formation of the E1a-CtBP complex scavenged CtBP away from its ZEB1 binding domain. So it became clear that the ZEB1-CtBP complex is indispensable for downregulation of the epithelial genes E-cadherin, desmoglein 2 and plakoglobin (Grooteclaes and Frisch, 2000). The functional implication of the ZEB1 dependency on CtBP is further established in a report by Peña et al., who discovered that the inverse correlation between ZEB1 and E-cadherin in colorectal tumor samples was more pronounced with increased levels of CtBP (Peña et al., 2005). The necessity of CtBP for ZEB1 functionality was additionally emphasised in a different study showing that downregulation of the BCL6 gene in germinal centre B cells was dependent on the CtBP interaction domain of ZEB1. Based on all these results, the region spanning amino acids 712–902 can be viewed as a general repression domain of ZEB1, as it is crucial for its interaction with CtBP. Likewise, the importance of CtBP for ZEB2 functionality has also been established. During neurulation in Xenopus, the CtBP-ZEB2 interaction is necessary for upregulation of the neuronal marker Sox2. When a fusion construct comprising CtBP2 and the N-terminal region of ZEB2 (NZF-mCtBP2) was used, neurulation was still able to take place. This further emphasises the importance of CtBP. Depleting CtBP levels in neuronal precursors in vivo abolished neurulation (van Grunsven et al., 2007). Interestingly, induction of the neuronal markers N-CAM (Neural Cell Adhesion Molecule) and NRP (Neuropilin 1) does not seem to depend on the CtBP domain of ZEB2, since deletion of the N-terminal domain could abolish upregulation of these genes. This indicates the necessity of alternative domains in addition to the CtBP interacting domain (CID) for full neuronal regulation (Nitta et al., 2007).

The view that transcriptional repression by ZEB1 and ZEB2 is purely dependent on the interaction with CtBP has been challenged in a report of Van Grunsven et al. These authors observed that the E-cadherin promoter could be derepressed by mutating the
CtBP interaction sites of a peptide comprised of the ZEB2 C-terminal domain fused to the CID (CZF-CID\textsubscript{mut}). Interestingly, when the same CtBP interacting sites were mutated in full length ZEB1 and ZEB2, the E-cadherin promoter was not derepressed. This suggests that other repressing mechanisms independent of CtBP are responsible for the inhibition of the E-cadherin promoter in the full length proteins (van Grunsven et al., 2003). Recent evidence shows that the functionality of ZEB1 as a repressor is not always dependent on its interaction with CtBP. Mutating the CtBP interaction domain of ZEB1 does not fully derepress the E-cadherin promoter in MCF7 and SW480 cell lines, indicating the presence of alternative repressing mechanisms. Sanchez-Tillo et al. have shown that this additional modulation is mediated by the SWI/SNF chromatin-remodeling protein BRG1, which can bind the N-terminal domain of ZEB1. Further proof for the functionality of this novel interaction is the derepression of the E-cadherin promoter by coexpression of the N-terminal part of ZEB1 together with the complete protein, which indicates that BRG1 was scavenged and so was unable to perform its function as a corepressor (Sanchez-Tillo et al., 2010).

The ZEB1-CtBP interaction forms the basis for a much larger and more intricate repression complex. When trying to elucidate by mass spectrometric analysis the molecular basis of CtBP mediated gene modulation, Shi et al. discovered that CtBP can form a complex with ZEB1 in HeLa cells. Additional binding factors identified in this experiment were CoREST, LSD1, LCoR and Pc2 (Shi et al., 2003). Other data suggesting the formation of this complex were provided by Wang et al., who found that LSD1, LCor, Pc2 and CtBP had the same expression pattern as ZEB1 during postnatal development of the murine pituitary. Further analysis showed that a ZEB1-Pc2-CtBP-LDS1-LCoR repression complex binds to the major repressive promoter element of growth hormone \textit{in vivo} (Wang et al., 2007). Interestingly the polycomb protein Pc2 is a SUMO E3 ligase involved in gene silencing (Geiss-Friedlander and Melchior, 2007; Kagey et al., 2003). Furthermore, Pc2 can recruit both the CtBP1 and CtBP2 corepressor to the multimeric PcG (polycomb group) bodies and sumoylate both factors. This post-transcriptional modification on K428 of CtBP1 is necessary for nuclear localisation and hence for its corepressor function. Mutation of K428 to R428 excludes CtBP from the nucleus and results in derepression of the E-cadherin promoter in mouse embryonic fibroblasts (Lin et al., 2003). Moreover, it has been demonstrated that Pc2 can regulate the transcriptional
activity of ZEB2. Repression of E-cadherin transcription was inhibited by a ZEB2 sumoylation null mutant when compared to the wild type. Also coexpression of Pc2 and ZEB2 attenuates ZEB2 driven E-cadherin repression (Long et al., 2005).

Not only CtBP, but also ZEB1 and ZEB2 have been proven to be substrates of Pc2, adding an extra level of regulation to the ZEB complex. Indeed, the protein bands of ZEB1 and ZEB2 in western blots appear higher than the calculated molecular weights. Long et al. have demonstrated that sumoylation is mainly responsible for this shift and identified the 390-IKTE-393 sequence of ZEB2 as a major Pc2 dependent sumoylation site. Strikingly, this site has been conserved in both ZEB1 and ZEB2 throughout evolution, indicating its importance in molecular signalling. Sumoylation of ZEB2 leads to its recruitment in PcG bodies and hence to the loss of its interaction with CtBP (Long et al., 2005). This finding points to the importance of sumoylation in regulation of the functionality of the ZEB proteins and in turn to the control of EMT.

ZEB2 as well can be found in a larger complex (Figure 5C). Affinity purification and subsequent mass spectrometry revealed the association of ZEB2 with the nucleosome remodelling/histone deacetylating NuRD complex. Interestingly, purification of the NuRD complex in other model systems did not identify ZEB2 as an interaction partner, which indicates that the ZEB2-NuRD interaction is context dependent and transient. An additional reason might be that the NuRD interaction motif is not fully conserved in ZEB2 in comparison with other interacting transcription factors, leading to a less stabilized association between ZEB2 and NuRD. Further investigations showed that the N-terminal domain of ZEB2 is responsible for this interaction. This finding provided a molecular mechanism for specific ZEB2 alterations leading to the Mowat-Wilson syndrome, a congenital disease characterised by facial anomalies, mental retardation, heart defects and Hirschprung’s disease. Furthermore, the NuRD member Mi-2β is necessary for repression of the E-cadherin promoter and for neurulation in the *Xenopus* embryo (Verstappen et al., 2008). Additional experiments showed that ZEB2 functionality as a repressor depends on deacetylase activity. Inhibition of HDAC by TSA treatment abrogated ZEB2 mediated neurulation in the *Xenopus* embryo (van Grunsven et al., 2007). Because both the NuRD complex and the cofactor CtBP have been shown to interact with HDAC, the cellular context selecting the proper deacetylase complex remains to be investigated.
1.3.6 The interplay of the ZEB transcription factors with TGFbeta signalling

It is widely accepted that the transforming growth factor beta signalling cascade has a major influence on the induction and maintenance of EMT in various \textit{in vitro} and \textit{in vivo} models. The first report on this subject was by Miettinen et al., who reported that TGFbeta can induce a mesenchymal transdifferentiation in the mammary epithelial NMuMG cell line (Miettinen et al., 1994). Since then, numerous studies have broadened our understanding of how TGFbeta influences EMT in both physiological and pathological situations (Akhurst and Derynck, 2001; Xu et al., 2009). In addition, several downstream mediators of the TGFbeta pathway, the SMAD proteins, can form complexes with the ZEB proteins. As mentioned above, ZEB2 (SIP1) was originally identified as an interactor of Smad proteins. Further experiments demonstrated that ZEB2 can interact with the MH2 domain of Smad1, -2 and -5. Strikingly, only receptor activated Smads seem to be able to bind to ZEB2 (Verschueren et al., 1999). It has furthermore been demonstrated that activated SMAD3 can bind both ZEB1 and ZEB2 in osteoblasts (Postigo et al., 2003). Remarkably, in \textit{Xenopus laevis} SMAD3 seems to directly interact with ZEB2, whereas binding with ZEB1 can only take place when p300 is part of the complex (van Grunsven et al., 2006). Binding of ZEB1 to SMAD3 leads to TGFbeta mediated transcriptional activation, whereas in the case of ZEB2 this results in repression. A model explaining this differential effect is based on the observation that only the N-terminal domain of ZEB1 is able to bind the acyltransferases p300 and P/CAF. The functions of both p300 and P/CAF in this complex are dual. On one hand, interaction with ZEB1 leads to acetylation of the lysine residues located after the CtBP interaction domain and results in displacement of the CtBP corepressor from the complex. On the other hand, p300 and P/CAF are also needed for SMAD3 mediated transcriptional activation (Postigo, 2003). This differential influence of ZEB1 and ZEB2 on TGFbeta mediated transcription is still debated, because knockdown of ZEB1 in TGFbeta-stimulated NMuMG cells results in upregulation of E-cadherin promoter activity. This indicates that ZEB1 can function as a transcriptional repressor in TGFbeta treated cells (Shirakihara et al., 2007). Additional evidence that opposes the differential effect of ZEB1 and ZEB2 in
TGFbeta signalling comes from developmental studies in *Xenopus laevis* in which both ZEB1 and ZEB2 were able to bind to p300 and P/CAF (van Grunsven et al., 2006). The existence of a ZEB1-SMAD3 complex has been confirmed by Nishimura et al., who showed that ZEB1 is a key intermediary of TGFbeta mediated differentiation of vascular smooth muscle cells. This process depends on the formation of a ZEB1-SMAD3 complex, which, together with the transcription factor SRF, is responsible for activating the alpha smooth muscle actin promoter. Interestingly, apart from activating SMAD proteins, TGFbeta signalling can also induce ZEB1 transcription (Lindley and Briegel; Nishimura et al., 2006). This suggests that activation of alpha smooth muscle actin and ZEB1 is SMAD dependent (Nishimura et al., 2006). Additionally, in the TE7 oesophagus carcinoma cell line, TGFbeta treatment leads to strong induction of ZEB2 expression, which subsequently leads to an EMT phenotype (Rees et al., 2005). However, it is clear that not all TGFbeta mediated effects on EMT depend on ZEB1 and ZEB2. For example, when ZEB1 and ZEB2 are both knocked down in NMuMG cells, TGFbeta can still induce the mesenchymal genes fibronectin and N-cadherin, but E-cadherin is no longer downregulated and actin fibres are still formed (Shirakihara et al., 2007). Furthermore, knockdown of ZEB1 in lung epithelial cells only partially diminished the activity of the TGFbeta responsive 3TP promoter upon TGFbeta stimulation (Postigo, 2003).

1.3.7 Conclusions

The ZEB transcription factors play essential roles during different stages of embryonic development. In the mouse embryo, ZEB1 is mainly involved in the further development of the neural crest derivatives and the musculoskeletal system and in selection of T-lymphocytes, whereas ZEB2 is expressed in the neural crest cells and neural tube and is responsible for the fate of neuronal progenitor cells (Takagi et al., 1998; Van de Putte et al., 2003; van Grunsven et al., 2006). Although both ZEB proteins have very similar structures, they have distinct molecular functions. The difference in function might be explained by examining their evolutionary history. Duplication of the ancestral ZEB gene occurred with the appearance of the vertebrates during evolution and suggests the
existence of evolutionary pressure for this event, resulting in the development of a more
complicated body plan that is characteristic of vertebrates. For instance, analysis of the
Smad binding domain (SBD) and homeodomain of both proteins in man reveals only
minor sequence similarity, suggesting distinct sets of functions for the two ZEBs. It is
striking that several important characteristics of the ZEBs emerged only in vertebrates.
For instance, the CtBP interacting domain (CID), Smad binding domain (SBD) and
distinct sumoylation sites are only found in the ZEBs of vertebrates, suggesting that upon
duplication, more intricate levels of regulation became necessary for their correct spatio-
temporal expression. These additional modes of regulation have resulted in generation of
many cofactors that directly interact with both ZEB proteins. Although several nuclear
factors, such as CtBP and the Smad proteins, interact with both ZEB1 and ZEB2, other
factors, e.g. the activating cofactor p300, can only interact with ZEB1 (Postigo and Dean,
1999; Postigo et al., 2003). Other proteins that interact directly with ZEB1 are members
of the CoREST complex, SRF and Nc2 (Ikeda et al., 1998; Nishimura et al., 2006; Shi et
al., 2003; Wang et al., 2007). For ZEB2, a specific interaction with the NuRD repression
complex has been shown (Verstappen et al., 2008). In addition to the physiological role of
ZEB factors, evidence is emerging for their involvement in the development of malignant
cancer. The same mechanisms used by ZEB1 and ZEB2 during embryonic development
are the driving forces behind the pathological onset of EMT in carcinogenesis (Berx et al.,
2007). Understanding the different modes of actions that form the basis of the spatio-
temporal regulation of both ZEBs will provide deeper insight into the complex
mechanisms that are responsible for the malignant behaviour of cancer cells. An
alternative approach for the treatment of carcinomas could be modulation of the
interaction partners of ZEB proteins in order to manipulate ZEB functionality.
The molecular basis for epithelial mesenchymal transition

1.4.1 Introduction

The establishment of the complex body plan that arises during metazoan development, requires the appearance of a second major cell type next to the epithelium. These cells are referred to as mesenchymal cells and stem directly from the primitive epithelium. In contrast to epithelial cells, mesenchymal cells display an elongated morphology, only form focal contacts with neighbouring mesenchymal cells and have the capacity to invade as single cells or as sheets through extracellular matrices of both epithelial or mesenchymal origin (Hay, 1995). This epithelial mesenchymal transition (EMT) can be observed during major events of tissue remodeling such as mesoderm formation during gastrulation, neural crest development, formation of the heart valve and the secondary palate (Yang & Weinberg, 2008).

The earliest EMT during embryonic development can be observed at the first establishment of the mesoderm during gastrulation. The induction of the mesoderm starts at a specific site in the primitive ectoderm termed the primitive streak in birds and mammals and the ventral furrow in Drosophila. The mesoderm arises when epithelial cells of the primitive streak undergo an EMT and as such are able to migrate underneath the existing ectoderm. The first stages of this EMT are characterized by a local invagination of epithelial ectodermal cells at the primitive streak, which is accompanied by the loss of apico basal polarity of the cells. Subsequently, these cells locally degrade the ectodermal basal membrane and ingress as sheets underneath the existing ectoderm. Organs and tissues that later develop from the mesoderm are muscle, bone and gut mesenchyme (Baum et al, 2008). The development of the mesoderm is tightly regulated at a spacio-temporal level by several major signaling pathways, including Nodal (via activin and Vg1), fibroblast growth factor (FGF), canonical Wnt and bone morphogenetic protein (BMP) (Kimelman, 2006).

The generation of the neural crest (NC), which is restricted to the development of vertebrates, is another event during embryogenesis where EMT plays a pivotal role. The
NC is a migratory, multipotent cell population that gives rise to several highly specialized types of tissue. Examples are peripheral neurons and glia, melanocytes and craniofacial structures (Scherson et al, 1993). The presumptive NC cells arise at the boundary between the neural plate and the epidermal ectoderm. With the progression of neurulation, the NC precursors are incorporated at the neural folds and the dorsal side of the neural tube, from which they will subsequently migrate. This migratory phenotype is accomplished by the loss of cell-cell adhesions and cytoskeletal rearrangements allowing the delamination from the neuroepithelium. Furthermore, modulation of matrix-metalloproteinases, cell surface receptors such as FGFR, integrines and other transmembrane receptors are observed during EMT these cells are undergoing (Sauka-Spengler & Bronner-Fraser, 2008a). From the moment NC cells arrive at their destination, they tend to self-aggregate during the first stages of terminal differentiation (Le Douarin & Kalcheim, 1999). This indicates the plastic nature that exists between epithelial and mesenchymal cells.

Although EMT is thus a pivotal mechanism at the cellular level in order to obtain a complex organization of tissues and organs in metazoan organisms, aberrant activation of the EMT program often leads to the development and progression of disease. In particular, recent evidence is emerging that EMT is able to contribute to the advance of carcinoma. One of the key features of EMT, both during mesoderm formation and NC delamination, is the decreased expression of the intercellular adhesion protein E-cadherin (Butz & Larue, 1995; Takeichi, 1991). Strikingly, loss of E-cadherin is considered to be a marker for low differentiation and poor prognosis during cancer development. The inverse correlation that thus exists between EMT in physiological conditions and expression of E-cadherin might thus be indicative for an emerging role of EMT in the frame of malignant cancer progression.

1.4.2 Mechanisms that lead to impaired E-cadherin functionality during cancer progression

Together with its function as an intercellular adhesion protein, the broad spectrum of signaling pathways that are modulated by E-cadherin suggest that the stability of the adherens junctions is crucial for the maintenance of the epithelial phenotype and
homeostasis of tissues. During the process of malignant cancer formation, several mechanisms have been described that impair the functionality of E-cad. These include mutations, epigenetic and transcriptional silencing, increased endocytosis and proteasomal degradation (Berx & van Roy, 2009).

The first inactivating mutations of E-cadherin were discovered in diffuse type gastric cancer or cancer with both diffuse or intestinal components. However, E-cadherin mutations have not been reported in the intestinal type of gastric cancer. In all found variants, exons were skipped that encoded for calcium binding regions and as such severely compromising the function of E-cadherin as an adhesion protein. In addition, the same type of alterations were found in tumor cells infiltrating the lymph nodes (Becker et al, 1993). Next to their presence in diffuse gastric carcinoma, E-cadherin mutations have also been characterized in lobular breast carcinomas. The predominant mutations occurring in this type of cancer give rise to a truncated form of the E-cadherin protein whereby the mutations are scattered throughout the whole extracellular domain (Berx et al, 1998). Furthermore, mutations of E-cadherin have been described in metastatic prostate cancer, in particular a novel S270A missense mutation in exon 6 (Ikonen et al, 2001). Apart from inactivating mutations, methylation of the 5' CpG islands in the E-cadherin promoter has been described. More specifically, a progressive increase in E-cadherin promoter hypermethylation has been shown to occur during the development of ductal breast carcinoma. Analysis of tumors during the stage of carcinoma in situ revealed that hypermethylation occurred in 30% of the lesions, while this corresponded to nearly 60% in metastatic lesions (Nass et al, 2000). Apart from ductal breast carcinoma, the correlation between promoter hypermethylation and E-cadherin dysfunctionality has been described for carcinoma of the prostate, oral cavity, colon and rectum (Chang et al, 2002; Graff et al, 1995; Kanazawa et al, 2002).

The nature of hypermethylation during carcinogenic progression seems to fit in the frame of a larger change in gene expression. Genome wide expression analysis in cancer cell lines displaying E-cadherin promoter hypermethylation or carrying inactivating E-cadherin mutations revealed hypermethylation occurred together with large changes in gene expression, while this was not the case for cells bearing E-cadherin mutations. Moreover, cells with a methylated E-cadherin promoter had a fibroblastic morphology, while cells with mutated E-cadherin still were epithelial like. These findings strongly
suggest that promoter hypermethylation of the E-cadherin promoter is part of cellular reprogramming that results in an epithelial mesenchymal transition (EMT) (Lombaerts et al, 2006).

1.4.3 Regulation of EMT at the transcriptional level

The expression of specific EMT inducing transcription factors forms the basis of a complete cellular reprogramming that allows epithelial cells to make a transition towards a mesenchymal phenotype (Figure 6 and table 1). The transcription factor Snail was the first protein described having EMT inducing capacities. Since its discovery, more then 20 members of the Snail family have been described in metazoan organisms (Barrallo-Gimeno & Nieto, 2005). Already from the beginning of the 1990s the spacio-temporal expression of Snail during mesoderm formation in the *Drosophila melanogaster* embryo had been observed (Alberga et al, 1991). The first suggestion of the pivotal role for the Snail genes in the process of EMT came form a report studying the effects of Slug inhibition on mesoderm formation in the chick embryo (Nieto et al, 1994). However, it was Snail that was first identified as a marker of malignancy during cancer progression by showing that the cell-cell adhesion molecule and marker for differentiation, E-cad, could be transcriptionally repressed by Snail. This effect is accomplished by the binding of Snail at specific sequences, designated as E-boxes (CACCTG), in the proximal promoter of E-cadherin (Cano et al, 2000b; Rodrigo et al, 1999). Moreover, cells overexpressing Snail obtained a fibroblastic morphology and subsequently gained migratory and invasive capacities (Cano et al, 2000b). Gene expression profiling indeed revealed that inducing Snail in epithelial cell lines leads to complete reprogramming of the cell, bringing them into a mesenchymal like state. Apart from E-cad, other proteins necessary for the establishment of cellular junctions such as members of the claudin family, plakophilin 2 and epithelial membrane protein 1 (EMP1) are significantly downregulated by Snail. In addition, proteins that regulate the constitution of the actin cytoskeleton, including Epithelial Protein Lost In Neoplasm β (EPLIN), gelsolin (GSN) and Capping Protein Gelsolin like (CAPG), were also expressed to a lesser extent upon Snail induction (De Craene et al, 2005).
Figure 6: General overview of the mechanisms leading to E-cadherin disfunctionality. Enhanced internalization through endocytosis, mutations and transcriptional silencing are the main mechanisms leading to inhibition of E-cadherin functionality. When comparing tumor samples with E-cadherin mutations with samples which lack E-cadherin due to transcriptional repression, it has been found that the latter condition is associated with a mesenchymal like reprogramming (EMT) of the cells. Several EMT inducing transcription factors, eg. ZEB1, ZEB2, Snail, Slug and Twist have been described and shown to be in a reciprocal functional interaction loop with several signalling pathways such as the TGFbeta, Notch, MAPK, NFkB and Wnt cascades. TF: transcription factors.
The discovery of Snail as an EMT inducer and direct modulator of E-cadherin was followed by a report of Comijn et al. whereby the transcription factor ZEB2 was also identified as a direct repressor of E-cadherin and EMT inducer (Comijn et al, 2001). In addition, its family member and close homologue, ZEB1, was shown to have similar capacities (Eger et al, 2005). Since then, several alternative EMT inducing factors have been described. These include members of the bHLH family of transcription factors, such as the E12/ E47 isoforms of the TCF3 gene, the E2-2A/ E2-2B isoforms of the TCF4 gene and TWIST1/2 (Peinado & Cano, 2008). Strikingly, in parallel with Snail, initial studies have shown that these factors might also be implicated in malignant cancer progression (Slattery et al, 2008; Sobrado et al, 2009a; Yang et al, 2004). This abundance of EMT inducing transcription factors might suggest that a significant functional overlap exists between these proteins. However, in vitro cDNA micro-array analysis of MDCK cells expressing Snail, Slug and E12/ E47 show that, apart from an existing overlap, almost 70% of the modulated genes seems to be linked to a specific transcription factor. This indicates that different EMT inducing transcription factors are able to induce common and specific EMT related programs and hence might be responsible for a differential role in malignant cancer progression (Moreno-Bueno et al, 2006).

Some scepticism however still exists as to whether EMT has the potential to be a driving force for malignancy in developing carcinomas. The basis of this debate lies in the fact that a real conversion at the morphological level has not been extensively documented and is rarely observed in tumours that have undergone a transition from a carcinoma in situ towards an malignant lesion. Even in the most advanced stage IV lesions with systemic metastases, clear evidence of a stable mesenchymal conversion is rarely seen. In addition, it might be expected that metastases that have arisen via the process of EMT, display several properties of mesenchymal like cells. This does not seem to be the case, since in most cases secondary tumours share similar histopathologic properties with their primary origin (Lee et al, 2006; Tarin, 2005; Thompson & Newgreen, 2005). These findings have urged the oncologist field to re-orientate the role of EMT in carcinoma progression (Savagner).

Compelling evidence of EMT occurring in an in vivo context has been gathered during recent years. The criticism that cells are unable to display plasticity regarding their
epithelial and mesenchymal characteristics (Tarin, 2005) has been elegantly disproved by Trimboli et al., whereby the authors developed an in vivo lineage tracing model. By making use of a the MMTV-myc transgenic mouse tumor model carrying a LacZ reporter construct under the control of WAP-Cre, the origin of stromal cells surrounding the primary tumour could be traced back to the epithelial cells of the tumour (Trimboli et al, 2008). In humans, one of the first reports showing a relation between malignant cancer progression and EMT in vivo came from Blanco et al. When looking at breast carcinoma, the authors showed that Snail expression is inversely correlated with grade of differentiation and E-cadherin expression. Moreover, Snail was shown to be overexpressed in infiltrating ductal carcinoma (IDC) (Blanco et al, 2002). Other types of tumours where expression of Snail is inversely correlated with reduced E-cadherin expression and/or stage are synovial sarcoma (Saito et al, 2004), head and neck squamous cell carcinoma (Yang et al, 2007), endometrial cancer (Blechschmidt et al, 2007) and colon cancer (Pálmer et al, 2004; Roy et al, 2005). In addition to Snail, the transcription factor Twist1 seems to be implicated in in vivo EMT. Micro-array analysis on human breast carcinoma samples revealed that Twist1 expression clustered together with invasive lobular carcinoma (ILC). Interestingly, a negative correlation could be observed between Twist1 expression and levels of E-cadherin (Yang et al, 2004).

Also for the members of the ZEB family, a link with the in vivo occurrence of EMT has been observed. Persuasive evidence was delivered by investigating the mechanisms used by colorectal adenoma to form metastases. While in the bulk of the tumour, the basal membrane remained intact, this was not the case in certain sites at the edge of the tumour. Breaching of the basal membrane integrity was shown to be the result of decreased expression of basal membrane constituents, in particular members of the laminin family. Moreover, cells that were capable of disturbing this barrier were shown to be overexpressing ZEB1, which could not be detected in the cells of the tumour bulk (Spaderna et al, 2006). In addition to these findings, ZEB1 was also found to be overexpressed in high grade endometrial carcinoma, while this was not the case in low grade lesions. Moreover, ZEB1 expression was inversely correlated with E-cadherin expression (Singh et al, 2008a; Spoelstra et al, 2006).
<table>
<thead>
<tr>
<th>EMT inducing TF</th>
<th>General target genes</th>
<th>Associated cancer type</th>
<th>Clinico-pathological features</th>
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<td>Snail</td>
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<td>Lymphnode metastasis, distant metastasis, tumor recurrence</td>
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<td>Slug</td>
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<td>Metastasis and recurrence</td>
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<td>Melanoma</td>
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Table 1: General overview of the main different EMT inducing transcription factors and their associated clinico pathological features.
1.4.4 Signaling pathways that lead to induction of EMT

1.4.4.1 TGFβ signaling

One of the best studied pathways that was already early identified as having EMT inducing capacities during embryologic development, is the TGFβ signaling pathway. Indeed, a multitude of TGFβ like ligands have been shown to be necessary for correct embryonic development. For instance, members of the Nodal/activin and BMP subfamily are responsible for the generation of the dorsal ventral body axes and the subsequent patterning of tissues across these axes during embryogenesis. In addition, Nodal is also required for the induction of the three germ layers in vertebrates (Wu & Hill, 2009). Clear evidence that the TGFβ pathway is a major inducer of a mesenchymal transition can be found in the development of the heart valves. Invasion of endocardial cells that originate from the atrioventricular canals into the heart cushion is a key step in the formation of heart valve structure. A distinct role for the TGFβ-2 and -3 ligands has been observed during this process. Indeed, while TGFβ-2 binds to the type I receptor and activates the endothelial cells by inducing cell separation, it is the TGFβ-3 ligand that is necessary for the subsequent invasion of the cells into the heart cushions (Mercado-Pimentel & Runyan, 2007). These findings were also found by the generation of TGFβ-2 knock out mice, showing severe heart abnormalities, including valve deformations (Sanford et al, 1997).

Furthermore, the EMT initiated during palatogenesis also seems to be dependent on TGFβ signaling. The formation of the palate during embryonic development occurs when the two opposing palatal shelves grow towards each other beneath the nasal septum up to the point where they make contact with each other. At this point, the medial edge epithelium (MEE) becomes receptive to EMT inducing signals, resulting in the fusion of the two originating plates into the mature palate (Nawshad et al, 2007). By adding oligonucleotides or neutralizing antibodies targeting different TGFβ family members to murine palate organ cultures, TGFβ-3 was identified as the key signaling ligand responsible for palate fusion. Inhibition of TGFβ-3 always led to a cleft palate phenotype,
which could be restored by supplementing the palate culture with recombinant TGFβ-3. Interestingly, fusion of the two palatal shelves did not seem to be dependent on TGFβ-1 and -2, indicating the highly specific nature of this process (Brunet et al, 1995). Moreover, during palatal fusion, expression of TGFβ-3 is highly upregulated, subsequently resulting in the downregulation of E-Cadherin (Nawshad et al, 2007).

In parallel, numerous in vitro studies have confirmed that the TGFβ pathway is a major inducer of EMT. One of the first reports claiming this biological role came from Miettinen et al., whereby stimulation of murine NMuMG cells with TGFβ indeed resulted in a mesenchymal phenotype. This was accompanied by the loss of E-cad, ZO-1, desmoplakin I and II and upregulation of the mesenchymal marker fibronectin. This furthermore resulted in a complete reorganization of the vimentin intermediary filament network, which was reflected in the formation of fibres across the cell (Miettinen et al, 1994). Since then, numerous in vitro and in vivo studies have been conducted to deepen the understanding of TGFβ induced EMT, which has led to the elucidation of the canonical TGFβ pathway (Oft et al, 1996; Piek et al, 1999; Portella et al, 1998; Zavadil & Böttinger, 2005).

TGFβ signaling occurs via a heteromeric cell surface complex of two types of transmembrane serine/ threonine kinases, designated as type I and type II receptors. As of today, three type I receptors are known, while only one for the type II has been reported. After binding of the TGFβ ligand to the receptor complex, the type II receptor (TGFβRII) will phosphorylate its type I counterpart (TGFβRI). This subsequently leads to autophosphorylation of TGFβRI and phosphorylation of downstream mediators. When considering the canonical pathway, the main downstream effectors are Smad-2 and -3. Both Smad2/3 are able to form a transient interaction with the autophosphorylated TGFβRI, which is stabilized by the Smad Anchor for Receptor Activation protein (SARA). Smad-2 and -3 are as such phosphorylated on their C-terminals by the receptor and subsequently dissociate into the cytoplasm to form a heterotrimeric complex comprising two receptor activated Smads and the regulatory Smad-4. This complex is then shuttled to the nucleus where binding to specific DNA sequences results in the modulation of different TGFβ target genes (Akhurst & Derynck, 2001). In addition, Smad-6 and -7 are known to have an inhibitory effect on TGFβ signaling, which is accomplished by competitive binding to the TGFβRI. As such, the effector Smad2/3 are
unable to bind to the receptor, preventing their phosphorylation. TGFβ signaling is known to induce Smad7, providing the existence of a negative feed-back loop (Shi & Massagué, 2003).

Although Smad-2 and -3 share 92% sequence identity, they do not seem to be functionally equivalent. For example, several promoters have been found to be responsive to TGFβ signaling, including PAI-1 and JunB. Transcription of these genes is activated exclusively by the Smad-3/Smad-4 complex, which directly binds to CAGA-like sequences in the proximal promoter areas. However, these CAGA motifs do not seem to confer any binding affinity towards the Smad-2/Smad-4 complex. These differences might be explained by an additional insert at the N-terminal domain of Smad-2 immediately before the DNA binding hairpin (Shi et al, 1998). Additional mechanisms that give rise to TGFβ signaling specificity can be explained by differential interaction of the Smads with different transcription factors. Indeed, the transcriptional modulators CBP/p300 have been found to act as activating elements by directly binding to the C-terminal activation domain of both Smad-2 and -3 (Shen et al, 1998). Furthermore, activation of the collagenase I promoter by Smad-3/Smad-4 can only occur in the presence of the c-Jun/c-Fos binding to a proximal AP-1 binding site (Zhang et al, 1998). Cooperation of the Smad-2/Smad-4 complex with the transcription factor FAST-1 or FAST-2 is necessary for activation of the Mix.2 and goosecoid promoters, respectively (Chen et al, 1997; Labbé et al, 1998). Next to transcriptional activation, TGFβ signaling can in addition lead to repression. For example, Smad-2 is able to bind to the homeodomain containing repressor TGFIF. Interaction of these two factors lead to the recruitment of HDACs via Sin3a and CtBP co-factors, subsequently resulting in repression of the Mix.2 promoter (Wotton et al, 1999). Additional co-repressors that are able to form a complex with the Smads are Evi-1, c-Ski and SnoN (Akhurst & Derynck, 2001) (Figure 7).

These findings add an extra level of complexity to the seemingly uncomplicated canonical TGFβ pathway. Furthermore, recent findings strongly point to the fact that TGFβ signaling is capable of functionally interacting with several other pivotal cellular pathways. For example, cross talk has been shown to exist with the Ras-MAPK pathway. TGFβ has been shown to rapidly activate Ras, ERK1/2 and Sapks/JNKs within 3-5 minutes after TGFβ addition to HEK293T cells (Edlund et al, 2003). In addition, TGFβ is
able to generate a positive feedback loop, leading to the production of additional TGFβ. An activated Ras-MAPK pathway has been shown to be indispensable for this effect (Yue & Mulder, 2001). When looking to signaling of BMP, a member of the large TGFβ family of ligands, phosphorylation of Smad-1 is almost completely inhibited when transfecting cells with dominant negative Ras or treating them with Ras inhibitors (Yue et al, 1999). In contrast to these findings, oncogenic Ras activation has also been found to inhibit TGFβ mediated signaling by preventing the shuttling of Smads into the nucleus in epithelial lung and mammary cancer cells. This is accomplished by the fact that oncogenic Ras is able to phosphorylate four Erk kinase sites (PXS/TP) in the linker region of Smad-1 (Kretzschmar et al, 1999). How exactly the distinction between activation or repression of the TGFβ pathway by Ras occurs, remains to be elucidated. In addition to displaying crosstalk with the Ras-MAPK pathway, TGFβ signaling seems to functionally interact with the p38 MAPK/ JNK cascade. Indeed, activated JNK is known to enhance Smad phosphorylation upon TGFβ stimulation. Interestingly, the transcription factors c-Jun and ATF2, both implicated in guiding the specificity of TGFβ signaling at the promoter level of target genes via cooperation with Smad-3, are known substrates of JNK (Hocevar et al, 1999). TGFβ signaling interaction can be further extended towards the small GTPases RhoA and RhoB as being a positive mediator of their stability. Apart from this effect, TGFβ signaling seems to be strongly dependent on RhoA activity in specific cellular contexts in order to exert its function as an EMT inducing pathway. Indeed, treating NMuMG cells with TGFβ and subsequently transfecting them with the inhibitory Smad-7 or dominant negative Smad-3 only results in a minimal decrease of EMT related characteristics. On the contrary, inhibiting RhoA in these cells completely inhibits the EMT inducing potential of TGFβ (Bhowmick et al, 2001). Activation of RhoA seems to take place directly via the TGFβRI, since RhoA activation does not occur in cell lines lacking this receptor (Azuma et al, 1996). The influence of the TGFβ pathway on general cell signaling is further reflected in the fact that extensive interaction with WNT signaling exist. As exemplified during formation of the mouse palate, TGFβ3 is able to induce the transcription factor LEF-1. Apart from this upregulation, TGFβ3 signaling in addition results in activation of LEF-1, which is also the case when an interaction with β-catenin occurs in the nucleus.
Figure 7: **Overview of TGFβ signalling.** Depending on which Smad complexes are activated upon TGFβ ligand binding to its receptor, several downstream responses can be elicited by binding of these complexes to additional transcription factors. In addition to the canonical Smad signalling, TGFβ has been shown to activate several other pathways, including WNT, Ras, Rho and ERK signalling. TA: Transcriptional activation.

In extension to these findings, also the Smad-3/Smad-4 complex is able to form an interaction with LEF-1 and has been shown to be necessary for the transcription of Twin (XTwin) in *Xenopus*. Deleting specific Smad or LEF-1 binding sites in the proximal
promoter of Xtwin resulted in an abrogation of upregulation (Labbé et al, 2000). Interaction between Smad-4 and LEF-1 has been further confirmed in vivo during the formation of Spemann's organizer in the Xenopus embryo (Nishita et al, 2000). Furthermore, in Mv1Lu mink lung epithelial cells, TGFβ signaling has been shown to be responsible for the degradation of APC. Since APC is necessary for degradation of cytoplasmic β-catenin in the absence of WNT signaling, TGFβ signaling leads to increased levels of β-catenin. A functional effect of these findings seems to be that these higher β-catenin levels enhance the TGFβ mediated G1 cell cycle arrest (Satterwhite & Neufeld, 2004).

1.4.4.1 The role of TGFβ during cancer progression

The fact that TGFβ signaling is able to directly or indirectly influence a multitude of crucial signaling pathways, resulting in an EMT phenotype in specific models in vitro and in vivo, may explain the dual role TGFβ plays during cancer progression. It was indeed originally reported that TGFβ signaling led to potent growth inhibition in a multitude of cell types (Coffey Jr et al, 1988). Moreover, treatment of murine lymphoma cells led to the induction of apoptosis (Perlman et al, 2001). These findings led to the assumption that TGFβ was a pivotal tumor suppressor. However, treatment of mammary adenocarcinoma cells with TGFβ resulted in an increased invasive and metastatic potential (Welch et al, 2000). In parallel with these data, inhibition of TGFβ signaling in an MDA-MB231 xenograft model led to a decrease in the formation of bone metastases and increased TGFβ1 expression was found to be associated with malignant colorectal carcinoma progression (Tsushima et al, 1996; Yin et al, 1999). This apparent discrepancy mediated by TGFβ signaling can be explained by the fact that during cancer progression the growth inhibiting properties of TGFβ can be canceled out. For example, continuous stimulation of the MAPK cascade has been shown to ablate the growth suppression of TGFβ. As such, its functionality as tissue homeostasis controlling factor is lost, while the EMT modulating properties of TGFβ remain intact (Kretzschmar et al, 1999).

Evidence that the ZEB proteins are necessary for maintaining the functionality of TGFβ signaling was delivered by showing that the differentiation of vascular smooth muscle
cells during the development of the murine vasculature was highly dependent on the formation of a ZEB1/Smad-3/SRF multimeric complex (Nishimura et al, 2006).

Apart from the transcription factors of the Snail and ZEB family, also Twist seems to be implicated in TGFβ signaling. Mutations in the Twist gene are the cause of the Saethre-Chotzen syndrome, which is characterized by the presence of a cleft palate (Kress et al, 2006). Since a clear link between the TGFβ pathway and the formation of the palate has been established, a connection with Twist was soon found. Indeed, Twist expression is elevated in the palatal mesenchyme and is relatively fast restored to undetectable levels just after fusion of the palatal shelves has taken place. Silencing of Twist in in vitro tissue cultures resulted in a delayed fusion, while using a TGFβ3 neutralizing antibody prevented the upregulation of Twist and perturbed the fusion of the shelves (Yu et al, 2008). This induction of Twist by TGFβ seems to be mediated by the High Mobility Group A2 (HMGA2) protein (Thuault et al, 2006).

1.4.4.2 Wnt signalisation

The Wnt pathway is a highly conserved signaling cascade shared between both vertebrates and invertebrates that plays an indispensable role in the embryonic development and tissue homeostasis. This is exemplified in Wnt loss of function mutation models. For instance, in the mouse embryo, loss of Wnt-1 results in severe defects during brain development. Furthermore, Wnt-4 and Wnt-7a loss of function leads to defects in the kidney and a strong ventralization of the limbs, respectively (Cadigan & Nusse, 1997). The first notion of the Wnt signalisation was reported in a study of the mouse mammary tumor virus (MMTV), whereby it was found that tumor initiation was strongly enhanced by the preferential integration of the virus in the Integration 1 (Int1) gene, leading to its subsequent activation (Nusse et al, 1984). In parallel, it was discovered that Int1 was the homologue of the Drosophila gene Wingless (Wg), which controls segment polarity during larval development (Nüsslein-Volhard et al, 1984a; Nüsslein-Volhard et al, 1984b). The designation of the Wnt gene was based on the concatenation of Wg and Int1.

To date, three distinct pathways seem to be activated upon Wnt receptor activation, namely the canonical Wnt/β-catenin cascade, the noncanonical planar cell polarity
pathway and the Wnt/ Ca\textsuperscript{2+} pathway (Clevers, 2006). The most elaborately studied aspect of Wnt mediated receptor activation in the frame of EMT is the canonical form of the pathway. In the absence of Wnt signaling, the cytoplasmic form of $\beta$-catenin is phosphorylated by a multimeric protein complex, comprising axin, glycogen synthase kinase 3$\beta$ (GSK3$\beta$), adenomatous polyposis coli (APC) and CK1$\alpha$. The kinases CK1 and GSK3$\beta$ subsequently phosphorylate $\beta$-catenin near its N-terminus, leading to the recognition of the phosphorylated protein by the F box repeat protein $\beta$-TrCP. As this latter is a component of an E3 ubiquitin ligase complex, phosphorylated $\beta$-catenin is ubiquitinated and subsequently degraded by the proteasome. However, in the presence of a Wnt ligand, this destruction is prevented (Clevers, 2006). Binding of Wnt ligands to the members of the Frizzled (Fzd) receptor family results in the recruitment of the lipoprotein-related receptor proteins -5 and -6 (LPR-5 and -6), functioning as a co-receptor. This newly formed complex binds Dishevelled, leading to the phosphorylation of LPR-5/-6 by the membrane bound kinase CK1$\gamma$, allowing axin to bind and subsequently eliminating the destruction complex. As such, levels of cytoplasmic $\beta$-catenin can be sustained at a high level. This permits $\beta$-catenin to be translocated to the nucleus, where it interacts with the transcription factor LEF-1, as such forming a transcriptional activation complex. In the absence of $\beta$-catenin, LEF-1 is bound to Groucho in a complex that is unable to transactivate target genes (Heuberger & Birchmeier, 2010). Examples of Wnt target genes are Snail, Twist, fibronectin and MMP-7. For an exhaustive list of target genes, the “Wnt homepage” can be consulted (Yee et al). Apart from activating Wnt members, three classes of antagonizing secreted factors exist: secreted Frizzled-related protein (sFRP) family, Dickkopf (Dkk) family, and Wnt inhibitory factor 1 (WIF1). Binding of these proteins to the Fzd or LPR receptors results in internalization via endocytosis, as such disturbing any existing Wnt signalisation (Glinka et al, 1998; Hoang et al, 1998; Leyns et al, 1997).

The connection between the Wnt pathway and EMT has been made in several reports. Overexpression of the transcription factor LEF-1 in epithelial DLD-1 cells results in a significant increase in nuclear $\beta$-catenin and subsequent induction of EMT. However, when MDCK epithelial cells were treated with Wnt-1, a less pronounced EMT phenotype could be observed (Kim et al, 2002). Transfection of the bladder carcinoma NBT II cell line with stabilized $\beta$-catenin resulted in an increased migratory potential in vitro (Müller
et al, 2002). The link between EMT and Wnt has been further emphasized by the fact that downregulation of GSK3β activity can lead to induction of Snail1 (Katoh, 2006). Increased levels of Snail1 expression can also be detected in the region of the primitive streak of embryoid bodies and has been shown to be the direct consequence of local activation of the Wnt pathway. Performing an ectopic Dkk1 treatment could inhibit this effect (ten Berge et al, 2008). Wnt signaling also has been described to modulate EMT occurring during heart cushion formation. As described in the previous section, one of the drivers that are responsible for this process is TGFβ3. Remarkably, an interplay seems to exist between the TGFβ pathway and β-catenin/LEF-1 signaling, since mice that are deficient in endocardial β-catenin display a significantly reduced formation of the heart cushions. Using a Wnt reporter mouse, it was further established that heart cushion formation was extensively accompanied by β-catenin/LEF-1 signaling (Liebner et al, 2004).

The transcription factor Twist1 has also been described to be upregulated upon Wnt-1 stimulation of murine mammary carcinoma cells. Moreover, in mammary tumors of the transgenic Wnt-1 mouse, 70% showed a significant enrichment for Twist1 (Howe et al, 2003). In addition, a connection between EMT and prostate cancer has been shown to exist. The majority of invasive prostate cancer cell lines shows low levels of the Wnt inhibitor WIF1 due to promoter hypermethylation. However, ectopic overexpression of WIF1 results in downregulation of Slug and Twist1. This was further reflected in xenograft experiments, whereby PC3 cells overexpressing WIF1 showed high levels of E-cadherin, while vimentin was significantly reduced (Yee et al). The significance of Wnt signaling during the progression of malignant cancer is further exemplified by the fact that tamoxifen resistant MCF-7 cells display an increase in nuclear β-catenin. Strikingly, these cells have reduced levels of E-cadherin, have lost the ability to form cell cell contacts and show higher rates of in vitro migration and invasion (Hiscox et al, 2006).
Figure 8: **Overview of the influence of Wnt signalling on EMT inducing transcription factors.** A positive feed-back loop seems to exist between Snail and Wnt5a signalling. Furthermore, binding of ZEB2 to the Sfrp1 promoter, an inhibitor of Wnt signalling, results in repression. In addition, in prostate cancer cells, the promoter of the Wnt inhibitor WIF1, is hypermethylated, leading to transcriptional repression. Overexpression of WIF1 results in the upregulation of E-cadherin and downregulation of Twist and Slug in PC3 cells.

Apart from being able to directly induce EMT modulating transcription factors, a reciprocal interaction between EMT and Wnt signaling seems to exist. In A431 squamous carcinoma cells for example, ectopic expression of Snail1 results in the upregulation of the Wnt-5a ligand. This subsequently leads to expression of ZEB1, further establishing the EMT phenotype. However, the connection between Wnt signaling and EMT seems to be highly specific, since overexpression of Snail in A431 cells also leads to decreased expression of the Wnt-4a ligand (Taki et al, 2003). In extension to this, ZEB2 is also able
to positively modulate Wnt signaling. In the hippocampus region of the mouse brain, ZEB2 directly binds to the promoter of the Wnt inhibitor Sfrp1 (Miquelajauregui et al, 2007). A general overview of the different signaling pathways that contribute to the establishment of an EMT phenotype is given in Figure 9.

1.4.4.3 Growth factor mediated EMT

One of the first discoveries that growth factors are able to impose a partial or full mesenchymal transition on epithelial cells was delivered by the report that human embryonic lung fibroblast secreted Scatter Factor (SF), which enhanced the mobility of MDCK cells. In addition to this, Scatter Factor also seemed to enhance DNA synthesis in hepatocytes. In a reciprocal way, Hepatocyte Growth Factor (HGF), also was found to induce migratory capacities in epithelial cells. Conversely, adding an anti-HGF antibody to the conditioned medium of human lung fibroblasts canceled this effect and as such revealed that HGF and Scatter Factor (HGF/SF) were actually identical (Furlong et al, 1991; Konishi et al, 1991). These findings were later confirmed in the bladder carcinoma cell line NBT-II where treatment of FGF led to a full EMT, reflected in the dissociation of cell contacts, a transition from keratin IF's to a vimentin IF network and induction of cell motility (Vallés et al, 1990). The receptor for FGF/ SF was found to be the c-Met proto-oncogene, which had already been found to be implicated in the onset and progression of malignant cancer. Indeed, c-Met is frequently overexpressed in several types of carcinomas and is a marker for poor prognosis (Birchmeier et al, 2003). Binding of FGF to c-Met results in the activation of a multitude of downstream effectors, including PI3K, STAT, Ras, ERK pathways and the pro-migratory GTPases (Naran et al, 2009). Furthermore, c-Met activation has also been shown to induce and stabilize nuclear β-catenin in hepatocytes, indicating an additional role for c-Met in malignant cancer progression (Monga et al, 2002). Activation of these pathways upon FGF stimulation has been shown to result in elevated levels of Snail and Slug. Indeed, the scattering phenotype that is observed in HGF treated MDCK cells is the direct consequence of Egr-1 dependent Snail upregulation, since knocking down Snail could completely inhibit this effect (Grotegut et al, 2006).
Apart from the TGFbeta signalling cascade, also the WNT-, Notch and NF-κB pathway have been shown to contribute to the establishment of EMT. In addition, several growth factors including HGF, EGF and IGF are able to induce an mesenchymal transition. Activation of these pathways results in upregulation of several EMT inducing transcription factors.
In addition, the transcription factor Slug is upregulated in MDCK cells that undergo a partial EMT during HGF induced tubulogenesis in vitro and is furthermore necessary for the survival of these cells (Leroy & Mostov, 2007). Next to Snail and Slug, HGF has been shown to induce ZEB1 in hepatocarcinoma derived cell lines within a distinct subpopulation of cells that had undergone an EMT (Ding et al, 2010).

Apart from HGF, additional growth factors have been shown to have the capacities of inducing EMT. Indeed, treatment of NBT-II cells with EGF and TGFα also results in an EMT and has been further confirmed in additional in vitro studies (Gavrilović et al, 1990; Hay, 1995). The EMT phenotype due to EGF stimulation has been shown to be the result of the induction of Snail, Slug and Twist (Hipp et al, 2009; Lee et al, 2008; Lo et al, 2007; Smith et al, 2009b). Interestingly, the upregulation of these transcription factors due to EGF receptor (EGFR) activation can be established via different signaling cascades. For example, an autocrine EGF-EGFR loop lies on the basis of the invasive behavior of SKOV3 ovarian carcinoma cells. The EMT phenotype of these cells occurs via an activation of p38MAPK and subsequently results in higher levels of Snail and Slug (Cheng et al, 2010). However, this does not seem to be the case in the invasive prostate cancer cell lines DU145 and PC3. Here, EGFR mediated Snail upregulation is dependent on activation of Akt. In addition, inhibition of the ERK pathway resulted in elevated EGFR signaling, subsequently leading to enhanced Akt activation (Gan et al). In sharp contrast to these findings, the increased levels of Slug due to EGF stimulation of cultured trophoblasts is completely dependent on activation of ERK (Zhao et al, 2010). EGF mediated downregulation of E-cadherin and occludin in human keratinocytes has also been shown to be dependent on PKC-δ. In addition, Src activity seems to be indispensable for this process (Singh et al, 2009). Another pathway that is necessary for EGF mediated EMT has been shown to be the JAK2/ Stat3 signaling cascade. Indeed, in ovarian cancer cell lines, EGF stimulation results in the upregulation of the mesenchymal markers N-cadherin and vimentin. Using a specific JAK2 inhibitor could abolish this effect. EGF mediated JAK2 activation does not seem to be the result of additional intricate cellular signaling, because activation of the JAK2/ Stat3 pathway already occurs several minutes after addition of EGF to the cells (Colomiere et al, 2009).

The fact that growth factors are able to induce an EMT phenotype in several model systems implies the existence of a seemingly contradiction to the contemporary paradigm
that EMT and proliferation are mutually exclusive. Evidence exists that the status of c-Src is able to modulate the outcome in growth factor stimulated epithelial cells. Indeed, when c-src activation results in an EMT phenotype of epithelial bladder carcinoma cells when treated with growth factors, while proliferation can be induced in the absence of c-Src (Rodier et al, 1995). In the case of FGF, it has been shown that cell density is able to determine whether an EMT or a mitogenic effect will take place. Treatment of sparsely seeded NBT-II cells with FGF results in an EMT phenotype, while conducting the experiment with confluent cells leads to stimulation of the cell cycle. Further investigations resulted in the discovery that the levels of c-AMP are responsible for this effect. Indeed, the intracellular concentration of c-AMP has been shown to be higher in sparsely seeded cells and is directly responsible for the growth factor mediated EMT phenotype (Boyer & Thiery, 1993).

Figure 10: Overview of growth factor mediated mechanisms leading to EMT. Binding of SF/HGF to its receptor, c-met, is known to activate several downstream pathways that are known to induce an EMT phenotype in different cell systems. Specifically, The SF/HGF mediated activation of Ras and ERK results in the upregulation of EGR, which is a direct activator of Snail. Furthermore, binding of EGF to its receptor has been shown to induce Snail expression via activation of p38MAPK, JAK2 and Akt.
1.4.4.4 Alternative mechanisms leading to EMT

Apart from the relatively well established EMT inducing pathways described above, an increasing amount of evidence is being uncovered supporting the fact that a multitude of additional mechanisms are able to induce an EMT. Indeed, the importance of NF-κB, Notch and Hedgehog signaling in the establishment of an EMT phenotype is becoming increasingly clear. Moreover, these pathways have been shown to interact with each other. These findings have led to the unraveling of a more and more expanding gene regulatory network that is able to modulate EMT during development and disease (Huber et al, 1996).

1.4.4.4.1 Notch signaling

Both Notch ligands (Delta-1, -4, -4 and Jagged-1, -2) and receptors (Notch1-4) are membrane bound proteins. Binding of one of the ligands to a receptor of a neighboring cell results in proteolytic cleavage by γ-secretase of the receptor at the extracellular side and at the transmembrane domain. As such, the cleaved cytoplasmic part of the Notch receptor is able to translocate to the nucleus, where it binds and activates the transcription factor CSL. In the absence of Notch signaling, CSL is bound to a corepressor complex (CoR). Binding of Notch to CSL displaces CoR, turning the complex into an activator (Grego-Bessa et al, 2004). The significance for Notch signaling in the process of mesenchymal transitions has first been described when studying the development of the heart valves. Mice with mutated Notch-1 or CSL display severely attenuated levels of Snail1 in the heart valve, leading to a disrupted endocardial EMT. Furthermore, overexpression of the Notch-1 intracellular domain in aortic endothelial cells induces a mesenchymal transition as the direct consequence of Snail1 upregulation (Timmerman et al, 2004). In extension to this, hypoxia induced Snail1 in ovarian SKOV-3 carcinoma cells also has been shown to be dependent on Notch signalisation (Sahlgren et al, 2008). Additionally, deregulation of the Notch pathway contributes to malignant characteristics of MCF-10A breast cancer cells. Activation of Notch-4 results in higher levels of Slug and subsequently leads to induction of an EMT. Moreover, inhibition of Notch-4
correlates with slower tumor growth and the formation of less metastases in MCF-10A xenografts (Leong et al, 2007). Malignant properties of several pancreatic carcinoma cell lines can also be attributed to Notch, since inhibition of this pathway resulted in a partial mesenchymal epithelial transition (MET). This was shown to be the direct consequence of diminished levels of Snail, Slug and ZEB1 (Wang et al, 2007b). Interestingly, recent evidence revealed a strong correlation between Notch and ZEB1 in several pancreatic cancer cell lines. Moreover, members of the microRNA family miR200 are able to target both Notch pathway members, such as Jag-1 and Maml2/3, and ZEB1 (Brabletz & Brabletz, 2010).

Compelling experimental data has shown that Notch signaling is intricately intertwined with the TGFβ pathway. Indeed, treatment of epithelial cells from the mammary gland, kidney tubules and epidermis with TGFβ, leads to initial Smad-3 dependent induction of the Notch pathway members Jag-1 and Hey-1. Interestingly, the EMT phenotype could be blocked by inhibition of Jag-1 and Hey-1 separately (Zavadil et al, 2004). Further evidence of the functional interaction between the two pathways is reflected in the response of TGFβ treated kidney epithelial cells. Similar to the previous study, Jag-1 and Hey-1 were upregulated prior to the induced EMT. However, activation of the Notch pathway in these cells could modulate both E-cadherin and vimentin, other TGFβ targets genes, such as α-smooth muscle actin remained unaltered (Nyhan et al, 2010). In parallel with these findings, mice with the artificially induced fibrotic skin disease scleroderma showed hyperactivation of the Notch pathway in fibrotic lesions. This could additionally be confirmed in sclerotic lesions of patients. Inhibition of ADAM-17, a proteinase involved in Notch activation, could significantly reduce the fibrotic lesions in the mice (Kavian et al, 2010a; Kavian et al; Leask).

1.4.4.2 NFκB signalisation

NFκB was first discovered to be a binding factor of the κ light chain immunoglobulin enhancer in lymphoid cell lines (Sen and Baltimore, 1986). Since then, the NFκB pathway has been found to play an important role in the control of cell growth, differentiation, apoptosis and inflammation (Sarkar & Li, 2008). Mammals have five
different NFκB members, including RelA (p65), RelB, Rel, NFκB1 (p50), and NFκB2 (p52). When activated, NFκB is found to be a heterodimer consisting of the factors p65-p50 or p52-RelB. In the absence of an external stimulus, NFκB is tightly bound to its inhibitor IκBα and p100 proteins. Activation of NFκB occurs through phosphorylation of IκBα by IKKβ or p100 by IKKα, leading to degradation of IκBα or processing of p100 into its smaller form p52, respectively. As such the two forms of NFκB are allowed to enter the nucleus and subsequently serve as transcriptional regulator of a myriad of target genes, including IL-2, IL-6, IL-8, TNFα, IL-2R and TCR (Min et al, 2008). Activation of the NFκB pathway mainly occurs through ligand binding of the TNF family of receptors, toll-like receptors, IL-1 receptor, T- and B-cell receptors (Ruland & Mak, 2003).

The role NFκB plays during malignant cancer progression is becoming increasingly clear. In a large panel of tumor cell lines and primary human tumors, high levels of NFκB were found. This is furthermore reflected in primary human breast cancer, where c-Rel, RelA, RelB, p50, p52 or Bcl-3 are often constitutively expressed (Wang et al, 2007b). One of the first indications that NFκB is implicated during the onset of EMT has been elucidated in Drosophila, where Snail is directly induced by the NFκB homologue Dorsal (Ip et al, 1992). Furthermore, insulin like growth factor (IGF) mediated EMT in MCF-10A cells is dependent on NFκB mediated upregulation of Snail (Kim et al, 2007). Similar results have been obtained in pancreatic carcinoma cell lines (Maier et al). One of the mechanisms that links NFκB activity to increased levels of Snail, is the fact that NFκB is necessary for the induction of COP9 signalosome 2 (CSN2). This in turn prevents the ubiquitination and subsequent degradation of Snail (Wu et al, 2009). In addition to its stabilizing role, NFκB is able to associate with the Snail promoter in zebrafish, leading to its direct activation (Liu et al, 2009). Furthermore, MCF-10A cells overexpressing the NFκB subunit p65 show elevated levels of ZEB1 and ZEB2, subsequently leading to the induction of an EMT. Treatment of MCF-10A cells with TNFα or IL-1α, two inducers of the NFκB pathway, also results in elevated levels of ZEB1 and ZEB2 (Chua et al, 2006).

In addition, epithelial NMuMG cells overexpressing the TGFβIII receptor undergo an EMT as the consequence of NFκB activation, which in turn is leading to induction of the transcription factors Snail, Slug, ZEB2 and Twist1 (Criswell & Arteaga, 2007). Remarkably, a negative feed-back loop between Twist and NFκB has been shown to exist. Indeed, mesoderm formation in the Drosophila embryo has been shown to rely on both
Twist1 and Twist2 expression in a RelA dependent manner. However, both Twist1 and Twist2 have been shown to interact with RelA, leading to the formation of an inhibitory complex that interferes with physiological NFκB signaling (Šošić et al, 2003). In addition, RelB, a component of non canonical NFκB signaling, has been shown to be inversely correlated with the estrogen receptor in breast cancer. Moreover, breast cancer cells of the basalB phenotype which have been associated with an EMT like status are also estrogen receptor negative. Expression of RelB in differentiated cancer cells leads in vitro to the establishment of an EMT phenotype (Wang et al., 2007).

Apart from being able to modulate EMT inducing transcription factors, a reciprocal signaling loop with NFκB seems to exist. Indeed, NFκB is a direct regulator of Slug during mesoderm formation in the *Xenopus* embryo. Subsequently, expression of Slug indirectly upregulates the NFκB subunits RelA, Rel-2 and Rel-3 (Zhang et al, 2006). In extension to these findings, overexpression of Snail in the colon adenocarcinoma cell line SW480 contributes indirectly to NFκB activation. Interestingly, the mechanism used to accomplish this effect is directly related to the expression status of E-cadherin. A direct interaction between E-cadherin and p65 sequesters NFκB to the cell membrane, strongly perturbing NFκB signalization (Solanas et al, 2008). Additionally, Snail is responsible for the induction of several pro-inflammatory cytokines in oral keratinocytes, further indicating its role in NFκB activation (Lyons et al, 2008).

### 1.4.4.3 Expression of microRNAs control the epithelial/ mesenchymal status of cells

Since the discovery of microRNAs in 2001 in *C. elegans*, a significant progress has been made in elucidating their specific functionality. In 2008, the microRNAs of the miR200 family were found to have a profound impact on the development of EMT. The miR200 family of microRNAs consists of at least three members that are located on different clusters. The members miR200-a and -b can be found in the same cluster located at Chr1p36.33, which also contains miR429. In addition, miR200-c is present in a cluster together with miR141 at Chr12p13.31 (Uhlmann et al). The first notion that these
microRNAs were implicated in EMT was found by a micro-array analysis of MDCK cells transfected with the EMT inducing tyrosine phosphatase Pez. Remarkably, a negative correlation was observed between the EMT status of the cells and the expression levels of the microRNAs of the miR200 family. Further investigations led to the discovery that the members of the miR200 family were specifically regulating both ZEB1 and ZEB2 and that ectopic expression of miR200 family members could reverse the EMT phenotype and drive mesenchymal cells towards an epithelial fate (Gregory et al, 2008; Korpal et al, 2008; Park et al, 2008). In the case of the ZEB1 3'UTR, at least eight sites can be identified that correspond to the seed regions of several members of the miR200 family (Brabletz et al). Conversely, a reciprocal interaction between the ZEB proteins and the miR200 family of microRNAs exists. Several ZEB responsive binding sites are present in the promoter of both microRNA clusters. More specifically, two and three conserved ZEB binding sites have been described in the miR200 cluster of chromosome one and twelve, respectively (Brabletz et al). The existence of this reciprocal loop has the consequence that relatively low levels of the microRNAs or ZEB1 are able to drive the cells into an epithelial or mesenchymal state. As such, the microRNAs of the miR200 family can be considered as strong stabilizing agents for the epithelial phenotype (Gregory, 2008 #35).

Apart from being responsible for inhibiting the ZEB proteins, conserved miR141 binding sites have also been identified in the 3' UTR of TGFβ2. Indeed, overexpression of miR141 in the mesenchymal like cancer cell lines SW480 and Panc-1 results in a dramatic decrease of TGFβ2 mRNA levels. These findings could be confirmed at the protein level in MDA-MB231 cells (Burk et al, 2008a). Similar conclusions can be made for miR200-a in a renal fibrosis model (Akkina & Becker). Besides the existence of the miR200 family as stabilizing factors of the epithelial phenotype, miR488 and miR125-a have recently been described to have a similar function. Expression of miR488 is inversely correlated with the chemotherapeutic induced EMT status in MCF-7 cells. Furthermore, miR488 overexpression in the mesenchymal like breast cancer cell line MDA-MB231 results in a mesenchymal epithelial transition and in significant lower expression levels of Twist1. Strikingly, miR488 can be mapped to the fourth intron of HRT2C, which is a target gene of NFκB. Repression of HRT2C by NFκB thus results in decreased miR488 levels, providing an additional mechanism for destabilizing the epithelial phenotype (Li et al). In parallel with these findings, overexpression of miR125-
a in the mesenchymal like DOV13 ovarian carcinoma cell line could induce a mesenchymal epithelial transition. Interestingly, miR125-a is negatively regulated upon EGFR activation and thus implies a novel mode of action for growth factor induced EMT (Cowden Dahl et al, 2009).

In contrast with the miR200 family and miR488, additional microRNAs exist that exert the opposite function, i.e. destabilizing the epithelial phenotype in favor of a mesenchymal status. For example, expression of miR21 is associated with increased invasive capacities and has already been found to be overexpressed in nine different types of solid tumors. Furthermore, miR21 expression is directly correlated with increased tumor stage (Jazbutyte & Thum, 2010). At least one E-box sequence is identified in the promoter that is responsible for ZEB1 mediated induction of miR21 and knocking down ZEB1 or miR21 resulted in a significant decrease of in vitro invasion of MDA-MB231 cells. Furthermore, the ZEB1-miR21 cascade is under the control of the inhibitory Smad-6, since Smad-6 overexpression results in lower levels of ZEB1 and subsequently in a decrease of miR21(Du & Pertsemidis, 2010). Recently, in MCF-7 cells overexpressing Snail, miR661 was found to be highly upregulated. Antagonizing miR661 does not lead to a reversal of the mesenchymal morphology, but significantly reduces in vitro migration and invasion. However, levels of E-cadherin and claudin 3 were upregulated in comparison with the control condition. High levels of miR661 can be found in the mesenchymal like MDA-MB435S and MDA-MB231 cell lines. Also in these cell lines, inhibition of miR661 did not lead to an epithelial mesenchymal transition, although invasion and migration were strongly reduced (Vetter et al, 2010).
1.5 RNA interference: a novel technique that facilitates the elucidation of gene functions

1.5.1 A short history of RNA interference

The discovery of RNA interference (RNAi) was preceded by the insight of reversible cosuppression in plants. Overexpression of chalcon synthase (CHS), one of the enzymes that is responsible for the violet color in petunias, did not lead to an increase in prevalence of violet petunias, but instead resulted in an overrepresentation of white flowers. Further investigation revealed that levels of CHS in the transgene plants were 50 fold lower than in their wild type counterparts. Because both the transgene and the endogenous gene were silenced, the term “cosuppression” was introduced (Napoli et al, 1990). A similar effect was observed when transfecting Neurospora crassa with the carotenogenic genes al-1 and al-3 resulted in a depigmentation of 36% of the treated fungi (Romano & Macino, 1992). In addition, a widespread technique used for gene silencing at that time was the use of antisense mRNA treatment, whereby it was thought that hybridization with the endogenous mRNA lead to destruction of the double stranded RNA (dsRNA). However, it was soon found that also the use of sense mRNA led to the same outcome (Guo & Kemphues, 1995). The exact mechanism leading to these specific types of gene silencing has remained elusive for a relative long period. In 1998 two reports were published where dsRNA was found to be responsible for these suppressive effects. In the manuscript of Waterhouse et al., the authors discovered that transgene plants that expressed both the sense and antisense mRNA strand of specific viral genes were much more protected against viral infection in comparison to plants overexpressing only the sense or antisense strand alone (Waterhouse et al, 1998). Similarly, in the nematode C. elegans, gene suppression was also found to be much more efficient when making use of dsRNA (Fire et al, 1998). These findings opened up the view that the sense strand mediated silencing in cosuppression events was dependent on an RNA dependent RNA polymerase (RdRp). This RdRp is then responsible for the generation of the antisense strand that is able to specifically bind to the target sequence. For example, in transgene Arabidopsis that carry mutant RdRp, cosuppression is severely compromised
the discovery that upon transgene induced suppression, RNA molecules with a uniform length of 25 nucleotides could be detected (Hamilton & Baulcombe, 1999). These findings were later confirmed in Drosophila and implied that long dsRNA molecules had to be cleaved into shorter substrates in order to obtain gene silencing (Hammond et al, 2000). Up until then, antisense strategies could not be used in mammalian cells, since long dsRNA was known to elicit a strong interferon response, leading to the induction of aspecific effects (Schlee et al, 2006). However, with the advent of these newly discovered short dsRNAs, termed siRNAs, specific gene silencing could also be used in mammalian cells without eliciting interferon mediated effects.

1.5.2 RNA interference is mediated by the ribonucleases dicer and "slicer"

The discovery that small dsRNAs are a necessary intermediate for gene silencing, provided the first evidence of the existence of a universal biochemical pathway leading to the degradation of mRNA. Since these small dsRNAs originate from longer fragments and are characterized by a 5' phosphate and a 3' hydroxyl terminus, an RNaseIII type of enzyme seemed to be responsible for the generation of these fragments. In Drosophila, an enzyme having these characteristics was soon discovered and was termed Dicer (Bernstein et al, 2001). Dicer is highly conserved among eukaryotes and generates dsRNAs with a length of 22 nucleotides. This is achieved by binding of Dicer as a monomer to the dsRNA substrate terminus, which is then followed by two endonuclease reactions cutting 21 nucleotides further downstream (Figure 11). Interestingly, these newly created siRNAs are marked with a two nucleotide overhang at the 3' side. Once a new terminus is created, the enzyme binds to this novel site and initiates a new cycle, as such producing several siRNAs out of long dsRNA (Hammond, 2005). Dicer homologues have been found in all organisms in which RNA interference is reported (Sontheimer, 2005). Several known domains can be found in Dicer, including an RNA helicase motif, a Paz domain, two tandem repeats of RNaseIII catalytic domains and one dsRNA binding motif (dRBM). Although the function of the helicase domain has long remained elusive, recent experiments suggest a role in the production of endo-siRNAs.
that have been mapped to regions in the genome capable of forming dsRNA, including retrotransposons. Strikingly, the functionality of exo-dsRNA and siRNA mediated silencing does not seem to be influenced by the presence or absence of the helicase function in Dicer (Welker et al, 2010). This is further illustrated in *Drosophila*, where two forms of Dicer can be found that only differ in the presence of the helicase domain. Dicer-2, lacking the helicase domain has been shown to be responsible for siRNA mediated silencing, while Dicer-1, with active helicase motif, is responsible for endogenous miRNA processing (Lee et al, 2004). Both the dRBM and the Paz domain are implicated in the DNA binding properties of Dicer. While the dRBM does not seem to have a specific target binding site, the Paz domain recognizes the 3' overhangs from dsRNA or from fragments generated by Dicer itself. After binding of Dicer to a dsRNA substrate, both the RNaseIII catalytic domains are positioned in a way that enables them to cut 22 nucleotides further downstream of the Paz recognition site. Both domains act as an intramolecular dimer and have been shown by means of mutational studies that they work independently from each other. Their relative position is shifted two base pairs so that mature siRNAs are generated with an overhang of two nucleotides at the 3' end (Zhang et al, 2004). In order for the RNAi pathway to be functional, a second protein complex that works independently of Dicer needs to be present and is referred to as the RNA induced silencing complex (RISC). By making use of high-speed centrifugation of *Drosophila* embryo extracts, it was shown that dsRNA processing and target mRNA degradations were two biochemically separated events (Bernstein et al, 2001). The composition of the RISC depends on the source where it is isolated from, since different complexes have been identified that range from 160 to 550 kDa in size. However, two general properties are common. All complexes are able to bind to the small regulatory RNAs and at the core of the RISC, a member of the argonaute (AGO) protein family can be found. These two components have been shown to be necessary to form the minimal RISC complex. The argonaute proteins have been identified to be responsible for the cleavage of target mRNA strands and are therefore often referred to as “slicer”. In order to function as an effector of siRNA mediated silencing, the argonaute protein first has to form a complex with the small double stranded RNAs that have been previously processed by Dicer. Next, the passenger or sense strand is ejected from the complex, while the remaining strand serves as a guide for the mRNA target.
Figure 11: **General mechanism of RNA interference.** The RNase III Dicer is able to bind to double stranded RNA and cleaves it to shorter fragments of ~22 nt termed siRNAs. These siRNAs are incorporated into the RISC complex and subsequently the passenger strand is ejected from the complex. As such, the remaining guide strand leads the RISC to the corresponding mRNA, which is then cleaved by the AGO2 protein, present in the RISC.
During slicing, the target sequence is cleaved at the phosphate that is opposite to the position between nucleotides ten and eleven of the guide strand as counted from the 5' side (Hammond, 2005). Four domains can be recognized in the argonaute family of proteins: the N-terminal, Paz, MID and PIWI domain. Structural analysis has revealed the existence of a bilobular structure of the argonautes that is established by the MID and PIWI domain on one hand, and the N-terminal and Paz domains on the other hand. Due to its highly similar architecture to the RnaseH fold, it was soon discovered that the PIWI domain is responsible for the slicer activity. Mutation of the crucial Asp-Asp-His motif of PIWI abrogated the argonaute slicer function. In humans, four argonautes have been identified, while only AGO2 can act as a slicing protein (Jinek & Doudna, 2009). In order to be functional, the argonaute member of the RISC has to be loaded with a guide RNA. This is accomplished by the RISC loading complex that consists of an argonaute protein, Dicer and the protein TRBP. This ternary complex is able to load the correct guiding strand and to perform the slicing function. The strand that has the thermodynamically least stable 5' end of the duplex is strongly favored to be incorporated into the RISC (Schwarz et al, 2003).

1.5.3 RNAi as a tool for genome wide functional genomic screenings.

The progress that was made with genomic sequencing during the first decade of the 21st century and the ease of synthesizing short RNA duplexes, permitted to develop siRNA libraries with the aim of performing high throughput screenings. One of the first RNAi screenings was carried out in *C. elegans* in an attempt to elucidate the functions of predicted genes of chromosome one (Fraser et al, 2000). In a second screening where the authors were interested in cell cycle modulators, all open reading frames on chromosome three were targeted with RNAi (Gönczy et al, 2000). Both screenings however, did not make use of siRNA, but instead were done by introducing long dsRNA into *C. elegans*. With the discovery that siRNAs could circumvent the interferon response, screening became possible in mammalian cell systems. The first screenings in mammalian cells were performed using a short hairpin RNA (shRNA) approach, developed by Brummelkamp and colleagues (Brummelkamp & Bernards, 2003). By suppressing 50
human de-ubiquitinizing enzymes, several novel modulators of NF\(\kappa\)B were identified (Brummelkamp et al, 2003). In 2003, the first true siRNA screening was performed by Aza-Blanc et al. in an attempt to identify new modulators of TRAIL induced apoptosis in HeLa cells. A total of 510 genes were silenced and delivered a multitude of novel candidates able to influence apoptosis (Aza-Blanc et al, 2003). During the following years, the collection of siRNA library steadily grew, finally culminating in the possibility of performing genome wide screenings. For example, it became possible to generate siRNAs from \textit{in} \textit{vitro} transcribed long dsRNA ranging from 100 to 500 bp and subsequently cutting them with an endoribonuclease. These siRNAs are termed endoribonuclease siRNAs (esiRNA) and have the property of being able to target one gene with different RNA substrates (Kittler et al, 2004). Furthermore, the introduction of specific barcodes in shRNA vector libraries enabled researchers to perform genome wide pooled screenings and identify the hits subsequently with the aid of qPCR technology (Brummelkamp et al, 2006). Together with the development of platforms for high content analysis, siRNA screenings are as such able to produce large amounts of in depth data.

However, with the fast establishment of siRNA as a tool to silence gene expression and the upcoming use of siRNA libraries, some disadvantages soon became clear. Indeed, it was found that siRNAs are able to cross react with targets of limited sequence similarity. For example, micro-array analysis of independent transfections of different siRNAs targeting the IGF receptor in HeLa cells revealed distinct expression patterns. Only a minority of genes were found to be modulated in common by all siRNA duplexes, indicating that these are IGF receptor dependent. The majority of the modulated genes however can thus be considered as sequence specific off target effects (Jackson et al, 2003). Further investigation led to the discovery that the seed region of siRNAs is in large part responsible for these off target effects, which is a similar mode of action as can be found in miRNAs (Jackson et al, 2006b). Since sequence dependent off target effects increase with concentration, one of the ways of mitigating this response is by making use of pools of siRNA. As such the concentration of the individual siRNAs can be kept low, while knock down efficiency is least affected. Keeping the individual siRNA concentration below 10 nM has been shown to significantly reduce the number and magnitude of off target silencing (Jackson & Linsley, 2010). A phenotypic screening comparing individual siRNAs with pools indeed confirmed the beneficial effect of pools.
concerning off target effects and showed that the use of pools in a greater phenotypic penetrance (Parsons et al, 2009). A second measure that significantly reduces off target silencing is the chemical modification of the siRNA backbone. Indeed, a 2'-O-methyl group modification at position one and two of the sense strand inhibits incorporation of this strand in the RISC. Furthermore, the same modification at position 2 of the anti-sense strand severely reduced sequence dependent off targets by enhancing the specificity of seed region binding to the mRNA target (Jackson et al, 2006a).

Also sequence independent effects have been reported when treating cells with siRNAs. Although the use of short double stranded RNAs as silencing agents greatly reduce induction of the interferon-γ response, several reports suggest activation of type I interferons might still take place. Membrane bound receptors in non-immune cells that are able to recognize siRNAs are the dsRNA-activated protein kinase (PKR) and 2′5′-oligoadenylate synthetase that are both able to initiate general protein synthesis inhibition and are furthermore implicated in activation of NF-κB and interferon regulatory factor 3 (IRF3). In addition, both immune and non-immune cells, such as endothelial cells, fibroblasts and hepatocytes, express Toll like receptor 3 (TLR3), which is able to direct an interferon I response (Marques & Williams, 2005). These findings severely compromise the use of siRNA in vivo due to the possibility of activation of the immune system (Sioud, 2010). Additional off target effects might arise when using higher concentrations of siRNA due to saturation of the endogenous miRNA pathway. By comparing 151 transfection experiments, Khan et al. concluded that several targets of endogenous miRNAs were significantly higher expressed in siRNA transfected cells. These findings further urge the critical interpretation of siRNA generated data, in particular when looking to miRNA-dependent regulatory mechanisms (Khan et al, 2009).
2 AIM OF THE PROJECT

The transcription factor ZEB1 has been shown to be implicated in the establishment of EMT *in vitro* and *in vivo* during the progression of carcinoma (Eger et al, 2005; Spaderna et al, 2006). Aberrant activation of the EMT program, which is crucial during several phases of embryonic development, has been shown to contribute to the invasive capacities of cancer cells (Berx et al, 2007). This is furthermore reflected by the fact that ZEB1 expression is correlated with bad prognosis and unfavourable grading in several types of cancer, including cancer of the endometrium and ovaries (Hurt et al, 2008; Spoelstra et al, 2006). Moreover, a connection between ZEB1 expression and the regulation of stem cell like properties has been discovered recently. These observations stress the importance of understanding how exactly ZEB1 acts as a transcription factor and the need to unravel novel insights into the diverse spectrum of techniques that are used by cancer cells to display malignant behavior. In addition, identification of novel pathways that are necessary to mediate the correct function of ZEB1, might open up new opportunities in the development of new treatments against advanced carcinomas. Indeed, it has been shown that the onset of EMT can result in the acquisition of drug resistance of the cancer cells (Singh et al, 2008b). Modulation of the EMT inducing transcription factors, specifically ZEB1, might thus provide new avenues to sensitize chemo therapeutic resistant cancer cells for treatment.

The **primary aim** of this project is to elucidate what molecular mechanisms are used by ZEB1 in order to exert its function as an EMT inducing transcription factor. Although several transcription factors and cofactors have been shown to physically and functionally interact with ZEB1, these experiments were not carried out in an EMT related background and could thus not provide information about the role of ZEB1 during the progression of carcinoma. For this purpose, we intend to use an *in vitro* siRNA approach. This implies the need to develop a robust screening system which can deliver an unambiguous readout. To this extent, several cell lines will be equipped with an inducible ZEB1 construct and assessed whether these are capable of undergoing an EMT. Furthermore, several approaches for a screening readout will be considered, rigourously tested and subsequently implemented in the screening cell line.
The **secondary aim** is to further deepen our understanding of the discovered hits on the ZEB1 induced EMT. For this purpose, we intend to examine the effects of the hits on transcription of EMT related genes, migration and invasion. On the basis of these findings, we aim to further investigate the functional relation between one of the major hits and ZEB1. As such, we hope to uncover novel mechanisms that give us further insight into ZEB1 functionality in the frame of EMT.
3.1 Optimization of the *in vitro* siRNA screening model

3.1.1 Introduction

In order to exert its correct function in a spatio-temporal manner, the EMT inducing transcription factor ZEB1 has been shown to have a myriad of interaction partners. However, to date, only a limited amount of functional information exists concerning these ZEB1 interactors during the onset of epithelial mesenchymal transition (EMT). In order to gain further insight into the molecular aspects of ZEB1 during EMT, we developed an *in vitro* siRNA screening model based on inducible ZEB1 expression. Here, proof of concept is delivered for the robustness and feasibility of the screening model. First, a cell line with stable, conditional ZEB1 expression had to be developed that was capable of undergoing an EMT upon induction. Second, a proper screening readout had to be implemented, delivering fast and unambiguous results. Taking into account these considerations resulted in the development of a ZEB1 inducible MCF-7 breast cancer cell line instrumented with an EMT sensor capable of measuring in a sensitive manner EMT.

3.1.2 Development of a ZEB1 inducible cancer cell line

We decided to chose the two epithelial cancer cell lines A431 and MCF-7 to serve as the basis of our *in vitro* models for the siRNA screening. The inducible expression system that was used to implement ZEB1 expression in both cell lines is based on the KRAB epigenetic repressor, as described in the report of Sculz et al. (Sculz et al., 2006). Briefly, by introduction of the KRAB repressor domain fused to the Tet repressor of *Escherichia coli* (tTRKRAB) a fusion protein is created that is able to bind to the TetO sequence in the responsive promoter of the gene of interest. Next to steric hindrance from the Tet repressor domain, the KRAB domain recruits a multimolecular complex that leads to deacetylation and methylation of the target DNA, resulting in a negative modulation of
expression. Indeed, in the absence of doxycyclin, the tTRKAB fusion protein is allowed to bind to the TetO sequence of the target promoter, implying inhibition of expression. However, treating the cells with doxycyclin perturbs the binding of tTRKRAB to the promoter, resulting in expression of the gene of interest (Sculz et al., 2006). By lentiviral cotransduction of the tTRKRAB plasmid and the expression plasmid with ZEB1 under the control of the Tet responsive EF1alpha promoter, inducible ZEB1 expression was accomplished in both the A431 and MCF-7 cell line (further referred to as A431 ZEB1 and MCF-7 ZEB1).

In order to obtain the most homogeneous induction of ZEB1, both cell lines were FACS sorted and subsequently clones were picked. Both lentiviral plasmids that were used for the creation of the conditional cellular system are equipped with a fluorescent selection marker. The tTRKRAB is followed by an IRES sequence and the fluorescent marker dsRED (Discosoma striata Red fluorescent protein), while the ZEB1 expression cassette is followed by an IRES-GFP (Internal Ribosomal Entry Site – Green Fluorescent Protein) sequence (Figure 12).

Figure 12: Overview of the used constructs to create the A431 ZEB1 and MCF-7 ZEB1 cell lines. Both plasmids were lentivirally transduced in A431 and MCF-7 cells. The tTR-KRAB repressing protein is followed by an IRES-DsRed cassette, allowing the cells to be sorted. Similarly, the C-terminal myc tagged ZEB1 open reading frame is followed by an IRES-GFP cassette.
After cotransduction, both non-induced cell lines were FACS sorted for dsRED. As such, all retained cells were expressing the KRAB repressor. These cells were subsequently induced with doxycyclin and sorted for GFP. However, by the application of FACS, a population of cells remain that still have a certain variability in the expression levels of ZEB1. In order to obtain a screening model that has homogeneous ZEB1 expression levels, clones were picked from the remaining cell population. Analyzing several clones of both A431 ZEB1 and MCF-7 ZEB1 indeed resulted in a cell population able to conditionally and homogenously express ZEB1, which was checked by a staining of the C-terminally located myc tag of the ZEB1 open reading frame (Figure 13, Figure 14).

However, the newly selected cell lines are screening systems that are based on overexpression of ZEB1 and might thus not reflect the endogenous expression levels occurring in mesenchymal like cell lines. In addition, due to the fact that individual siRNAs might not be able to overcome a ZEB1 response that is too strong, an additional rise of false negative results might be expected. To overcome this, we compared the ZEB1 expression levels of several selected clones with the ZEB1 mRNA levels in the metastatic breast cancer cell line MDA-MB231 which has acquired a stable mesenchymal phenotype (Figure 15a). In this cell line it has been shown that ZEB1 is the driver of the mesenchymal properties. Knocking down ZEB1 in MDA-MB231 leads to increased E-cadherin levels, upregulation of polarization complexes and inhibition of *in vitro* cell mobility (Aigner et al, 2007a).
Figure 13: **Inducible MCF-7 ZEB1 cell line.** Clone of the MCF-7 ZEB1 cell line that was picked after FACS sorting. The cells were treated with doxycyclin for 96h. **a.:** ZEB1 induction leads to the occurrence of an EMT at the morphological level. **b.:** The C-terminal myc tagged ZEB1 could be detected in 100% of the cells, leading to loss of E-cadherin at the plasma membrane.
Figure 14: **Inducible A431 ZEB1 cell line.** Clone of the A431 ZEB1 inducible cell line that was picked after FACS sorting. The cells were treated for 96h with doxycyclin. **a.:** Induction of ZEB1 leads to an EMT at the morphological level. **b.:** The C-terminal myc tagged ZEB1 can be detected in 100% of the cells and disrupts the E-cadherin organisation at the plasma membrane.
When looking at the relative expression levels of ZEB1 in the induced MCF-7 ZEB1 cells, two clones show similar expression levels of ZEB1 in comparison to the MDA-MB231 cell line (Figure 15b). Despite these findings, vimentin mRNA expression in the induced MCF-7 ZEB1 cells could not reach the same levels as in MDA-MB231 (Figure 15b). A possible explanation for this apparent discrepancy is the fact that, besides ZEB1, additional EMT inducing factors are expressed in MDA-MB231. In particular, mRNA of the transcription factor Slug and SIP1/ZEB2 can be detected (see PhD thesis of Dr. Cindy Vandewalle). Although mRNA of both proteins is expressed at lower levels than that of ZEB1, it cannot be excluded that they contribute to the mesenchymal state of the MDA-MB231 cells. In the case of the A431 ZEB1 cells, a similar conclusion can be made (Figure 15)

Figure 15: qPCR analysis of ZEB1 expression levels in selected clones of MCF-7 ZEB1 and A431 ZEB1. a.: Levels of ZEB1 mRNA in MDA-MB231 are set to one. For induced MCF-7 ZEB1, two clones could be selected that show similar expression levels in comparison with MDA-MB231. For induced A431 ZEB1 cells, this was limited to one clone. b.: A similar analysis showing the relative expression levels of vimentin.

Once several clones were picked, we further characterised the inducible screening system in relation to EMT. Therefore, we assessed whether ZEB1 expression is also capable of
modulating several epithelial and mesenchymal markers at the protein level. Western blot and immunocytochemical analysis revealed that the epithelial markers E-cadherin, occludin, desmoglein and desmoplakin could be significantly downregulated at the protein level of induced MCF-7 ZEB1 cells. In addition, expression of vimentin was upregulated after ZEB1 induction. Treatment of the cells with siRNA targeting ZEB1 resulted in the conservation of the epithelial phenotype even when ZEB1 was induced (Figure 16).

Figure 16: Characterisation of the MCF-7 ZEB1 cell line at the protein level. a.: Western analysis of epithelial and mesenchymal markers. Transfecting the cells with siRNA targeting ZEB1 could inhibit the mesenchymal transition of the MCF-7 ZEB1 cells. b.: Immunocytochemical analysis of MCF-7 ZEB1 showing the upregulation of the mesenchymal marker vimentin (Vim) in the induced cells and the decreasing protein levels of the epithelial markers E-cadherin (E-cad) and occluding (Occ). Functional levels of these proteins are restored upon siZEB1 transfection.
Interestingly, we were also able to show the ZEB1 inducibility in an *in vivo* context. For this purpose, the MCF-7 ZEB1 cells were injected in combination with matrigel into the mammary fat pad of immune compromised female mice. The tumors were permitted to grow for eight weeks after which doxycyclin was administered to the drinking water. The mice were sacrificed five days post induction and the tumors were subsequently analysed (Figure 17:). No effect on tumor growth could be observed due to doxycyclin administration. Staining for the myc-tag revealed that ZEB1 can indeed be induced *in vivo*. The myc-tag can be detected in the nucleus in analogy with the *in vitro* situation. Furthermore, several cells that have a mesenchymal like appearance also stain for myc, indicating that these have undergone a mesenchymal conversion. This is further exemplified by the fact that ZEB1 induction also leads to severe downregulation of E-cadherin and a marked upregulation of vimentin (Figure 17:). These findings pave the way for a possible *in vivo* validation of the future siRNA hits. Since the mice were sacrificed after five days of drinking doxycyclin containing water, we were not able to assess whether the formation of metastasis was influenced. In addition, local invasion did not seem to be affected during this short timeframe. However, since after five days of induction, several tumor cells already display a mesenchymal phenotype, it is tempting to speculate that indeed the cells will become (locally) invasive over a more extended period of doxycyclin induction. To this end, a new experiment has been set up in order to investigate this.
Figure 17: In vivo validation of the MCF-7 ZEB1 cell line. Cells were injected in the mammary fat pad of immune compromised mice. After eight weeks, doxycyclin was administered to the drinking water and tumors were analysed after five days. Treating the mice with doxycyclin leads to induction of ZEB1 (myc-tag), subsequently resulting in the downregulation of E-cadherin (E-cad) and upregulation of vimentin.
3.1.3 Selection of a reporter assay that is able to deliver a simple and unambiguous readout

Considering the existing expertise that exists in the Berx laboratorium with the luciferase reporter system, we opted for this system to be used as a cheap and sensitive EMT sensor for the screening readout. Several EMT controlled promoters were cloned in front of the luciferase reporter and were, in parallel with the development of the inducible ZEB1 cell lines evaluated for their responsiveness upon stimulation of the A431 ZEB1 and MCF-7 ZEB1 cells. Both A431 and MCF-7 cell lines were transiently cotransfected in a 24 well plate with the pCS3 or pCS3 ZEB1 vector together with the different promoter constructs (Figure 18a). The activity of the E-cadherin promoter was reduced in both cell lines under the influence of ZEB1 (Figure 18b). However, the small relative difference and the relatively weak absolute signal did not permit to use this reporter construct in high throughput screening conditions. Since the screening is to be performed in a 96 well format, the absolute signal is expected to drop significantly in comparison with these transient promoter assays that were performed in a 24 well setup. The same issues can be observed for the promoters of the mesenchymal genes N-cadherin and S100A4, making them not suitable for screening purposes. When looking to the vimentin promoter, the absolute value of the luciferase signals are much higher in comparison with the other tested promoters. Furthermore, especially in the MCF-7 cells, the signal to noise ratio is high enough in order for the assay to be performed under screening conditions. Note that due to the higher activity of the vimentin promoter in the control condition of the A431 cells, the signal to noise ratio is less optimal for screening conditions (Figure 18b).
Figure 18: **Transient promoter assays in MCF-7 and A431 cells.**

*a:* Overview of different promoter constructs used. All promoters were cloned in the pGL3 vector.

*b:* Transient transfection assays reveal that the vimentin promoter is most suited as screening readout in comparison with the other tested promoters. Normalisation of the luciferase signal was done via measurement of the total protein content. E-cad: E-cadherin promoter, S100A4: S100A4 promoter, Vim: Vimentin promoter, N-cad: N-cadherin promoter.

Additional findings concerning transfection efficiency and viability of the cells under screening conditions as discussed above suggested that the A431 cell line was not suitable for screening purposes. The next step was thus to stably transfect the MCF-7 ZEB1 cells with the vimentin reporter. However, of crucial importance is the fact that the readout signal stays as stable as possible under screening conditions. Signal variation might arise due to the specific integration site of the vimentin reporter in the genomic DNA of the MCF-7 cells. Regions that are prone to DNA methylation or deacetylation might provide
a weaker signal over time. Furthermore, it has to be taken into account that due to specific siRNAs that target the DNA methylation or acetylation machinery of the cell, might as such influence the luciferase signal that is not specific in relation to ZEB1 induced EMT. To circumvent these issues, we decided to flank the vimentin-luciferase cassette with insulator sequences. First described in *Drosophila*, insulators are sequences that are able to shield the promoter activity from nearby regulatory elements, whether these contribute to activation or repression. The chicken β-globin insulator was found to be part of a 1.2 kb fragment at the 5’ end of the β-globin locus. Further investigations refined the insulator sequence to be 250 bp of length with a GC rich core and multiple short internal repeats. Most important, a minimal of canonical transcription factor binding sites are present in this fragment (Chung et al, 1997). After flanking the vimentin-luciferase reporter with the chicken β-globin insulator sequences, the whole cassette was transferred into a modified pGL3 plasmid containing the selection marker blasticidin (pGL3 Vim Luc). Comparing the non-flanked vimentin promoter with its flanked version in a transient transfection assay in induced MCF-7 ZEB1 cells showed that although the absolute amplitude of the luciferase signal of the flanked promoter was significantly lower, the overall stability was highly increased in a 96 well plate setup. However, this drop in absolute value of the reporter activity still allowed us to clearly distinct the positive and negative controls (data not shown). We explicitly did not opt for the integration of the reporter cassette into a lentiviral vector, since it is known that the long terminal repeats (LTR) in the lentiviral vectors are able to exert transcriptional activity on their own. As such the luciferase readout could be disturbed, altering the optimal signal to noise ratio we observed during the transient transfection assays (Figure 18).

In addition to the vimentin reporter, we decided to implement a viability marker. When only the activity of the vimentin promoter is taken into consideration, a hit would be selected when a significant drop of luciferase activity would be detected compared to the control condition. However, this could be the result of aspecific effects due to targeting of the siRNAs of survival pathways or critical proteins that are essential for cell survival. To detect these false positive hits, an additional viability marker based on secreted alkaline phosphatase (SEAP) has been developed. As the viability sensor, we have used the elongation factor alpha (EF1alpha) promoter. EF1alpha is responsible for the binding of aminoacyl-tRNA to the A site of the ribosome mRNA complex (Uchiumi & Ogata, 1986).
Since this is a general process that is strictly necessary for cell survival and homeostasis, we propose to use the EF1alpha promoter activity as a reporter for cell viability. The EF1alpha-SEAP cassette was cloned into the vector with the vimentin-luciferase reporter, and was as such transfected into the MCF-7 ZEB1 cells. Blasticidin selection yielded a population of cells that was capable of delivering an increased vimentin promoter activity upon ZEB1 induction (Figure 19a). This newly created cell line will from now on be referred to as MCF-7 ZEB1 vim-EF1alpha. Strikingly, when inducing the cells, the activity of the EF1alpha promoter was downregulated at 40% of its initial value in the non induced control. To verify whether this reduced activity could still be used as a reporter for cell viability under screening conditions, we treated the cells with the protein synthesis inhibitor cycloheximide (CHX). CHX acts by inhibiting the binding and release of tRNA from the donor site of the ribosome. Applying CHX at both low and high concentration still leads to significant repression of the EF1alpha promoter in the ZEB1 induced cells, indicating its functionality as a viability reporter is not critically affected (Figure 19b).

Figure 19: Screening readout for the vimentin promoter and EF1alpha promoter activity in the stable MCF-7 ZEB1 clones. a.: Luciferase assay on stable MCF-7 ZEB1 Vim-EF1 cells showing the increase in vimentin promoter activity upon ZEB1 induction
after 96h. b.: SEAP assay of the first clone of fig. 5a. showing that upon 96h of ZEB1 induction the activity of the EF1 alpha promoter is downregulated to 40% of its initial value. As a positive control for reduced cell viability, cells were treated with low (10 µg/ml) and high (30 µg/ml) concentrations of CHX, showing the SEAP reporter is indeed functional.

3.1.4 Optimalization of transfection efficiency under screening conditions

The two principle methods for bringing in siRNA into the cell are electroporation and lipofection. Although electroporation has proven to be a potent mechanism to transfec cells, its use in high throughput applications is unfavorable due to its high cost. For this reason, we decided to optimize the transfection conditions for the screening on the basis of lipofection. At first instance, lipofectantia from different companies were tested with the aid of fluorescently labeled siRNAs targeting cycloplilin B (siCyc). On the basis of these findings, the Dharmafect I reagens came out as the most favourable. During the next optimization steps, the silencing efficiency and overall cell survival were tested for both the A431 and MCF-7 cell lines (Figure 20). Strikingly, although the fluorescent siRNAs could be detected in an equally high percentage of cells in both MCF-7 and A431 (Figure 20a), the knockdown efficiency was less optimal in the A431 cells. In contrast, a silencing efficiency of > 90% could be observed in the MCF-7 cell line over a period of 120h. As can be concluded from the MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, this initially leads to a minor reduction in metabolic activity after 24h, but the cells are fully recovered after 96-120h (Figure 20b).
Figure 20: **Optimization of siRNA transfection efficiency.** a.: Both MCF-7 and A431 cells were transfected with fluorescently labeled siRNA targeting cyclophilin B. For both cell lines, the siRNA molecules could be detected in 80-90 % of the cells. b.: qPCR analysis verifying the knock down efficiency of the optimized protocol. In parallel, an MTT assay was performed to assess the viability of the transfected cells.

Although the use of short antisense RNA for silencing purposes has been shown to elicit only a very weak interferon response, we still wanted to assess whether our optimized transfection protocol was capable of inducing an interferon related aspecific response. For this purpose, MCF-7 cells were transfected with siCyc and checked for upregulation of the interferon sensitive genes IFITM and OAS. Expression analysis 96h after transfection revealed that no upregulation of one of these genes could be detected, suggesting the absence of an interferon response (Figure 21). Interestingly, when the same analysis was performed on less optimal transfection conditions that demanded higher concentrations of siRNA (> 50 nM) to obtain an efficient knock down, a significant increase in expression of both IFITM (Interferon induced transmembrane protein) and OAS (oligoadenylate synthetase) could be observed (data not shown). This indicates that although siRNAs
severely reduce an interferon response in comparison with the use of long double strandes RNAs, a sufficiently high siRNA concentration might still be able to activate endogenous interferon.

Figure 21: qPCR analysis of the interferon responsive genes upon siRNA transfection. MCF-7 cells were transfected with 10 and 20 nM of siCyc. Lysates were made 96h after transfection and a qPCR analysis was performed for the interferon responsive genes IFITM and OAS. The synthetic polymer pI:C was used as a positive control.

Having determined and validated the optimal transfection parameters in parental MCF-7 cells, we tested these conditions in the screening cell line MCF-7 ZEB1 vim-EF1alpha. By using siRNA targeting ZEB1 (siZEB1), this also permitted us to assess the efficiency of our positive control that will be used during screening. siZEB1 treatment of the cells leads to an efficient knock down of ZEB1 in the induced cells. This is furthermore reflected by the fact that vimentin expression is severely inhibited as a response of the siZEB1 treatment of the induced cells (Figure 22a). In addition, siZEB1 treatment is able to counteract the morphological changes that are associated with ZEB1 induced EMT (Figure 22b). These findings are reflected in the promoter activity of both vimentin and EF1alpha (Figure 22c). Both high and low concentrations of siZEB1 were able to downregulate the activity of the vimentin promoter in the induced cells. Furthermore, a restoration of the EF1alpha promoter activity could be observed, indicating that the
siZEB can be used as an effective positive control in our screening. In addition, we tested whether siZEB1 transfection in induced cells could reproducibly lead to a significant downregulation of the vimentin promoter activity under screening conditions. For this purpose, we transfected MCF-7 ZEB1 vim-EF1alpha cells with 20 nM of siZEB1 in a 96 well plate setup and subsequently induced the cells with doxicyclin. Luciferase measurements after 96h show that a relatively good reproducible signal could be attained under screening conditions (Figure 22d).

A frequently used parameter that tests the robustness of a screening assay is the so called Z' factor. Introduced in 1999 by Zhang et al. the Z' factor aims to give a measure for the overlap between the positive and negative control in a screening setup. This can be calculated by the use of the following formula:

\[
Z' = 1 - \frac{3(\sigma_n + \sigma_p)}{\mu_n - \mu_p}
\]

Hereby, \(\sigma_p\) and \(\sigma_n\) denote the variability, while \(\mu_p\) and \(\mu_n\) stand for the average values of the positive and negative control, respectively (Zhang et al, 1999). In general, the outcome of the Z' calculation is divided in three categories. If \(Z'=1\) the screening assay is ideal and an overlap between the positive and negative control will never occur. With \(0.5 < Z' < 1\) the screening is considered to be very robust with only a minor chance that the positive and negative control will overlap. The more \(Z' < 0.5\), the less robust the screening assay is. If \(Z' < 0\), the positive and negative control will always overlap, meaning that the screening is practically not feasible. In our case, repeated determinations of the Z' factor yielded a result of 0.43 ± 0.05. This value is smaller than 0.5 and thus indicates our screening readout might still benefit from some improvements. However, since we aim to perform the screening in triplicate and additionally make use of a viability marker, the detection of false positives or negatives due to overlapping signals is reduced to a minimum and should thus allow us to perform a robust screening. Moreover, we aim to perform a secondary screening with independent siRNAs for the hits that came out of the primary screening, further reducing the risk of delivering false positive data.
Figure 22: Analysis of the MCF-7 ZEB1 vim-EF1alpha cell line under screening conditions. 

a.: ZEB1 and vimentin mRNA expression analysis via qPCR after siZEB1 treatment of the MCF-7 ZEB1 vim-EF1alpha cells. Lysates were made after 96h. 

b.: siZEB1 treatment is able to disturb the morphological changes that are associated with ZEB1 induced EMT. 

c.: Effect of siZEB1 treatment on vimentin and EF1alpha promoter activity. Both high and low siZEB1 concentrations are able to restore the system into its epithelial state, even when ZEB1 is induced. 

d.: Reproducibility test of the MCF-7 ZEB1 vim-EF1alpha screening system by multiple transfections in a 96 well plate. Subsequently, cells were induced with doxicyclin and luciferase activity was after 96h.
3.1.5 Selection of a suited siRNA library

As briefly touched upon in the introduction, different types of siRNA libraries exist that determine the interpretation of the delivered data and subsequent setup of the secondary screening. Since our *in vitro* cell system is not suited to perform a pooled shRNA screening, our initial choice was directed to a library consisting of individual or siRNA pools. Both approaches have their advantages and disadvantages. When using individually arrayed siRNAs it has to be taken into account that performing the screening in triplicate will increase the workload, since in general three siRNAs per target are supplied. This is not an issue with pooled siRNAs, since the different oligos targeting the same gene are supplied to the same well. Concerning off target effects, the use of pools has the advantage that the separate siRNAs are used at a lower concentration, while the silencing efficiency is maintained. It has been shown that the presence of off target effects significantly decreased if the siRNA concentration drops below 20 nM. When performing the screening with pools at a concentration of 20-30 nM, this means the concentration of the individual siRNAs will vary around 10 nM, which is well below the 20 nM threshold (Semizarov et al, 2003). Strikingly, it has also been shown that the phenotypic penetrance of screening hits is stronger when pools are used. An explanation for these findings might lie in the fact that insufficient knock down of individual siRNAs is translated into more efficient silencing with a combination of siRNAs (Parsons et al, 2009). The disadvantage of a pooled setup however, is that if a hit is detected, it is not known whether this is supported by all or only by one or two siRNAs in the pool. When using individual siRNAs it is immediately known which siRNAs are responsible for the phenotype, facilitating the choice for the secondary screening setup. In addition, since the phenotype penetrance is more pronounced with the use of pools, it might be expected that a larger fraction of the hits will be false positives in comparison with the individual siRNAs (Parsons et al, 2009). However, this automatically implies that a greater percentage of false negatives will be missed with the individual siRNAs. Since false positives will be filtered out by performing a secondary screening and false negatives will de facto never be detected, the increased hit rate from the primary screening with pools can hardly be viewed as a big disadvantage. Considering the above mentioned arguments, our primary choice was to perform the screening with siRNA pools. Together with our aims in relation
to ZEB1 induced EMT, the acquired library targets all known human transcription factors. The entire collection consists of 1040 pools of siRNAs, meaning that the screening can be performed manually in a semi-high throughput setup.

### 3.1.6 Discussion

Our primary aim is to identify novel functional interactors for the transcription factor ZEB1 in relation with its EMT inducing capacities. The development of siRNA as a technique for functional genomics and the commercial availability of specific libraries, rendered us the possibility to systematically screen for candidate genes that are necessary to mediate ZEB1 induced EMT. For this purpose, an *in vitro* screening system needed to be created. One of the first prerequisites was that the screening needed to be performed in a cell line that is capable of undergoing an EMT under the influence of ZEB1. We stably introduced inducible ZEB1 expression in the two cancer cell lines MCF-7 and A431 and observed that in both cases ZEB1 was indeed capable of inducing an EMT phenotype after 72-96 h. Because we could not resolve the relatively poor siRNA transfection efficiency in A431, we decided to work further only with the MCF-7 cell line. For the screening readout, several EMT related gene promoters were tested, whereby the vimentin-luciferase reporter came out to be the most effective based on its signal to noise ratio. In addition, we implemented a survival sensor on the basis of the EF1alpha-SEAP cassette. These two reporter systems were stably transfected into the ZEB1 inducible MCF-7 cell line, providing us with a cell system wherein our screening could be conducted. A quality test with siRNA targeting ZEB1 and subsequent calculation of the Z’ factor learned that, although our system might still benefit from some improvements to augment the robustness of our screening system, our MCF-7 cell model is fit enough to perform an siRNA screening.
3.1.7 Materials and methods

Cell culture

Both the MCF-7 and A431 cell lines were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 0.01 mg/ml bovine insulin, 4mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. ZEB1 expression was induced using doxycyclin (1 µg/mL, Sigma, St. Louis, MO). For the FACS sorting, cells were treated with trypsin/EDTA for 10 min and prior to sorting brought into suspension into 50% inactivated FCS in PBS at 4 °C. After sorting, the cells were seeded in their normal culture medium supplemented with 20% FCS.

Immunocytochemistry

Cells (4E5) were seeded on coverslips in a 24 well plate and induced with doxycyclin. Ninety six hours post induction, cells were fixed in 2% paraformaldehyde (PAF) for 20 min. Next, cells were washed three times with PBS and permeabilized for 5 min in 50 mM NH₄Cl. Subsequently, the cells were again washed three times for 5 minutes with PBS, treated with 2 % Triton X100 and finally washed with PBS. Primary antibodies were dissolved in a 0,5 gelatine solution in PBS and put on the cells for 1-2 h. After washing, the secondary antibody was put on the cells for one hour. Cells were washed a final time, mounted and examined under the fluorescent microscope.

Luciferase and SEAP assay

Luciferase was used as a reporter for the activity of the vimentin promoter and hence to quantify the EMT status of the cells during the screening. The luciferase activity was measured with a Tropix Galacto-Star kit (Applied Biosystems, Bedford, MA).
Normalization was done by quantification of the protein content of the wells with the Bio-
rad DC protein assay (Biorad Laboratories, Hercules, CA).

Quantification of the secreted alkaline phosphatase (SEAP) was performed using the
Chemiluminescent SEAP Reporter Gene Assay (Roche Applied Science, Mannheim,
Germany). Luminescence of both the luciferase and the SEAP assay were measured with
the GloMax plate reader (Promega, Madison, WI).

qPCR analysis

Cells were lysed with the Trizol reagent (Sigma-Aldrich, St. Louis, MO) and processed
according to the manufacturers protocol. Subsequently, the obtained mRNA was reverse
transcribed with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). QPCR analysis
was performed using the Lightcycler 480 SYBR green I master mix (Roche, Rotkreuz,
Switzerland).

MTT assay

In a 96 well plate, 10000 cells were seeded per well in a total volume of 100 µl. Four
hours prior to measuring, 20 µl of 5 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-
diphenyltetrazolium bromide (MTT) was added and the cells were left for incubation in
the dark. After four hours, the medium was removed and cells were lysed in 100 µl
DMSO, dissolving the formed formazan crystals. Finally, the absorbance was measured at
a wavelength of 633 nm.

Transient transfection assays

Cells were seeded 24 h prior to transfection in a 6 well plate. As lipofectants, the Fugene
6 reagent was used (Roche, RotKreuz, Switzerland) and instructions according to the
protocol were followed. Twenty four hours post transfection, the medium was refreshed.
siRNA transfection protocol

Cells were seeded 24 h prior to transfection. The Dharmafect I (Dharmacon, Lafayette) lipofectans was used for both the A431 and MCF-7 cell line. Unless specified elsewhere, the used siRNA concentration was 25 nM. As fluorescently labeled siRNA, the siGLO oligo targeting cyclophilin B was used (Dharmacon, Lafayette). The cells were refreshed 10 h post transfection.
3.2 The primary and secondary siRNA screening reveal novel ZEB1 modulating genes

3.2.1 Introduction

In the previous section, we reported the development of an *in vitro* screening system in order to indentify novel functional interactors of the transcription factor ZEB1 during the process of EMT. The used siRNA library targets all known human transcription factors and consists of 1040 siRNA pools. A general overview of the different steps that were carried out in order to come up with a short list of highly potent hits is depicted in Figure 23.
Carrying out the primary screening identified several potential novel functional interactors with a ‘hit ratio’ of 3%. We subsequently carried out a deconvoluting secondary screening. Here, the siRNA pools were replaced by four independent individual siRNAs per target gene. A confirmation of the hits from the primary screening means that at least two out of the four, but preferably three individual siRNAs, could reproduce the phenotype found with the siRNA pools. Furthermore, an expression analysis was performed in order to assess whether our candidate hits can modify ZEB1 induced EMT at the transcript level for EMT marker genes. This experimental setup allowed us to perform a cluster analysis in order to discriminate between major and minor modulators of ZEB1 induced EMT.

To get a better view on the functionality of the hits, we examined how these genes are modulated upon ZEB1 induction in our MCF-7 breast cancer cell system. Finally, with specialized software, a network analysis was carried out showing us the transcriptional or direct interactions between the major hits. As such, we postulate that ZEB1 is the central node of a large signaling complex that is necessary to mediate its function as an EMT inducing transcription factor. A general overview of the discovered hits can be found in Figure 24.
Starting from 1040 siRNAs targeting all known human transcription factors, 27 hits were found in the primary screening. Of these 27 hits, 22 were confirmed in the secondary screening. Nine of these remaining hits were able to significantly alter the ZEB1 induced EMT profile, while four of this latter list could inhibit the ZEB1 induced in vitro migration.

3.2.2 The primary and secondary screening uncovers novel functional interactors of ZEB1

On the basis of our MCF-7 ZEB1 vimentin-EF1alpha cell model, we were able to develop a screening protocol that allows us to investigate novel functional interactors of ZEB1 in relation to its EMT driving capacities. Briefly, 24 h prior to transfection, 10 000 cells are seeded in a well of a 96 well plate. Lipofection is done with 25 nM of siRNA pools and the medium is refreshed after 10h. Subsequently, ZEB1 expression is induced by treating the cells with doxicyclin and both the luciferase and SEAP activity are measured 96h post induction. The complete screening is performed in triplicate, targeting all known human transcription factors. In each screening plate, multiple controls were incorporated: the
positive control with siRNA targeting ZEB1, a negative control with siRNA specifically
developed not to target any gene and a mock transfection where cells are only treated with
the lipofectans. A general setup of the complete screening can be viewed in Figure 25.
After completing the primary screening we found 27 hits that were able to downregulate
the vimentin promoter activity by at least 50% in comparison with the control siRNA
(Figure 26).

Figure 25: General lay out of the primary siRNA screening. a.: Setup of a screening
plate. The first two columns are reserved for the controls. siZEB1: siRNA targeting ZEB1,
siCon: non targeting siRNA, Mock: lipofectans used only. b.: Working scheme of the
screening. Cells are seeded 24h prior to transfection. Medium is refreshed and
doxicycline is added 10h after transfection. The readout is done 96h post induction.

For the majority of the found hits, the EF1alpha promoter does not show reduced activity
in comparison with the non targeting siRNA (siCon) and mock transfection. However,
when looking to the siRNA pools of Pou2F2 and Pou5F1, SEAP values indicate that the
viability of the cells is affected and that the reduction of the vimentin promoter activity is
potentially an aspecific effect.

Interestingly, for the hits Elk4 and FoxN1 the EF1alpha promoter activity could be
restored to the levels that are observed in the positive control. Furthermore, from all the
hits, only siRNA targeting Elk4 was able to counteract the morphological changes that are
associated with ZEB1 induced EMT in our cell system. Quantifying the circularity of the
cells with the software package ImageJ indeed revealed that the average circularity was significantly higher in the cells that were treated with siRNA targeting Elk4 in comparison with the control condition (Figure 27a). At first sight, these findings implicate that Elk4 might be a master regulator of ZEB1 functionality.

Figure 26: Readout of the primary screening. Vimentin-luciferase and EF1alpha-SEAP readout of siRNAs that are capable of reducing the vimentin promoter activity in comparison with the non-targeting siRNA (siCon). Twenty-seven hits out of a total of 1040 siRNA pools were identified. For most of them, the SEAP values were comparable with the non-targeting control. Even more, for the hits Elk4 and FoxN1, the levels of EF1alpha promoter activity were similar in comparison with the positive control siZEB1, furthermore suggesting the functional connection between these factors and ZEB1.

The specificity of the novel candidate hits is further assessed in a secondary, deconvolution screening. Here, the pools are replaced by four independent siRNAs for each identified hit. We consider a hit to be specific when a minimum of two out of four siRNA are able to copy the phenotype that was produced by the siRNA pools. However, preferably, at least three out of four individual siRNAs should be able to mimic the initially found phenotype (Figure 27). As an additional control, we also determined the knock down efficiency of each individual siRNA (data not shown). When handling the
same criteria as the primary screening, namely a 50% reduction of the vimentin promoter activity, 24 out of 27 hits could be confirmed whereby at least two out of four siRNAs fulfilled the criterion. However, from these 24 hits, it has to be noted that the hits BATF and CDX4 are borderline. We have nevertheless included them as positive hits.

Figure 27: **Phenotype analysis and readout in the primary screening and secondary screening.**  
**a.** In the primary screening, only the cells that were transfected with the siRNA pool targeting Elk4 were able to counteract the ZEB1 induced mesenchymal like morphology. This is further reflected in circularity measurements of the cells.  
**b.** For the 27 hits out of the primary screening, a secondary, deconvoluting screening was performed. Instead of using siRNA pools, cells were transfected with four individual siRNAs per target. The colored lines depict the stringency level to score a hit, with green showing low stringency and red, high stringency. NT: non transfected.
3.2.3 Discrimination between hits that are vimentin specific or general EMT regulators

Since the screening is solely based on the vimentin promoter readout, it has to be assessed to what extent the confirmed hits are able to modulate the ZEB1 induced EMT program or act on the vimentin promoter alone. For this purpose, we performed an mRNA expression analysis on genes that are known to be influenced by ZEB1 induction. These genes were identified on the basis of a micro-array analysis of 48h induced MCF-7 ZEB1 cells compared to their non induced counterparts (data not shown). Genes that are significantly downregulated are E-cadherin, Rab25, Eva1 (Epithelial V-like antigen 1), IRF6 (interferon regulatory factor 6), Hook1 and Muc1 (transmembrane mucin 1). Interestingly, an inverse correlation between vimentin and Rab25 has been described in breast cancer cell lines (Vuoriluoto et al., 2011). In addition, Hook1 has been shown to directly interact with different members of the Rab family (Luiro et al., 2004). Strong upregulation is observed for the mesenchymal genes vimentin and Col5a2. Expression of these genes was analyzed for each of the individual siRNAs used in the secondary screening and compared with the siCon and mock controls. Looking to the expression of the vimentin mRNA, only the hits BATF, CDX4, Elk3, Elk4, HoxB6, HoxB7, HoxB9, LHX3, NeuroD6, Pou2F2 and Prop1 fulfill the criteria to be considered as a hit. Thus 11 out of 27 hits are retained. Comparing this to the functional readout of the secondary screening in Figure 27 where 22/27 hits were confirmed, shows that mRNA expression analysis is a more stringent method to assess the specificity of the identified hits from the primary screening. An explanation for these differences can be given by the fact that the readout of our screening model was only based on a relatively short fragment of the human vimentin promoter. Undoubtedly, a myriad of elements that regulate vimentin expression lie outside this region and are taken into account when performing an mRNA expression analysis. A further reduction of the hits is achieved when taking into account the E-cadherin expression profile. Indeed, if our aim is to identify genes that are responsible for ZEB1 induced EMT, assessing the status of E-cadherin is of crucial importance since this can be considered as a critical parameter for the establishment of epithelial phenotype in MCF-7.
Strikingly, only a minority of the hits confirmed in the secondary screening are effectively able to upregulate E-cadherin expression in the presence of ZEB1. These are BAZ1B, Elk3 and Elk4. In combination with the screening data, these findings strongly suggest that these genes are implicated in the modulation of ZEB1 induced EMT in MCF-7 cells.

To get a better overview of the data presented in Figure 28, we constructed a heatmap by taking the average expression value of the four individual siRNAs. This permitted us to cluster the expression profiles of the different hits. As such, it becomes clear that the collection of primary hits can be divided into two main groups, namely the hits that are capable of disturbing a relatively large proportion of the ZEB1 induced gene expression and a second group that only has a minor influence on this EMT profile (Figure 29a). Overall, apart from being able to modulate the E-cadherin expression, silencing of Elk3 and Elk4 seems to have the most significant influence on the ZEB1 induced EMT profile. Indeed, Elk3 and Elk4 knock down is able to disturb the ZEB1 gene modulation in seven out of eight cases, strongly resembling the epithelial or non induced profile. Only modulation of the gene Hook1 does not seem to be affected by knock down of Elk3 and Elk4. Strikingly, within the group of hits that is able to strongly influence the ZEB1 induced expression profile, Elk3 and Elk4 form a separate category in relation to the other hits of the group. Indeed, the next best hit seems to be Prop1 being able to influence the modulation of five out of eight genes. Also, the transcription factor SRF seems to play a role in the functionality of ZEB1 as silencing results in modulation of four ZEB1 modulated genes.

Figure 28: mRNA expression analysis of ZEB1 modulated genes under influence of confirmed hits. MCF-7 ZEB1 cells were transfected with four individual siRNAs per hit and subsequently induced with doxycyclin. RNA lysates were made 96h after induction.
The rest of this group is only able to modulate three genes or less, indicating that these factors play a less outspoken role in the ZEB1 functionality in comparison with Elk3, Elk4, Prop1 and SRF. The second and largest group contains the hits which only seem to have a minor influence on the ZEB1 controlled gene expression profile. Indeed, several members of this group only seem to have an influence on vimentin mRNA expression and are likely not involved in the direct modulation of a general EMT program. These hits are: FoxN1, ZNF18, E2F5, LEF1, LHX1, YBX1, Baz1B and KLF1.

The cluster analysis gave us an idea about which hits are able to modulate the ZEB1 induced EMT program. However, at this point we did not know what the functional implications are when knocking down these targets. To assess this functional aspect, we performed an in vitro migration assay with two independent siRNAs per target that came out as the most efficient in the secondary screening and expression analysis. The principle of this particular migration assay is not based on the prevailing transwell method. Instead, cells are seeded in a 96 well plate in the presence of a so called “stopper” that prevents the cells from attaching to the inner, circular region of the well. After the cells have attached, the “stopper” is removed and migration can be visually quantified. By measuring the migrated area with image processing software and comparing this to the control conditions, a migration index is calculated (Figure 29b)
Silencing of C10Orf48, Elk3, Elk4, EN2, HoxB7, RelB, SRF and TCF3 had a negative influence on the migratory capacities of the induced MCF-7 ZEB1 cells. Five of these eight hits can also be found in the cluster of the genes that are able to significantly alter the ZEB1 induced expression profile (Figure 29a). Remarkably, three additional genes, C10Orf48, EN2 and HoxB7 that cannot be found in this list are nevertheless able to modulate the ZEB1 mediated migration. Furthermore, it has to be noted that for the hits Pou6F1, Pou5F1, ZNF142 and Prop1, which were also clustered in this group, no influence on the migration can be observed. This suggest that, although five hits were found to modulate the ZEB1 EMT profile on one hand and are able to alter the migratory potential of the induced MCF-7 ZEB1 cells on the other hand, the selected ZEB1 EMT expression profile might be too restricted in order to fully predict the influence of the discovered hits in the disturbance of ZEB1 functionality. To what extent we would have to expand this profile in order to have a more penetrant predictability remains unclear, since one has to take into account the possibility that the major driving forces of this migratory behavior are not necessarily to be found at the mRNA level. To further refine the functional implications that arise upon silencing of these hits, we decided to perform a western analysis on a limited set of proteins that are often implicated in a general EMT phenotype (Figure 30).

Figure 29: **Functional validation of the primary hits.** a.: Silencing of the hits reveals a differential expression profile of known ZEB1 modulated genes. Subsequent cluster analysis divided the primary hits into two groups, namely genes that had little or no influence on the expression profile and genes that were capable of modulating the ZEB1 induced EMT profile. b.: In vitro migration assay on ZEB1 induced MCF-7 cells. The two most efficient siRNAs were used to determine the effect on migration.
For this purpose, we assessed expression levels of the proteins E-cadherin, ZO-1 and vimentin when several hits that influence the migratory capacity of the induced MCF-7 ZEB1 cells were silenced. When looking to the expression levels of E-cadherin, it seems that the three hits RelB, EN2 and SRF are in part contributing to the downregulation of E-cadherin in the induced MCF-7 ZEB1 cells. For ZO-1, only an upregulation can be observed with the knockdown of EN2 and RelB. Silencing of SRF does not appear to result in a significant upregulation of ZO-1. Last, the regulation of vimentin protein expression is not dependent on RelB and EN2. For EN2 this is contradicting what we have found at the mRNA level while assessing the ZEB1 induced EMT profile. Here, knocking down EN2 indeed resulted in the downregulation of the vimentin mRNA. One possible explanation for this discrepancy might be that the regulation of vimentin at the protein level is a relatively slow process, reflected in a low turn over of this protein.

Figure 30: **Western analysis of several major hits.** MCF-7 ZEB1 cells were transfected with the selected siRNAs and subsequently induced. Lysates were made 96h post induction and stained for E-cad (E-cadherin), ZO-1 (Zona occludens 1), Vim (vimentin), ZEB1 and Tub (Tubulin) was used as loading control.
However, the knock down of SRF shows that this is enough to partially downregulate vimentin protein expression, which at first sight opposes the fact that regulation of vimentin is a slow process. It has to be noted that the protein SRF is a very general transcription factor, implicated in a wide variety of cellular processes. For instance, SRF is responsible for the regulation and activation of immediate early genes after stimulation of cells with different growth factors. This implicates a role for SRF in apoptosis, cell cycle regulation and cell differentiation. Indeed, the serum response factor regulatory element has been documented in well over 300 genes (Posern & Treisman, 2006). Furthermore, SRF has been implicated in the migratory behaviour of cells. SRF knock out mice are unable to form a mesoderm layer during embryogenesis (Modak and Chai, 2010). These findings implicate that SRF covers a broad spectrum of regulatory networks and might explain why indeed SRF is capable of regulating vimentin protein expression at the timepoint of 96h, while this does not seem to be the case for EN2. Western blot analysis of the transcription factor Elk4 will be discussed in the next chapter.

### 3.2.4 Kinetic expression analysis of the hits reveals their response to ZEB1 induction

An additional analysis that was performed was to assess the effect of ZEB1 induction on the expression levels of the discovered hits. Although we previously carried out a micro-array analysis where we compared induced MCF-7 ZEB1 cells with their non-induced counterparts, it is generally known that detection of low abundant mRNA as the ones coding for transcription factors might be difficult due to assay noise (Duggan et al, 1999). Therefore, we decided to perform a qPCR expression analysis where we evaluated the expression of the hits under the influence of ZEB1 at discrete time points (of E-cadherin mRNA, even when these additional EMT inducing factors are still present. Comparing this to the partial influence ZEB1 seems to have on the regulation of vimentin expression, suggests that although ZEB1 is a major contributor to the EMT phenotype, other regulatory mechanisms orchestrate the regulation of vimentin. The fact that E-cadherin can be upregulated by disturbing the functionality of one transcription factor, in this case
ZEB1, might reflect the epithelial origin of these cells. As is depicted in Figure 31a, the expression of both ZEB1 and vimentin mRNA increased in time after treatment of the MCF-7 ZEB1 cells with doxycyclin, indicating the functionality of the inducible cell line in this particular experiment. Interestingly, the increase of vimentin mRNA can only be observed after 48h, whereas ZEB1 induction can already be seen after 24h. This is indicative for the fact that vimentin is a target gene of ZEB1, although it is not known whether this is a direct or indirect effect. Induction of ZEB1 in the MCF-7 ZEB1 cells leads to a high increase of the levels of Elk3 mRNA. This also seems to be the case for the transcription factor TCF3 (E12/ E47), albeit to a lesser extent. Interestingly, TCF3 has already been extensively described as an EMT inducer (Sobrado et al, 2009b). As such, it becomes clear that in the inducible MCF-7 ZEB1 model a certain hierarchy seems to exist between different EMT inducing factors. Given the fact that other EMT inducing transcription factors were not picked up from the screening suggest that in our model only the ZEB1-TCF3 cascade seems to have functional implications in relation to EMT in our cell system.
Figure 31: Kinetic analysis of several major hits upon ZEB1 induction. a.: ZEB1 and vimentin mRNA levels both rise upon induction of the MCF-7 ZEB1 cells with doxycyclin, reaching a maximum at 72 h. b.: Time point analysis of the mRNA expression analysis of several hits which were able to disrupt ZEB1 enhanced migration when knocked down.

However, the strongest induction that could be observed upon induction of ZEB1 was for the transcription factor Elk3. Interestingly, in a panel of 51 breast cancer cell lines, increased levels of Elk3 mRNA could be detected in those particular cell lines that are part of basal B subgroup of breast cancer cell types. Breast cancer cells that are categorized in the basal B group display a significant shift towards a mesenchymal gene expression profile and have an increased invasive potential. Even more striking is the fact that all the cell lines of the basal B group have high levels of ZEB1 mRNA, further confirming our discovered connection between Elk3 and ZEB1 (Blick et al, 2008).

When looking to the other genes, these do not seem to undergo dramatic changes in mRNA expression levels in the ZEB1 induced cells. Interestingly, both Elk3 and Elk4 are
members of the ternary complex factors that form complexes with the transcription factor SRF and as such modulate growth factor signaling. The sequence similarity of both factors is 47%, whereby the A or ETS domains are 87% similar. Although both Elk3 and Elk4 are able to form a complex with SRF, the C-terminal domain of Elk3 has been shown to inhibit this interaction (Price et al, 1995a). Furthermore, Elk3 contains an additional inhibitory domain which is not present in Elk4 and it has as such been shown that Elk3 is a potent transcriptional inhibitor while Elk4 is considered to be an activator. These differences between Elk3 and Elk4 might thus explain the fact that only Elk3 gets upregulated under influence of ZEB1. However, in the presence of activated Ras, Elk3 can switch from a repressor to an activator and a direct interaction between Elk3 and TCF3 has been shown to exist in COS cells (Maira et al, 1996). Interestingly, both Elk3 and TCF3 show an upregulation upon ZEB1 induction.

Strikingly, RelB shows a significant decreased expression upon ZEB1 induction, which is counter intuitive for its putative function in ZEB1 mediated EMT. RelB is a family member of the NF-κB family of transcription factors and is involved in non-canonical NF-κB signaling (Bakkar & Guttridge, 2010). Its impact on invasive behavior of cancer cells has recently been uncovered. Indeed, overexpression of RelB in non invasive MCF-7 breast cancer cells induced a scattered and migratory phenotype. This was moreover associated with a loss of E-cadherin and an increase of vimentin expression at the protein level. Conversely, silencing of RelB in the invasive breast cancer cell line MDA-MB231 resulted in decreased migration associated with the upregulation of E-cadherin and downregulation of fibronectin at the protein level (Wang et al, 2007a). These findings certainly suggest a central role for RelB in the onset and or maintenance of the EMT phenotype and confirm the validity of being a hit in our siRNA screening. A possible explanation for the fact that RelB mRNA is nevertheless downregulated under influence of ZEB1 expression can be found when looking to the other members of the NF-κB family. It has indeed been described that a negative feed back loop exists that autoregulates the levels of active NF-κB, which is as such responsible for a fast turn over of an NF-κB response. Both direct and indirect mechanisms have been described that mediate this negative feed back (Hofmann & Schmitz, 2002; Jono et al, 2004). It might thus be plausible to state that a similar mechanism takes place with RelB in our induced cell system. Initial activation of the RelB functionality upon ZEB1 induction might thus
result in a negative feedback loop leading to dramatically lower levels of RelB. However, these low levels of RelB still seem to be high enough to exert a functional impact on ZEB1 induced EMT.

In addition to the kinetics experiment, we also wanted to investigate whether these findings hold true in a reverse setup. For this purpose, we knocked down ZEB1 with siRNA (siZEB1) in the mesenchymal like breast cancer cell line MDA-MB231, which has high endogenous levels of ZEB1 and also can be classified under the Basal B group. Lysates were made 96h after transfection and mRNA expression of the above described hits was assessed (Figure 32). Interestingly, while overexpression of ZEB1 in our MCF-7 ZEB1 cell model was enough to induce vimentin expression, the opposite statement does not seem to hold true in the MDA-MB231 cells. Indeed, silencing ZEB1 only partially resulted in a decrease of the vimentin mRNA levels (Figure 32a). The fact that other EMT inducing transcription factors such as ZEB2 and Slug are coexpressed in this cell line, might explain this partial effect (see thesis dr. Cindy Vandewalle). However, one has to take into account that this does not seem to be true for other EMT related genes. For instance, it has been reported and independently confirmed in our lab that knocking down ZEB1 in MDA-MB213 cells results in a dramatic increase of E-cadherin mRNA, even when these additional EMT inducing factors are still present. Comparing this to the partial influence ZEB1 seems to have on the regulation of vimentin expression, suggests that although ZEB1 is a major contributor to the EMT phenotype, other regulatory mechanisms orchestrate the regulation of vimentin. The fact that E-cadherin can be upregulated by disturbing the functionality of one transcription factor, in this case ZEB1, might reflect the epithelial origin of these cells. This further stipulates that downregulation of E-cadherin is a relatively difficult, but necessary step in order for epithelial breast cancer cells to undergo an EMT.

In contrast to what might be deduced from our forward MCF-7 ZEB1 cell model, knocking down ZEB1 in MDA-MB231 does not result in a significant decrease of Elk3 levels. This is also not the case for the EMT inducing transcription factor TCF3 (Figure 32b). Again, these findings can be explained by the presence of endogenous ZEB2 and Slug levels in the MDA-MB231 cells that prevent downregulation of these factors or are able to compensate for the loss of ZEB1. Strikingly, both expression of Elk4 and EN2
seems to be affected by ZEB1 silencing. On the basis of the forward experiments that were carried out in MCF-7 ZEB1, these findings were not expected. Since both Elk4 and EN2 mRNA expression was not influenced by induction of ZEB1 in the MCF-7 model, we presumed this would also not be the case in the reverse MDA-MB231 model. One explanation might be the ZEB1 dependence of MDA-MB231 cell survival that does not take place in MCF-7. Indeed, several reports have suggested that the occurrence of EMT during advanced stages of cancer development significantly increases the survival capacities of tumor cells (Barr et al, 2008; Thomson et al, 2005).

Figure 32: **Effect of ZEB1 knock down on mRNA expression of several hits in MDA-MB231 breast cancer cells.**

a.: Knock down efficiency of ZEB1 in comparison with a scrambled siRNA (siCon) is more than 80%. Levels of vimentin mRNA decrease partially upon ZEB1 silencing. Lysates were made 48h after siRNA transfection.

b.: Expression levels of several hits upon ZEB1 knock down.
In addition, different cell types that have undergone EMT are less prone to the effects of different chemotherapeutic substrates (Robson et al, 2006). Moreover, treatment of cancer cells with chemotherapeutic reagents has been shown to induce EMT itself. The underlying rationale behind this is that additional survival pathways are activated when cells undergo EMT and as such escape the apoptotic effect of the drugs (McMorrow et al, 2005). Furthermore, expression of ZEB1 has been shown to inhibit replecative senecence in cancer cells and in mouse models (Browne et al, 2010). Taking these findings back to the MDA-MB231 model suggest that the high expression of ZEB1 is responsible for these anti-apoptotic effects. Indeed, it has been confirmed in our lab that prolonged inhibition of ZEB1 in MDA-MB231 leads to a high incidence of cell death. In addition, the transcription factor Elk4 is widely known to be a mediator of MAPK and pro-survival signaling (Janknecht & Hunter, 1997). The fact that Elk4 expression is under control of ZEB1 in MDA-MB231 might thus reflect this pro-survival signaling that occurs during EMT. Since our MCF-7 ZEB1 model did not have to undergo an in vivo EMT as the MDA-MB231 cells did, there has been no selective pressure for these pro-survival pathways, thus resulting in the modulation of less effectors in comparison to the MDA-MB231 cells.

3.2.5 Pathway analysis of the hits suggests the existence of a multimeric protein complex with ZEB1 as a central player

Previous analysis has already shown some connections between ZEB1 and the discovered hits. However, to get a more clear picture of the functional interactions between the hits and ZEB1, pathway analysis was performed using the Cytoscape software package (Cline et al, 2007). The network analysis indeed revealed interesting connections (Figure 33) and suggests the existence of a multimeric complex with ZEB1 and Elk4 as central nodes.

It has already been extensively described that ZEB1 is able to interact with the CtBP cofactor. Interestingly, also Elk3 has been shown to form a complex with CtBP. Whether CtBP can act as an adaptor protein to establish a physical link between ZEB1 and Elk3 cannot be derived from the network analysis. The fact that CtBP is a protein that is used
by a multitude of transcription factors to exert their proper function according to the cellular context might indeed contradict the existence of a ZEB1-Elk3 complex. Indeed, both ZEB1 and Elk3 have been independently shown to bind to CtBP in order to exert transcriptional inhibition (Criqui-Filipe et al, 1999; Postigo et al, 2003).

Figure 33: In silico analysis of the discovered hits in relation to ZEB1. Cytoscape network analysis of several of the discovered hits. Full lines between nodes indicate a physical interaction has been reported. Arrows point to transcriptional activation. The dotted lines between ZEB1 and Elk3/Elk4 depict a physical interaction that was found by immunoprecipitation and will be discussed in the next chapter.

Elk3 or Net is a member of the Ets transcription factors whose transcriptional repressing capacities are partly dependent on CtBP. However, in the presence of activated Ras, Elk3 becomes an activator (Criqui-Filipe et al, 1999). These findings highlight the dualistic functionality of this transcription factor that might play an important role during cancer
progression. Indeed, many tumors harbor a constitutively activated Ras pathway. Apart from disturbing the normal growth homeostasis of the cell, active Ras enables the switch of Elk3 from general repressor to activator. Based on our findings coming out of the screening, this might have a significant influence on the further onset of EMT.

An additional major player in the *in silico* constructed network is Elk4. The structural connection with ZEB1 can be made via the transcription factor SRF. Indeed, Elk4 has been first described as a member of the ternary complex factors that are responsible for the correct function of SRF. Hence its alternative name, serum accessory protein 1 (Sap1). Interestingly, Elk4 is unable to bind to DNA autonomously and always requires SRF for this purpose. The region of SRF that interacts with Elk4 to form a ternary complex has been mapped to the DNA binding domain of SRF. For Elk4 to form a complex with SRF, the Ets domain and a conserved region of 50 amino acids at the C terminus are necessary (Dalton & Treisman, 1992). Furthermore, Elk4 has been described to be a central signaling protein in p38 MAPK and JNK signaling (Janknecht & Hunter, 1997; Price et al, 1995b). Target genes of the Elk4-SRF ternary complex are c-fos and several EGR genes (Clarkson et al, 1999). The fact that a multitude of growth factors of which the signalisation is relayed via the MAPK pathway, have been shown to provoke an EMT in several cell systems, further confirm the robustness of Elk4 as a hit coming out of our screening (see introduction).

Furthermore, a direct link between SRF and ZEB1 has been shown during the process of vascular smooth muscle cell differentiation (Nishimura et al, 2006). While ZEB1 on itself is not capable of transactivating the alpha smooth muscle actine promoter and SRF can only do this very weakly, the combination of both factors leads to a significantly higher promoter activity. The interaction domain of ZEB1 has been located to the C-terminal domain, while SRF interacts with ZEB1 via its MADS domain (Nishimura et al, 2006). Interestingly, alpha smooth muscle actin is considered to be a mesenchymal marker and has been extensively reported in cases of fibrotic disease and with TGFbeta induced EMT (Masszi et al, 2003; Zavadil & Böttinger, 2005). It has furthermore been shown that c-fos, together with SRF, is highly overexpressed during late stages of skin carcinogenesis and that H-Ras transgenic mice lacking c-fos are unable to display malignant transition of skin cancer (Saez et al, 1995; Zoumpourlis et al, 2000). Also, the capacity of SRE binding
of SRF is dramatically increased in aggressive carcinoma cell lines that have undergone an EMT. In addition to this enhanced activity, overexpression of SRF could be detected only in these mesenchymal-like cell lines (Psichari et al, 2002).

Elk4 seems to be the central node that connects several other hits with each other. For instance, a major target gene of Elk4 is the early response gene EGR1, which has been shown to be responsible for upregulation of the EMT inducing transcription factor Snail. By making use of a dominant negative form of EGR1, Snail induction and subsequent EMT could be abolished in HGF treated HepG2 cells (Grotegut et al, 2006).

Further connections between hits are established via the Id proteins. Indeed, Id1-3 have been shown to directly interact with Elk4. The Id proteins have been extensively studied in the frame of TGFbeta signaling and are generally considered as negative modulators of this pathway. Ectopic overexpression of Id2 in Nme cells is able to inhibit the TGFbeta induced EMT and growth delay (Kondo et al, 2004). Interestingly, the Id proteins are both negative modulators of the TGFbeta and SRF pathway. The physical interaction between the Id proteins and the ternary complex factors such as Elk4 occurs via the DNA binding domain of the latter. As such, the ternary complex comprising of Elk4 and SRF is inactive and has been shown to be incapable of binding to and activating the SRE located on the c-fos promoter. Similar observations have been found for Elk3 (Yates et al, 1999). The fact that no mediator of TGFbeta signaling came out of the screening suggests however that in our system TGFbeta does not play a central role in the maintenance or onset of ZEB1 mediated EMT.

Additionally, the EMT inducing transcription factor TCF3 is capable of physically interacting with both Id1 and Id3 and has been described during the process of muscle differentiation. The biological impact of this heterodimer formation is dual. While binding of TCF3 with Id3 prevents its DNA binding capacities, interaction with Id1 has been shown to prevent complex formation of TCF3 with MyoD during muscle differentiation (Lingbeck et al, 2008; Loveys et al, 1996). Whether the TCF3-MyoD complex has a significant implication on EMT has not been investigated yet. Furthermore, TCF3 has been found to form heterodimers with the transcription factor Twist1 (Connerney et al, 2008). Again here, Id proteins are able to disturb this interaction, since the Twist1-TCF3 binding affinity is smaller than that of the Id-TCF3. In the absence
if TCF3, Twist1 forms homodimers, which enhance osteogenesis in the cranial structures during development (Connerney et al, 2008).

In conclusion, the connection between several hits has been well established and indicates that the SRF-TCF axis is of major importance for ZEB1 induced EMT in our MCF-7 cell model. Interestingly, on the basis of our in silico network, the Id proteins seem to be central negative modulators of EMT in our screening system. However, our siRNA collection contained smartpools targeting Id1 and Id2, but not Id3. If these proteins would be major inhibitors of ZEB1 induced EMT, then silencing these factors would have a positive influence of the vimentin promoter activity. This was however not the case (see next section), indicating that in our case, Id1 and Id2 are not major EMT inhibiting proteins.

3.2.6 The primary screening identifies potential novel inhibitors of ZEB1 induced EMT.

The discovered hits we identified in the previous section are all considered to be functional interactors of ZEB1 and can therefore be considered as EMT enhancing factors. Indeed, they were selected on the basis of their capacity to reduce the vimentin promoter activity at least 50% when silenced. In contrast to these findings, we also discovered several genes that are able to enhance the vimentin promoter activity upon knock down. This implicates that these genes can be considered to be inhibitors of the vimentin promoter and in extension might be general EMT inhibiting factors (Figure 34). Since these candidate genes did not fall into the scope of this research project, we did not assess what their effect was on the EF1alpha promoter.

Of particular interest is the fact that knocking down estrogen receptor 2 (ESR2) results in upregulation of the vimentin promoter activity. In prostate cancer, ESR2 has been described as a general marker for differentiation. Indeed, the growth phase of the prostate during development is characterized by high expression of estrogen receptor 1 (ESR1). However, when the proliferative stage is terminated and the differentiation stage is initiated, a shift from ESR1 to ESR2 expression can be observed (Imamov et al, 2005).
Moreover, transgenic mice which express the SV40 early genes in a prostate specific context that were treated with a specific ESR2 agonist displayed a significantly higher number of well differentiated prostate cancers when compared to the untreated controls which showed a strong dedifferentiated phenotype (Mentor-Marcel et al, 2001).

Figure 34: Readout of primary screening for siRNAs showing upregulation of the vimentin promoter activity. siRNA targeting ZEB1 was used as a positive control. The vimentin promoter activity of the siCon (non targeting siRNA) condition of the induced MCF-7 Vimentin-EF1alpha cells was set at one and is comparable to the non transfected control (NT). Knock down of the listed genes resulted in a significant increase of the vimentin promoter activity. The readout was performed 96h post induction of the cells.

When looking to breast tissue, a similar conclusion can be made. In mammary gland tissue of human and mice origin, ESR2 is more abundantly expressed than ESR1. Moreover, ESR2 null mice display abnormal growth of breast epithelium, characterized by overexpression of the proliferation marker Ki67. In addition, mice lacking ESR2 are more prone to developing severe cystic breast disease as they age (Jensen et al, 2001). These findings indicate that ESR2 can play a protective role in the onset and development of cancer and can thus be regarded as a general tumor suppressor. Since cells that are undergoing an EMT are considered to enter a phase of dedifferentiation, ESR2 expression
in the induced MCF-7 ZEB1 cells might indeed have the potential to restrict the EMT inducing effects of ZEB1.

Apart from ESR2, silencing of the retinoic acid receptor gamma (RARG) shows a similar effect on the vimentin promoter activity. RARG is also regarded as a general marker and effector or cellular differentiation. Squamous carcinoma cells overexpressing RARG show growth inhibition and have a higher expression of the terminal differentiation markers involucrin, cytokeratine 1 and transglutaminase I. Moreover, expression of the EGFR was significantly lower upon RARG expression (Oridate et al, 1996). A similar effect can be observed for human embryonal carcinoma and neuroblastoma cells (Marshall et al, 1995; Moasser et al, 1995).

Another gene that can be found in this list is the max binding protein (MNT), which is a negative regulator of the transcription factor myc. Interestingly, myc has been shown to be implicated in the onset of EMT in diverse model systems. For instance, micro-array analysis of mammary tumors originating from MMTV-c-myc mice revealed an EMT like expression profile and morphology (Liu & Habener, 2009; Trimboli et al, 2008). Furthermore, myc has been shown to be a critical factor in Snail induction of TGFbeta treated NMuMG cells (Smith et al, 2009a). Also, myc is only able to bind E-box target sequences when it is bound to the cofactor Max. However, a Max-MNT interaction can sequester Max, preventing its binding with Myc, which impairs its functionality (Walker et al, 2005). Being a negative regulator of myc functionality, MNT can thus be considered as a factor that is able to mitigate or prevent the EMT inducing effects in our inducible cell system.

Whether these genes only have an effect on the vimentin promoter or are general inhibitors of ZEB1 induced EMT remains to be investigated. Alternatively, specifically in the case of activation of ESR2 and RARG, a general driving force towards differentiation might counteract EMT in a general way, which is aspecific in relation to ZEB1. The fact that several of the found hits are related to cellular differentiation already gives a good indication that these identified genes are indeed valid candidates that have EMT mitigating capacities. However, since our primary focus was set on the genes that are necessary for ZEB1 induced EMT, we did not further pursue these candidates that seem to have an opposite function in relation to ZEB1.
3.2.7 Discussion

We performed an in vitro siRNA screening with the inducible MCF-7 ZEB1 Vimentin-EF1alpha model. The siRNA library consisted of 1040 smartpools targeting all known human transcription factors. The primary screening yielded 27 potential functional interactors of ZEB1. Next, a secondary screening was carried out, whereby the siRNA pools were replaced by four individual siRNAs per target. Twenty four out of 27 primary hits could be confirmed, whereby at least two out of four siRNAs are able to suppress the vimentin promoter activity by at least 50%. However, the screening readout does not provide enough information to make the conclusion there is a general perturbation of the ZEB1 functionality. To identify the hits that only have a minor influence on the ZEB1 induced EMT related transcriptional reprogramming from those that are only able to influence the vimentin promoter activity we performed a qPCR analysis on genes that are known to be modulated by ZEB1. Clustering these different profiles for the remaining hits revealed the existence of two distinct groups. The largest group consists of hits that are only marginally capable of modifying the selected mRNA profile. In contrast, the second group, consisting of nine genes, is able to alter the ZEB1 dependent EMT profile to a significant extent. Additionally, an in vitro migration assay was performed in order to assess the functional implications silencing of the hits have. Seven hits were identified to be able to disrupt ZEB1 induced migration, whereby four of these can be found in the cluster of hits that are able to significantly modulate the ZEB1 induced profile.

Strikingly, only silencing the ternary complex factor Elk4 resulted in the conservation of the epithelial phenotype of the ZEB1 induced ZEB1 cells. This furthermore resulted in a significant restriction of the ZEB1 modulated EMT profile at the mRNA level and lead to inhibition of in vitro migration. These findings strongly suggest that Elk4 is a key signaling protein in order for ZEB1 to exert its full functionality. Together with the fact that the transcription factors Elk3 and SRF, also both members of the ternary complex, this suggests that the SRF signaling pathway is a central player in ZEB1 functionality.

However, the majority of the primary hits are only able to alter the ZEB1 induced EMT to a minor extent in our screening system. The changes occurring in the ZEB1 induced EMT profile due to silencing of these hits suggests that EMT cannot be considered as a discrete
system which can only occur in an on and off state. It seems that EMT is a gradual, plastic process that is dependent on the regulation of a multitude of different genes. Among these genes are major regulators, such as ZEB1 and Elk4 in our case, and less important genes that contribute in a very specific way to EMT. This is further reflected in the fact that overexpression of different EMT inducing factors in mammary carcinoma cells induce different mRNA profiles, while EMT nevertheless takes place in all cases. Interestingly, an EMT core signature could be detected that is similar for all used EMT inducing transcription factors, while a multitude of genes are still differentially regulated (Taube et al, 2010).
3.2.8 Materials and methods

Dissolving the siRNA library

The total amount of ordered siRNA pools was 0.5 nmoles and was delivered in a lyophilized form. Since the screening was to be performed in a 96 well format, dissolving the library in too high concentrations would imply very small pipetting volumes. For this purpose, we decided to work with a working concentration of 500 nM. The lyophilized siRNA pools were dissolved in 250 µl of 1X siRNA buffer (Dharmacon, Lafayette), leading to a stock concentration of 2µM. From this volume, 125µl was kept as a back-up, while the other half was used as working stock. For this purpose, the 2µM stock was diluted to 500 nM and aliquoted into five 96 well plates, which were subsequently used as working plates.

siRNA transfection under screening conditions

MCF-7 ZEB1 Vim-EF1alpha cells were seeded 24h prior to transfection in a 96 well plate. A total number of 10 000 cells were seeded in a total volume of 250 µl penicillin/streptomycin free medium. This initially higher volume of 250 µl of medium results in a homogeneous spreading of the cells. The medium was refreshed after 24h with a volume of 55 µl. For the preparation of the siRNA transfection master mix for one 96 well plate, 24 µl of Dharmafect I (Dharmacon, Lafayette) was added to 610 µl of serum free medium (SFM) and incubated for 5 minutes at room temperature. In parallel, a siRNA master plate was made by transferring 3,2 µl of siRNA pools out of the working plate of 500 nM to an empty round bottomed 96 well plate and 3,2 µl of SFM was added. After incubating 5 min at room temperature, a total volume of 6,4 µl of the Dharmafect I master mix per well was added to the siRNA master plate. The siRNA-Dharmafect mix was incubated for 20 min at room temperature and subsequently added to the 96 well plate with the seeded cells which were subjected to a final siRNA concentration of 25 µM. After 10h the transfection medium was refreshed with doxicyclin containing medium in order to induce
ZEB1 expression. For the deconvolution screening, the individual oligos were On Target Plus oligos (Dharmacon, Lafayette) which are chemically modified in order to minimize off target effects. However, due to suboptimal silencing efficiencies at 25 nM we decided to use a final concentration of 50 nM.

Determination of the ZEB1 induced EMT profile by means of qPCR analysis

Cells were lysed with the Trizol reagent (Sigma-Aldrich, St. Louis, MO) and processed according to the manufacturers protocol. Subsequently, the obtained mRNA was reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). QPCR analysis was performed using the Lightcycler 480 SYBR green I master mix (Roche, Rotkreuz, Switzerland). The primers were designed with the PrimerExpress 1.0 software (Life Technologies, Carlsbad, CA). The following primers were used:

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Cluster analysis

A hierarchical clustering analysis was performed on the mRNA expression data of the ZEB1 induced EMT profile. This was carried out with the statistical software package R (R development core team, Vienna, Austria). Briefly, the mRNA expression values of the individual siRNAs were averaged. As such, per hit, a vector of length eigth (the number of genes in the investigated EMT profile) was obtained. These hit specific vectors were used to calculate a distance matrix, which was used to perform the hierarchical clustering algorithm of the R software package (hclust).
Western blot analysis

Cell lysates were made in 1X Laemmli buffer (for 10 ml: 5 ml H2O, 1 ml glycerol, 230 mg SDS, 5 ml TrisHCl 0.125M) supplied with protease inhibitors (Complete inhibitor, Roche, Rotkreuz, Switzerland). Lysates were sonicated and protein concentration was measured with the Bradford protein assay (Bio-Rad, Hercules, CA). The following primary antibodies were used:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cadherin</td>
<td>HECD1 monoclonal</td>
<td></td>
</tr>
<tr>
<td>ZO-1</td>
<td>Rabbit polyclonal</td>
<td>Zymed Laboratories</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Rabbit polyclonal</td>
<td>Genetex</td>
</tr>
<tr>
<td>ZEB1</td>
<td>Mouse monoclonal</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Beta tubulin</td>
<td>Mouse monoclonal</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

In vitro migration assay

The Oris cell migration assay (Platypus Technologies, Madison, WI) was used in order to assess the migratory potential of the MCF-7 ZEB1 cells. The assay was carried out in a 96 well plate. The cells were detached and seeded at a density of 30 000 cells per well in the presence of the stopper mechanism that is provided in the kit. After attachment of the cells, the cells were transfected with the individual siRNAs as explained above. Ten hours post transfection, the cells were refreshed by medium containing 1% fetal calf serum (FCS) and doxycyclin to induce the cells and the stoppers were removed. At the measuring time point, the cells were visualised with calcein AM provided by the kit. Briefly, the medium was replaced by calcein AM solution (0.5 mM) in Ca2+ containing PBS and incubated at 37°C for 30 minutes. Subsequently, the cells could be visualized fluorescently using the green filter. After measuring, the calcein Am solution was replaced by normal cell medium with 1% FCS and doxycyclin until the next measuring time point.
With the image processing software Image J a migration index was calculated. At time point zero, a picture of the cells was taken and the non invaded area was measured (A0). The same was done at a specific measuring timepoint (A1). As such, the migration index was calculated by using the formula \((A0 - A1)/A0\).

Figure 35: *Schematics of the Oris cell invasion assay.* Initially, the wells are coated with the ECM substrate. After solidification, a stopper is applied to prevent cells from attaching to the substrate in the middle of the well. Next, the cells are seeded and after attachment to the ECM substrate, the stopper is removed and an additional layer of ECM substrate is added to bring the cells in a three dimensional scaffold.
3.3 The transcription factor Elk4 is a central player in ZEB1 induced EMT.

3.3.1 Introduction

In the previous section, several candidates were identified as being functional interactors of the transcription factor ZEB1. Although different hits were later confirmed in a secondary screening and in additional experiments, only knock down of Elk4 had the capacity to retain the epithelial morphology of induced MCF-7 ZEB1 cells. Accordingly, together with its family member Elk3, silencing of Elk4 had the most profound influence on a selected ZEB1 EMT related mRNA expression profile. Furthermore, the migratory capacities of the induced MCF-7 ZEB1 cells were severely compromised when levels of Elk4 were reduced. These findings strongly indicate a central role for Elk4 in ZEB1 induced EMT, which prompted us to further investigate its functionality. To assess the influence of Elk4, an initial western blot analysis was performed for key EMT markers in induced MCF-7 ZEB1 cells. This was further extended to dedifferentiated carcinoma cell lines with endogenous expression of ZEB1. In addition, in vitro migration and invasion assays were carried out in these cell lines in order to investigate to what extent knock down of Elk4 would influence these malignant properties. Finally, we wanted to know whether Elk4 and ZEB1 function as two parallel signaling cascades towards EMT or whether the possibility exists that these two factors can be found in the same signalisation pathway.

3.3.2 Elk4 is able to disturb the effect of ZEB1 on key EMT markers at the protein level

In order to deepen our understanding of the functionality of the transcription factor Elk4 in relation to EMT, we wanted to assess what effect silencing of Elk4 has on key EMT markers at the protein level. MCF-7 ZEB1 cells were lentivirally transduced with an shRNA vector targeting Elk4 (shElk4) and a control vector (shCon). Figure 36 shows that
by making use of the Elk4 shRNA construct, we could mimic the phenotype we observed during the primary siRNA screening. This is reflected in the preservation of the epithelial phenotype in the presence of ZEB1 expression. Quantification of this effect by measuring the circularity of both the shCon and shElk4 conditions indeed confirms our findings (Figure 36b).

![Image](image)

Figure 36: Morphology of induced MCF-7 ZEB1 cells with knock down of Elk4. a: MCF-7 ZEB1 cells were lentiviral transduced with a control vector (shCon) and an Elk4 silencing vector (shElk4). Subsequently, the cells were induced with doxicyclin and pictures were taken 96h later. b: Plot of the average circularity of both the shCon and shElk4 conditions of the induced MCF-7 ZEB1 cells.

The fact that the maintenance of the epithelial phenotype was confirmed by making use of one siRNA pool, 4 independent siRNAs and an shRNA construct, strongly indicates that the observed effect is indeed specific and not generated by off-target effects.

Next, we extended our investigation with immunocytochemic analysis in order to assess the functional effect of Elk4 knock down in the induced MCF-7 ZEB1 cells. We have previously shown that ZEB1 induction in our screening model leads to a reduction of E-cadherin. Furthermore, the residual E-cadherin is relocated from the cell membrane to the cytosol, indicating the loss of its adhesive function (Figure 13). Here, we show that this redistribution of E-cadherin is strongly reduced when the induced MCF-7 ZEB1 cells are transduced with the Elk4 shRNA (shElk4) vector in comparison with the control (shCon). The fact that E-cadherin remains at the cell membrane implies that its functionality is also maintained.
Figure 37a). In extension, an opposite effect can be observed for the mesenchymal marker vimentin. Induction of ZEB1 leads to the expression of vimentin in the MCF-7 ZEB1 cells when transduced with the shCon vector. Knocking down Elk4 leads to a drop in vimentin expression levels below the detection limit, further reflecting the epithelial state of the cells. As a control, a myc staining was performed in order to visualize that in both the shCon and shElk4 condition, ZEB1 nuclear protein is still present (Figure 37a). Next, a western analysis was performed on both E-cadherin and vimentin. In extension, we also looked at the epithelial marker occludin. As was shown via immunohistochemistry, knocking down Elk4 in the induced MCF-7 ZEB1 cells indeed resulted in an upregulation of E-cadherin and a downregulation of vimentin. Furthermore, occludin could be detected at higher levels in the shElk4 condition in comparison with the shCon condition (Figure 37b).

**Figure 37:** Effect of Elk4 silencing in induced MCF-7 ZEB1 cells. *a.* Immunocytochemical analysis of induced MCF-7 ZEB1 cells that were lentivirally transduced with a control vector (shCon) and a vector targeting Elk4 (shElk4). The cells were induced 24h post transduction and fixed 96h later. *b.* Western blot analysis of induced MCF-7 ZEB1 cells.
All together these experiments further confirm the conclusions of the primary siRNA screen and position Elk4 as a central player in ZEB1 induced EMT in our cell system. Although the ZEB1 bands do not show the exact intensity in the shCon and shElk4 condition, this does not seem to influence our conclusion. Indeed, the Zeb1 levels seem to be higher in the shElk4 condition. However, even with higher levels of Zeb1, knock down of Elk4 still results in the conservation of the epithelial phenotype. Additional experiments have furthermore shown a more equal distribution of the Zeb1 levels across both the shCon and shElk4 conditions, delivering the same conclusions as stated above.

3.3.3 Silencing of Elk4 leads to an mesenchymal epithelial transition (MET) in carcinoma cell lines

The obtained results in our MCF-7 ZEB1 model strongly suggest that the ZEB1-Elk4 axis is a major player in the establishment or maintenance of the EMT phenotype. To find more solid proof for this claim, we wanted to verify whether additional carcinoma cell lines from different origins are also dependent on Elk4 for their mesenchymal like properties. We opted for the MDA-MB435S, MDA-MB231 and Mia-PaCa2 cell lines, which were respectively isolated from high grade breast, breast and pancreas carcinomas. However, several indications point to the fact that MDA-MB435S might also be of melanoma origin (Chambers, 2009; Ellison et al, 2002). All three cell lines have an EMT like morphology in vitro and are known to express several EMT inducing transcription factors. For example, high levels of Snail, ZEB1 and ZEB2 have been shown in MDA-MB435S, corresponding with a strong expression of vimentin and N-cadherin (Blick et al, 2008; Cano et al, 2000a; Comijn et al, 2001). In addition, Slug, ZEB1 and ZEB2 can be detected in MDA-MB231 (see PhD thesis of Dr. Cindy Vandewalle), while ZEB1 and Snail are expressed in Mia-PaCa2 (Blick et al, 2008; Wellner et al, 2009a). The three cell lines were lentivirally transduced with an shRNA expressig plasmid that represses Elk4 (shElk4) and compared with a control vector (shCon). Strikingly, in all cases, knock down of Elk4 resulted in a shift of the mesenchymal like morphology towards a more epithelial resembling appearance. This effect was quantified by measuring the circularity of the cells, whereby a significant increase in roundness is measured under influence of Elk4.
knock down (Figure 38). These findings prompted us to investigate whether the morphological changes were accompanied by an increased expression of several epithelial associated proteins and a decrease in vimentin protein levels. As is shown in Figure 39, in all three cases, repression of Elk4 results in a severely reduced protein expression of vimentin both on western blot and immunocytochemical analysis. Accordingly, the general trend is that Elk4 knock down is associated with the upregulation of epithelial markers, although several differences exist. For example, while for MDA-MB435S and MDA-MB231, a clear upregulation of E-cadherin can be observed upon Elk4 silencing, this does not seem to be the case in Mia-PaCa2. A similar conclusion can be made when silencing ZEB1 in for instance MDA-MB231 and Mia-PaCa2, verifying the functional association between ZEB1 and Elk4 (Aigner et al, 2007b; Wellner et al, 2009b). Remarkably, in all three cases, this mesenchymal-epithelial transition (MET), occurred while ZEB1 was still present, confirming that the functionality of ZEB1 is strongly depending on the presence of Elk4.

In conclusion, the EMT modulating capacities of Elk4 have been shown to also exist in three carcinoma cell lines, originating from aggressive cancers. Given the fact that we initially picked up Elk4 from the \textit{in vitro} siRNA screening in our MCF-7 ZEB1 model, further indicates that the obtained hits are biologically relevant.
Figure 38: Morphological analysis of three aggressive carcinoma cell lines upon Elk4 knock down. Knocking down Elk4 in MDA-MB435S, MDA-MB231 and Mia-PaCa2 results in a significant switch towards a more epithelial like morphology. This is quantified by measuring the average circularity of the cells, whereby a value of 1 stands for a perfect circle.
Figure 39: Influence of Elk4 knock down on protein expression of epithelial and mesenchymal markers. Knocking down Elk4 results in an epithelialization of all three examined carcinoma cell lines. In MDA-MB231, the upregulation of E-cadherin is associated with localization to the membrane, indicating its functionality. The same conclusion can be made for ZO-1 in the case of MDA-MB435S and Mia-PaCa2.
3.3.4 Silencing of Elk4 leads to impairment of EMT at the functional level

In order to conclude that a complete reversal of the EMT phenotype takes place upon Elk4 knock down, it is necessary to verify whether this effect can be extended at the functional level. Therefore, we conducted three types of *in vitro* functional assays that will inform us about the following parameters: slow aggregation, migration and invasion in a 3D matrix.

In the slow aggregation assay, cells are seeded on top of an agar based matrix that prevents the cells from attaching to the substrate. As such, the cells can only establish cell cell contacts. In general, epithelial cell lines are able to form large clusters of cells 24h after seeding the cells on the agar matrix. In contrast, due to the lack of functional cell cell adhesion complexes, mesenchymal cells do not have this capability and form a scattered cell population.

![Figure 40: Slow aggregation assay of shElk4 transduced MDA-MB435S and MDA-MB231 cells. A total of 30,000 cells were seeded on a solidified agar based substrate in a 96 well plate. After 24h pictures were taken and revealed a enhancement of cell clustering in the knock down condition (shElk4) of both cell lines in comparison with the negative control (shCon).](image)

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For both the MDA-MB435S and MDA-MB231 cell lines, knocking down Elk4 results in a dramatic enhancement of the cell cell aggregation, indicating that the observed MET is indeed functional (Figure 40). However, since these slow aggregation assays were performed in a 96 well format, the formation of a meniscus of the agar substrate might exert a significant influence on the clustering of the cells in the center of the wells (Debruyne et al., 2010). In our case, this could imply that upon Elk4 knock down, the cells tend to roll easier towards the center of the well, rather than enhanced cell-cell adhesion takes place. To exclude this possibility, the tests should be repeated in a 24 or 6 well, where the meniscus formation is not as strong as in a 96 well.

The fact that an enhanced cluster formation could be observed upon Elk4 silencing prompted us to investigate whether this also had consequences onto in vitro mediated migration and invasion. For this purpose, we again used the Oris cell migration and invasion kit based on the “stopper” tool. For the migration assay, the cells were seeded in a 96 well plate which was equipped with the stopper tool, as such preventing the cells from attaching onto the central region of the well. Once the cells were firmly attached to the plastic substrate of the well, the stopper was removed and migration was assessed at fixed time points. The cells were visualized with the Calcein AM substrate that gets fluorescent properties when metabolized (Figure 41).

Figure 41: Schematics of the Oris cell invasion assay. Initially, the wells are coated with the ECM substrate. After solidification, a stopper is applied to prevent cells from attaching to the substrate in the middle of the well. Next, the cells are seeded and after attachment to the ECM substrate, the stopper is removed and an additional layer of ECM substrate is added to bring the cells in a three dimensional scaffold.
For the *in vitro* invasion assays, the cells are embedded in a three dimensional matrix, resembling the *in vivo* extracellular matrix. As is depicted in Figure 42, MDA-MB435S cells with knock down of Elk4 (shElk4) have less propensity to migrate towards the central area 24h after the stopper is removed. This is also reflected in the calculation of the migration index which is significantly lower in the shElk4 condition in comparison with the control (shCon). When looking to the invasion assay, the differences between the control and the Elk4 knock down condition are more outspoken than in the migration assay. Interestingly, also the morphological differences between the control and the shElk4 condition are significantly different, a feature that was less obvious in the migration assay. Indeed, at the edge of the invasion front of the control condition (shCon) the cells have a spindle shaped, mesenchymal appearance.

**Figure 42:** *In vitro migration and invasion assay of MDA-MB435S cells with knock down of Elk4.* Using a migration and invasion assay reveals that knockdown of Elk4 (shElk4) result in the loss of both the migratory and invasive capacity in comparison to the negative control (shCon). This has been quantified with the software package ImageJ by calculating the migration and invasion index.
Both single, detached cells can be observed as well as strings of cells that invade the three dimensional matrix. When comparing this to the Elk4 silencing condition, the cells remain clustered together at the edge of the invasive front and show no tendency to migrate towards the central area of the well. This indeed reflects the clustering of the shElk4 cells that could be observed in the slow aggregation assay. A similar conclusion can be made for the MDA-MB231 cells. Also here, knocking down Elk4 resulted in a strong reduction of the invasive capacities in comparison to the control (shCon) (Figure 43). These findings again confirm the crucial role Elk4 plays in the maintenance and functionality of the mesenchymal phenotype in cells that have undergone an EMT during carcinoma progression.

Figure 43: Invasion assay of MDA-MB231 cells with knock down of Elk4. Knocking down Elk4 (shElk4) strongly reduces the invasive capacities of MDA-MB231 cells in comparison to the control condition (shCon). This is quantified by calculation the invasion index for both conditions using the software package ImageJ.

3.3.5 Elk4 is necessary for the formation of metastatic lung nodules

To further investigate the influence of Elk4 on the functional level, we wanted to assess whether the results from previous sections could be translated into an in vivo environment. For this purpose, we injected MDA-MB231 shElk4 and shCon cells into the tail vein of nude mice and evaluated their capacity to form metastatic lung nodules. In order to follow the progression of the metastatic formation, the cells were additionally
lentivirally transduced with a luciferase expressing vector, designated MDA-MB 231 Luc shElk4 and MDA-MB231 Luc shCon. Both cell lines were injected in a group of five mice. By performing a bioluminescent assay on the mice, we were able to detect a positive signal in the lungs of the mice treated with the MDA-MB 231 Luc shCon cells 14 weeks post injection, whereas this was not the case for the mice injected with MDA-MB 231 Luc shElk4 (Figure 44a).

In total, 3/5 mice treated with the MDA-MB231 Luc shCon cells developed tumors compared to 0/5 in the shElk4 group. Isolation of the lungs indeed revealed the presence of several metastatic nodules in the shCon group, whereas no could be observed when Elk4 was silenced. These findings were also confirmed on H&E histochemical sections (Figure 44b and c). In conclusion, these findings strongly suggest that Elk4 is a necessary protein for the establishment of a metastatic phenotype in our mouse model. The view that metastatic foci can only grow to visible size is indeed dependent on the ability of the cells to cluster together. However, in our tail vein experiment, several additional aspects play a role in metastasis formation, including survival of the cells in the bloodstream and extravasation into the lungs. Knocking down Elk4 indeed results in an enhanced capability of clustering, while no metastatic foci seem to be formed. These seemingly conflicting findings can be explained by the fact that any perturbation in survival in the bloodstream and/or during extravasation provides a limiting step that prevents the cells from colonizing the lungs. One might thus speculate that knockdown of Elk4 reduces the viability of the cells and/or severely compromises extravasation.
Figure 44: The metastatic capacities of MDA-MB231 are dependent on Elk4. 

a.: MDA-MB231 Luc shElk4 and MDA-MB231 Luc shCon cells were injected in the tail vein of nude mice. Fourteen weeks post injection, bioluminescent measurements were performed. The presence of metastatic lung nodules was clear in the case of the shCon cells, whereas no signal could be detected in the shElk4 cells.

b.: Macroscopic analysis confirming the findings that are observed with the bioluminescent assay.

c.: H&E histochemical analysis showing the absence of metastatic nodules in the shElk4 condition when compared to the control shCon.
3.3.6 Micro array analysis associates Elk4 functionality with different pathways crucial for the establishment of EMT

In order to get a more detailed view on the functionality of the transcription factor Elk4 we performed a micro array experiment where we compared the MDA-MB231 cells transduced with the knock down vector of Elk4 (shElk4) with cells transduced with a control vector (shCon). The used Affimextrix chip was of the “Human genome U133 plus 2.0” type containing more than 54000 probe sets for 47000 different transcripts. If we set the cut off value for up- and downregulated genes at 2 and 0,5 fold respectively, an equal amount of both sets can be observed. Indeed, upon Elk4 knock down, 254 genes are downregulated, while 231 genes are expressed at a lower level. This indicated that Elk4 can be implicated in both transcriptional activation and repression. Since we discovered that Elk4 is a necessary factor for the functionality of ZEB1, this is contradictory to the fact that ZEB1 has generally been described as being a transcriptional repressor. (see introduction). A possible explanation for this divergent finding might be the fact that Elk4 and ZEB1 act in parallel of each other. In other words, Elk4 might be responsible for the modulation of additional cofactors that are necessary for ZEB1 functionality. However, this implies that these cofactors are highly likely to have been picked up as a hit in the screening. A comparison between the significantly modulated genes in the micro array experiment and the confirmed hits from the screening quickly reveals that no common genes can be found. If the above assumption would be correct, then this implies that these additional cofactors do not have a profound impact on the general EMT phenotype induced by ZEB1. It is thus highly likely that the parallel function of Elk4 in relation to ZEB1 only has a minor impact on the general EMT phenotype.

An alternative explanation could be that Elk4 is only responsible for the functionality of a subfraction of the EMT program induced by ZEB1 and that inhibition of this fraction is enough to provoke a mesenchymal epithelial transition in the MDA-MB231 cell line. Furthermore, it has to be noted that in addition to ZEB1, the EMT inducing transcription factors Slug and ZEB2 are also present in MDA-MB231. It is thus possible that these factors are also responsible for the modulation of a different part of the mesenchymal like phenotype that is observed in the MDA-MB231 cells.
An extensive list of the most significantly modulated genes can be found in the supplementary data (table S1). Strikingly, when analyzing these most strongly affected genes, it becomes clear that several key pathways are affected. This can be observed in table 1, where TGFbeta, Wnt and cadherin signalisation components are modulated. In addition, the functionality of angiogenesis and the proteolytic machinery seems to be disturbed.

<table>
<thead>
<tr>
<th>TGF beta pathway</th>
<th>Fold</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upregulated by Elk4 knock down</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ID2</td>
<td>3.3</td>
<td>Inhibitor of TGFbeta signaling</td>
</tr>
<tr>
<td>Foxo6</td>
<td>2.7</td>
<td>Cellular differentiation</td>
</tr>
<tr>
<td>FKBP1A</td>
<td>2.1</td>
<td>Adaptor for Smad7-Smurf complex</td>
</tr>
<tr>
<td>Downregulated by Elk4 knock down</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GDF15</td>
<td>0.18</td>
<td>Same as Placental TGFbeta</td>
</tr>
<tr>
<td>FoxA2</td>
<td>0.21</td>
<td>TGFbeta target gene, lung fibrosis</td>
</tr>
<tr>
<td>Cadherin signalisation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upregulated by Elk4 knock down</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDH1</td>
<td>5.2</td>
<td>E-cadherin, cell cell adhesion</td>
</tr>
<tr>
<td>PCDHB10</td>
<td>2.32</td>
<td>Protocadherin</td>
</tr>
<tr>
<td>PCDHB7</td>
<td>2.02</td>
<td>Protocadherin</td>
</tr>
<tr>
<td>PCDHB14</td>
<td>2.65</td>
<td>Protocadherin</td>
</tr>
<tr>
<td>PCDHB16</td>
<td>2.79</td>
<td>Protocadherin</td>
</tr>
</tbody>
</table>

| PI3K                      |      |                                                  |
| Downregulated by Elk4 knock down |      |                                                  |
| FoxA2                     | 0.21 | Phosphorylation substrate of PI3K                |
| IBP1                      | 0.13 | Regulatory unit of protein phosphatase2         |
| CCND2                     | 0.38 | Activated by PI3K signaling                     |

| Proteolytic activity      |      |                                                  |
| Downregulated by Elk4 knock down |      |                                                  |
| MMP1                      | 0.28 | Collagenase, degradation of ECM                  |
| MMP16                     | 0.49 | Activation of pro-gelatinase2                   |
| ADAMTS1                   | 0.19 | Protease contributing to invasion in breast cancer |
| Serpin1                   | 0.1  | Plasminogen activating inhibitor 1               |
| SerpinB2                  | 0.19 | Plasminogen activating inhibitor 2               |

<p>| Notch signaling           |      |                                                  |</p>
<table>
<thead>
<tr>
<th>Downregulated by Elk4 knock down</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Hes1</td>
<td>0.14 Target gene of Notch signalisation</td>
</tr>
<tr>
<td>Jag1</td>
<td>0.45 Transmembrane ligand for the Notch receptor</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Upregulated by Elk4 knock down</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD24</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Downregulated by Elk4 knock down</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44</td>
<td>0.61 Marker used for the detection of breast cancer stem cells</td>
</tr>
</tbody>
</table>

Table 2: Micro array gene ontology experiment of MDA-MB231 shElk4 vs MDA-MB231 shCon. The analysis was conducted using the online “PANTHER” classification system (Thomas et al, 2003). The genes that were inserted into the PANTHER algorithm was the top modulated fraction that was at least times upregulated or at least two times downregulated.

Interestingly, by performing a knock down of Elk4, several modulating genes of the TGFbeta pathway are strongly affected (Table 2). The mRNA of TGFbeta modulating proteins that is upregulated due to silencing of Elk4, in particular ID2 and FKBP1A, have been shown to be negative modulators of the TGFbeta response. Indeed, overexpression of ID2 in TGFbeta stimulated NMuMG cells could inhibit the TGFbeta mediated upregulation of ZEB1 and ZEB2. As such, the TGFbeta induced EMT could be abolished (Shirakihara et al, 2007). Furthermore, as depicted in Fig. 18, a direct association between Elk4 and ID2 has been found by performing a molecular pathway analysis on a subfraction of the discovered hits. Besides ID2, FKBP1A was upregulated twofold in the MDA-MB231 shElk4 cells in comparison with MDA-MB231 shCon. It has been demonstrated that FKBP1A is an inhibitor of TGFbeta signaling. More specifically, FKBP1A binds to the cytoplasmic part of the TGFbeta I receptor, as such preventing the TGFbeta receptor II mediated phosphorylation (Chen et al, 1997). In addition, FKBP1A is responsible for the interaction of Smad-7 with Smurf1 at the cytoplasmic region of the TGFbeta receptor 1. Smurf1 has been shown to ubiquitinate the TGFbeta receptor 1, which induces receptor degradation (Ebisawa et al, 2001). Disturbing FKBP1A functionality by treating the cells with the chemical ligand of FKBP1A, resulted in
disruption of the Smad-7 Smurf1 complex, leading to impaired ubiquitination of the receptor (Yamaguchi et al, 2006).

GDF15 is downregulated fivefold in the MDA-MB231 shElk4 cells in comparison to MDA-MB231 shCon and is a member of the TGFbeta superfamily. GDF15 has also been designated as placental TGFbeta. Although its specific receptor is unknown to date, it has been demonstrated that GDF15 can activate transcription of TGFbeta responsive promoters and that this effect could be abolished in Smad-4 deficient cells. In addition, GDF15 has been shown to activate Smad-2 and Smad-3 in neonatal cardiomyocytes. As a possible consequence of these findings, expression of GDF15 has been shown to be associated with poor prognosis in a several cancer types, including melanoma, colorectal, breast and pancreas cancers (Mimeault & Batra, 2010). Taken together, knock down of Elk4 in MDA-MB231 highly likely results in a negative modulation of the TGFbeta signaling pathway. Since TGFbeta signaling has been shown to induce EMT in vitro and in vivo (see introduction), disturbing this pathway fits in the frame of the mesenchymal epithelial transition (MET) that is provoked by Elk4 knock down. Furthermore, in specific cellular models, it has been shown that TGFbeta mediated EMT is dependent on ZEB1 expression (Shirakihara et al, 2007).

Apart from the TGFbeta pathway, two crucial players in the Notch signaling cascade are also affected by Elk4 knockdown in MDA-MB231. Both the Notch ligand Jag1 and the Notch target gene Hes1 are strongly downregulated due to silencing of Elk4. Activation of the Notch pathway and subsequent transcription of Hes1 has been shown to inhibit cell differentiation and keeping the cells in a proliferative or precursor stage (Kageyama et al, 2000). Recently, a connection between ZEB1 and the Notch pathway has been uncovered. Both in MDA-MB231 and Panc1 cells, knockdown of ZEB1 results in a downregulation of Jag1 and subsequent disruption of the Notch pathway (Brabletz & Brabletz, 2010). The finding that knocking down ZEB1 and Elk4 in MDA-MB231 has the same effect in MDA-MB231 strongly stipulates their functional interaction. The downregulation of Jag1 in MDA-MB231 due to ZEB1 knock down has also been shown to be dependent on several microRNAs of the miR200 family, which are known to be negatively influenced by ZEB1 expression (Brabletz & Brabletz, 2010). Interestingly, Notch signaling is also able to downregulate miR200 family members in an indirect way. One of the consequences of this event is that activation of Notch leads to an EMT in
mouse lung adenocarcinoma cells and furthermore leads to an enhanced metastatic potential of the cells (Yang et al, 2011). Together with the TGFbeta pathway, modulation of the Notch cascade can thus be considered as one of the mechanisms that depend on Elk4 expression in order to induce an epithelial mesenchymal transition.

Recently, a connection between EMT and the induction of cancer stem cell like properties is discovered. Mammary cancer cells that were transfected with vectors expressing Twist1 or Snail underwent an EMT and displayed a CD44 high/CD24 low profile which is associated with human breast cancer stem cells. Further investigations led to the conclusion that these cells that had undergone an EMT, gained several crucial features that can be found back in mammary stem cells, including an enhanced capacity to form mammospheres, enhanced self renewal capacity and a hierarchy whereby CD44 high/CD24 low cells gave rise to CD44 low/CD24 high non stem cell progenitors. Reciprocally, isolated mammary cancer stem cells display a range of EMT markers, including vimentin, Twist, ZEB2 and Snail (Mani et al, 2008). Interestingly, knocking down Elk4 in the MDA-MB231 cells results in an upregulation of the CD24 marker (x 2.26), while CD44 is moderately downregulated (x 0.61). Although the connection between EMT and cancer stem cells is still a matter of debate, these findings suggest that knocking down Elk4 might have an impact on the stem cell properties of the MDA-MB231 cells.

In addition to these findings, knock down of Elk4 also results in the downregulation of MMP1. Interestingly, in analogy with these findings, ZEB1 has been shown to be a strong positive regulator of MMP1 in MDA-MB231 cells. Indeed, overexpression of ZEB1 in MDA-MB231 leads to a 30 fold upregulation of MMP1, while knocking down ZEB1 results in a significant downregulation of MMP1. Upregulation of ZEB1 has been shown to result in an increase of c-fos binding to the MMP-1 promoter, inducing its subsequent expression (Hu et al, 2011). This provides a link with Elk4, which has been shown to be responsible for c-fos expression via binding to the SRE sequence of the c-fos promoter (Griffiths et al, 1998).
3.3.7 The epithelialization due to Elk4 knock down modulates the miR200 family of microRNAs

A reciprocal association between ZEB1 and the regulation of the microRNAs of the miR200 family has been discovered recently. By downregulating the epithelial stabilizing miR200 members, ZEB1 is able to potentiate its function as an EMT inducing transcription factor. Vice versa, in epithelial cells, the high levels of miR200 microRNAs inhibit ZEB1 protein expression and are as such capable of maintaining the epithelial phenotype (Burk et al, 2008b). Since we postulate that Elk4 is a functional interactor of ZEB1 in relation to its EMT inducing capacities, this prompted us to investigate whether knock down of Elk4 was able to modulate the expression levels several members of the miR200 family. Furthermore, in the previous section, we have seen that knock down of Elk4 can lead to impaired Notch signaling. Interestingly, Notch pathway activation has been shown to reduce miR200 expression levels, giving us a additional indication that Elk4 has the ability to modulate these miRs.

We determined the microRNA expression level of miR200 a, b and c in both the MDA-MB435S shElk4 and MDA-MB231 shElk4 cell line in comparison with their respective controls. Both miR200a and b can be found on the same cluster located on Chr 1p36.33, while miR200c is located at Chr. 12p13.31. As is depicted in Figure 45, there is a general tendency in both cell lines that knock down of Elk4 results in the upregulation of the miR200 members. However, this seems to be more outspoken in the MDA-MB435S cells, where both miR200a and b are significantly upregulated. For MDA-MB231, a dramatic upregulation can be seen for miR200b.

These results further demonstrate the necessity of Elk4 in order to maintain the mesenchymal phenotype and suggests that the ZEB1 mediated downregulation of the miR200 family is dependent on Elk4. Whether this is a direct or indirect effect is not immediately clear. Indeed, it has been shown that ZEB1 is able to bind and inhibit activation of the miR200 promoters (Bracken et al, 2008). It remains to be investigated whether also Elk4 can be associated with these promoters. If this is indeed the case, this would show that Elk4 is also capable of mediating transcriptional repression.
Figure 45: *Effect of Elk4 knock down on members of the miR200 family of microRNAs.* A qPCR analysis was performed on the basis of locked nucleic acid (LNA) primers. Knock down of Elk4 in both MDA-MB435S and MDA-MB231 results in upregulation of one or more miR200 family members.

When taking into account that a negative feedback loop exists between the miR200 family and the ZEB transcription factors, one might assume that the observed upregulation of the miR200 members due to Elk4 knock down results in a marked downregulation of ZEB1. However, this does not seem to be the case in our cell systems. Indeed, when looking to the protein expression levels of ZEB1 in the shElk4 condition of MDA-MB231, we could not observe a reduction (Figure 39). A possible explanation could be that, although the miR200 microRNAs in the shElk4 condition are significantly upregulated in comparison to the control, this increase is not high enough to influence ZEB1 expression. One possible way to verify this, is by making use of a miR200 reporter construct based on the ZEB1 3'UTR (Burk et al, 2008a).
3.3.8 MicroRNA profiling reveals a shift towards a differentiated phenotype under influence of Elk4 knock down in MDA-MB231 cells.

In order to further assess the influence of Elk4 on general miRNA expression, we performed a microRNA profiling based on the Megaplex principle that makes use of a pool of 769 stem loop primers (Chen et al., 2005; Mestdagh et al., 2008). The profiling was carried out on MDA-MB231 shElk4 cells in comparison with MDA-MB231 shCon. From a total of 769 screened miRNAs, 28 miRNAs were reduced by at least a factor of two, while 27 were upregulated minimally twofold. This can be viewed in table 3.

<table>
<thead>
<tr>
<th>Downregulated miRs</th>
<th>Upregulated miRs</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR</td>
<td>shElk4/ shCon</td>
</tr>
<tr>
<td>hsa-miR-886-5p</td>
<td>0,186</td>
</tr>
<tr>
<td>hsa-miR-183</td>
<td>0,223</td>
</tr>
<tr>
<td>hsa-miR-483-5p</td>
<td>0,255</td>
</tr>
<tr>
<td>hsa-miR-362-3p</td>
<td>0,298</td>
</tr>
<tr>
<td>hsa-miR-185</td>
<td>0,365</td>
</tr>
<tr>
<td>hsa-miR-373</td>
<td>0,370</td>
</tr>
<tr>
<td>hsa-miR-142-3p</td>
<td>0,370</td>
</tr>
<tr>
<td>hsa-miR-20b</td>
<td>0,381</td>
</tr>
<tr>
<td>hsa-miR-454</td>
<td>0,383</td>
</tr>
<tr>
<td>hsa-miR-339-3p</td>
<td>0,387</td>
</tr>
<tr>
<td>hsa-miR-139-5p</td>
<td>0,389</td>
</tr>
<tr>
<td>hsa-miR-146a</td>
<td>0,411</td>
</tr>
<tr>
<td>hsa-miR-149</td>
<td>0,414</td>
</tr>
<tr>
<td>hsa-miR-148a</td>
<td>0,433</td>
</tr>
<tr>
<td>hsa-miR-22</td>
<td>0,463</td>
</tr>
<tr>
<td>hsa-miR-301b</td>
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<td>hsa-miR-138</td>
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<td>hsa-miR-24-2#</td>
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<td>hsa-miR-532-3p</td>
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<td>hsa-miR-494</td>
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</tr>
<tr>
<td>hsa-miR-17</td>
<td>0,494</td>
</tr>
<tr>
<td>hsa-miR-19b</td>
<td>0,496</td>
</tr>
</tbody>
</table>
Table 3: *Overview of the differentially regulated miRNAs under influence of Elk4 knock down in MDA-MB231 cells.*

With the aid of miRo tool (miR-ontology database tool) we identified the miRNAs with experimentally validated targets. Among these were miR22 (x 0.463) which has been shown to target the estrogen receptor1 (ESR1) (Pandey & Picard, 2009). One of the characteristics of basal breast carcinomas, which have been associated with EMT, is indeed the lack of the estrogen receptor. Overexpression of miR22 in MCF-7 cells strongly impairs their proliferation rate and goes hand in hand with downregulation of ESR1 at the mRNA level (Pandey & Picard, 2009). This negative correlation between miR22 and ESR1 has furthermore been confirmed in a panel of breast cancer tissues (Xiong et al). Interestingly, for miR373 (x 0.370), miR190b (x 0.413), miR100 (x 0.474) and miR19b, ESR1 is predicted as a target gene by three independent algorithms (TargetScan, miRanda, PicTar). In addition to these findings, miR17 (x 0.494), is a direct regulator of the transcription factor NCOA3 (nuclear receptor coactivator 3), which has been shown to directly interact with ESR1 in a ligand dependent way. Transfection of NCOA3 in breast carcinoma cells results in increased estrogen signaling upon estradiol stimulation and is furthermore higher expressed in non invasive breast cancer cell lines (Anzick et al, 1997). These results are supported by the fact that we are indeed able to observe an upregulation of the estrogen receptor 1 at the protein level upon Elk4 knock down in MDA-MB231 cells (. From a therapeutic point of view, this suggests that via the above mentioned miRNAs, Elk4 is a crucial determinant for the inhibition of estrogen dependent cell growth in breast cancer cells. Inhibition of Elk4 functionality in estrogen receptor negative breast cancers might thus be a relevant strategy to regain estrogen dependent growth and as such allow therapy with SERMs (Selective Estrogen Receptor Modulators).

In breast cancer, the loss of the estrogen receptor can be accompanied by loss of the progesterone receptor and the Her2 protein and is referred to as triple negative cancer.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Expression</th>
</tr>
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<tbody>
<tr>
<td>hsa-miR-517c</td>
<td>0.502</td>
</tr>
<tr>
<td>hsa-miR-1269</td>
<td>6.628</td>
</tr>
<tr>
<td>dme-miR-7</td>
<td>7.723</td>
</tr>
<tr>
<td>hsa-miR-582-3p</td>
<td>10.237</td>
</tr>
</tbody>
</table>
Triple negative breast cancer occurs in 12-17% of all cases and is associated with poor survival due to their intrinsic aggressive nature and the fact that no endocrine based treatment is possible. Furthermore, triple negative breast cancers encompass the claudin low tumors, which have been found to be enriched with cells with increased stemness and with features of EMT (Foulkes et al). Interestingly, two miRNAs, namely miR532 (x 0.297) and miR362-3p (x 0.298), that are specifically associated with triple negative breast cancer are significantly inhibited under the influence of Elk4 knock down (Janssen et al). Nevertheless, no target genes for both microRNAs have been experimentally validated so far, but these findings further support the reversal from a malignant phenotype towards a more differentiated state.

Apart from having NCOA3 as a direct target gene, miR17 (x 0.494), a member of the miR17-92 cluster, has been associated with enhanced proliferation and invasion in both pancreatic and breast cancer (Li et al, 2010a; Yu et al, 2010). In addition, the expression levels of miR17 are significantly higher in MDA-MB231 in comparison with the epithelial like MCF-7 cell line. Overexpression of miR17 in MCF-7 cells results in a marked increase of the colony forming potential in soft agar and an enhanced invasive potential. Conversely, knocking down miR17 in MDA-MB231 cells strongly disturbs the colony forming potential, as well as the invasive characteristics (Li et al, 2010a). miR17 downregulation in our MDA-MB231 shElk4 knock down thus provides an additional explanation for the decreased *in vitro* invasion observed in Figure 43. Together with the fact that another member of the miR17-92 cluster, namely miR19b (x 0.496), is also downregulated upon Elk4 knock down, this suggests that additional functionalities of the cluster are affected. One of these features could be related to the stemness inducing capacities of miR17-92. For instance, in mixed lineage leukemia (MLL) expression of several members of the miR17-92 cluster are significantly enriched in the stem cell pool of the tumor cells. In addition, leukemias with high expression levels of cluster members are characterized by a less differentiated phenotype, shorter onset to disease in a leukemia mouse model and have an increased cancer stem cell pool (Wong et al, 2010). Although relevant in a leukemia background, these findings agree with the fact that in our MDA-MB231 model knock down of Elk4 results in a downregulation of CD44 and an upregulation of CD24 (section 3.3.6) and thus impairs the stemness of the cells. Together with the miRNA profiling data, this might suggest that Elk4 is able to modulate stem cell characteristics via several members of the miR17-92 cluster. In addition to the modulation
of stemness, the miR17-92 family has been found to promote angiogenesis by targeting thrombospondin 1 and connective tissue growth factor (CTGF), two anti-angiogenic genes (Wang & Olson, 2009).

When looking to the microRNAs that are upregulated by Elk4 knockdown, both miR206 (x 3,736) and miR335 (x 2,790) have been associated with the modulation of metastatic capabilities. Indeed, in an elegant study where metastasized breast cancer cells in bone and lungs were compared to the cells of the primary tumor, it could be observed that the metastatic foci had a strongly reduced expression of both miRs. Next, the authors overexpressed miR335 in MDA-MB231 cells, which resulted in a clearly decreased migration and invasion in vitro. Antagonizing miR335 in breast cancer cells isolated from pleural effusion from a patient lead to a significant diminishing of metastatic foci. In addition, breast cancer patients with low miR335 expression had a shorter time to relapse and were associated with very poor overall metastasis free survival. Further analysis revealed that miR335 is responsible for the suppression of TNC (Tenascin C), MERTK (c-Mer tyrosine kinase) and Sox4 (Tavazoie et al, 2008). Upregulation of these miRNAs thus strongly favors a less aggressive phenotype which corresponds to the MET related effect we observe in our MDA-MB231 shElk4 cells.

An additional interesting candidate for the modulation of the epithelial phenotype is miR7 (x 7,723). Recently, it has been shown that two of the direct targets of miR7 are epidermal growth factor receptor (EGFR) and Raf1, a crucial mediator of growth factor receptor signaling which is often constitutively activated in human cancers. Overexpression of miR7 in several cancer cell lines furthermore results in reduced activation of ERK1/2 and Akt (Webster et al, 2009). Interestingly, it is shown that Elk4 is implicated in growth factor mediated signaling. These results thus suggest the existence of a feed-back loop whereby growth factor receptors are able to activate Elk4, which in turn negatively modulates miR7, as such further enhancing growth factor mediated signaling. In addition to EGFR, another direct target of miR7 is the insulin like growth factor receptor (IGFR) and insulin receptor substrate 1 and 2 (IRS1/2), further emphasizing the role of miR7 in growth factor signaling (Jiang et al). Furthermore, also p21 protein activated kinase 1 (PAK1) is a direct miR7 target. PAK1 has been extensively shown to be implicated in migration and invasion of malignant cells. For instance, overexpression of PAK1 in colorectal carcinoma cells leads to enhanced migration and invasion, whereas
downregulation of PAK1 has the opposite effect (Li et al, 2010b). Similar effects have been described in gastric, urinary tract, ovarian and breast carcinoma (Cai et al, 2009; Coniglio et al, 2008; Kamai et al, 2010; Siu et al, 2010). Upregulation of miR7 thus seems to be an important event in order to establish a less aggressive, more epithelial like phenotype in (malignant) carcinoma cells. In relation to this, it has been found that expression of another miRNA that is upregulated upon Elk4 knock down, let-7e (x 4,860), is severely compromised in chemotherapeutically resistant pancreas carcinoma cells. Strikingly, these cells are characterized by a mesenchymal like morphology and express vimentin and ZEB1. Pancreatic carcinoma cells that are not resistant to treatment, still show high levels of let7-e and show a high degree of epithelial differentiation (Li et al, 2009). In general, the let7 family of microRNAs is considered to be markers for differentiation and los of let7 members has been connected with EMT and stem cell formation. For let7-e specifically, no target genes have been elucidated. However, all 12 let7 members have the same seed region and significant overlap between targets can thus be expected. As such, let7 targets that have already been identified, are Ras, c-myc and CDK6 (Li et al, 2009). Interestingly, this might provide a link with the downregulated members of the miR17-92 family, miR17 and miR19b, in the MDA-MB231 shElk4 cells, since it has been shown that c-myc is a transcriptional activator of this miRNA cluster (Li et al, 2010a).

For the other differentially modulated miRNAs, no experimentally validated targets or functions have been described. It is nevertheless useful to verify whether interesting target genes can be predicted by means of bioinformatic analysis. Only miRNA target genes that are predicted by three independent algorithms (TargetScan, miRanda and PicTar) are considered. From these predictions, it seems that the Notch pathway might be specifically affected by the Elk4 knock down. Indeed, both miR518-f (x 2,482) and miR875-5p (x 2,976), are predicted to have Notch4 and Notch1, respectively, as target genes. In addition, miR335 (x 2,790), has Jag-1 as a predictive target. Interestingly, these findings link Elk4 to the Notch pathway and are in accordance with the fact that ZEB1 is associated with Notch activation (Brabletz et al, 2010). Other modulated miRNAs that have at first sight relevant target genes in the frame of EMT are miR95 (x 3,088) which targets insulin like growth factor 1 (IGF1) and Smad2. A comprehensive overview of the modulated miRNAs under influence of Elk4 knock down can be seen in Figure 46:.
Figure 46: Overview of different modulated miRNAs by Elk4 in MDA-MB231 cells. Elk4 is responsible for the upregulation of several miRNAs that are known markers for malignancy or dedifferentiation. In particular, ESR1 is a known target of miR22. We can indeed observe that there is a negative correlation between Elk4 and ESR1 expression in MDA-MB231 cells. In addition, several miRNAs that have been shown to contribute to epithelial differentiation, are downregulated under influence of Elk4 expression. TNBC: Triple negative breast carcinoma.

3.3.9 Elk4 functionality is necessary to maintain the TGFbeta pathway

On the basis of the micro array data of MDA-MB231 shElk4 in comparison with MDA-MB231 shCon, it could be deduced that knock down of Elk4 is severely affecting key components of the TGFbeta pathway. Indeed, silencing of Elk4 leads to upregulation of
several known TGFbeta inhibiting factors such as ID2 and FKBP1A, while the mRNA levels of activators are negatively influenced. However, there is no clear proof that these changes effectively have an effect on the net functionality of the TGFbeta pathway.

Figure 47: **Influence of Elk4 silencing on the TGFbeta pathway activity in MDA-MB231.** *(a.)* The TGFbeta reporter pCAGA was transfected in MDA-MB231 shCon and MDA-MB231 shElk4 and subsequently treated with recombinant TGFbeta as positive control. Lysates were measured 72h post transfection and revealed silencing of Elk4 leads to a marked reduction of the pCAGA activity. *(b.)* Western blot analysis of several Smad proteins. No effect of Elk4 knock down can be observed for Smad-2 and phospho-Smad-2 (p-Smad-2). However, a significant upregulation for Smad-6, a negative modulator of TGFbeta signaling, can be observed upon Elk4 silencing.

In order to get a more detailed view on this matter, we transfected MDA-MB231 shElk4 and MDA-MB231 shCon cells with a TGFbeta reporter plasmid. The TGFbeta responsive element in this reporter is a tandem repeat of the CAGA box. Three copies of this sequence have originally been identified in the PAI1 promoter as being responsible for the TGFbeta transcriptional induction. Interestingly, the CAGA box is highly specific for TGFbeta and does not respond to BMP signaling. Furthermore, activation of the CAGA element by TGFbeta has been shown to be Smad dependent (Dennler et al., 1998). Transfection of both MDA-MB231 shElk4 and MDA-MB231 shCon reveals that TGFbeta signalisation is severely compromised upon knock down of Elk4 and thus
confirms the results that were obtained in the micro array experiment (Figure 47a). As a positive control, the cells were treated with recombinant TGFbeta. Both the shCon and shElk4 condition showed an increase in their TGFbeta reporter activity upon recombinant TGFbeta treatment, indicating the specificity of the response (Figure 47a). Further investigation learns that the inhibiting effect of the Elk4 knock down on TGFbeta activity is based on upregulation of the inhibiting Smad-6. No effect takes place on the levels of Smad-2 and phospho-Smad-2 (Figure 47b). As stipulated before, a possible reason for the lack of influence of Elk4 knock down on Smad-2 and p-Smad-2 might be the presence of ZEB2 and Slug in MDA-MB231 that allows the cells to compensate for the loss of Elk4. This possible compensatory effect is however not strong enough to overcome the effects of Elk4 knock down and thus still results in an MET. The above experiment does not learn us whether the effect Elk4 has on the TGFbeta pathway occurs in conjunction with ZEB1. In order to determine this, inducible MCF-7 ZEB1 cells were transfected with the TGFbeta reporter plasmid and subsequently transduced with an Elk4 silencing lentiviral vector. Inducing ZEB1 expression in these cells could thus determine whether knock down of Elk4 is able to inhibit ZEB1 induced activation of the TGFbeta pathway. As can be observed in Figure 48a, induction of ZEB1 expression in the MCF-7 ZEB1 cells leads to a significant increase in the TGFbeta reporter activity. The activation of the TGFbeta pathway by ZEB1 is furthermore reflected in the levels of the Smad proteins (Figure 48b). Upon ZEB1 expression, both Smad-2 and phospho-Smad-2 levels are increased. Furthermore, the inhibitory Smad-6 is downregulated at the protein level in the induced MCF-7 ZEB1 cells. The ZEB1 induced activation of the TGFbeta pathway can be inhibited by treating the cells with the TGFbeta signaling inhibitor SB431542, showing the specific nature of the response (Figure 48a). This effect could be mimicked by knocking down Elk4 in the induced MCF-7 ZEB1 cells. When looking to the modulation of the Smads, it can be observed that knocking down Elk4 has a strong negative effect on protein expression levels of Smad-2 and its phosphorylated form (Figure 48c). Although involved in BMP and not TGFbeta signaling, Smad-5 levels are strongly affected by silencing of Elk4. In extension, also phospho-Smad-5 levels are significantly reduced upon Elk4 knock down.

Since induction of ZEB1 leads to an upregulation of phosphorylated Smad-5, this suggests BMP signaling takes place. Similar to the TGFbeta pathway that uses both
Smad-2 or -3 to form a complex with Smad-4 to act as a signaling transducing complex, BMP signaling uses Smad-1 and Smad-5 to form a complex with Smad-4 (Derynck & Zhang, 2003). However, while activation of the TGFbeta pathway has been shown to be a potent driver of EMT, this is less clear for BMP signaling. For instance, it has been shown that BMP7 is able to counteract TGFbeta and cyclosporin A induced EMT in renal epithelial cells (Xu et al, 2009). In addition, expression of BMP6 in MDA-MB231 cells induces expression of E-cadherin by repressing ZEB1. Reciprocally, ZEB1 expression is able to repress BMP6 expression (Yan et al, 2009). In contrast, both BMP2 and BMP4 have been shown to initiate or enhance an EMT phenotype. Treatment of gastric cancer cells with BMP2 resulted in a decrease of E-cadherin levels, while Snail was induced. These alterations led to an increased migrational an invasive potential (Kang et al). Furthermore, addition of BMP4 to airway epithelial cells has been shown to result in a mesenchymal morphology and a downregulation of several cell adhesion proteins (Molloy et al, 2008). Remarkably, BMP2, 4 and 7 are all known to signal via Smad-1 and -5 (von Bubnoff & Cho, 2001). It remains however unclear how cells can discriminate between the different BMPs and the EMT promoting or inhibiting effect. One possible explanation may be that the members of the BMP family are also able to activate different additional pathways next to the Smad cascade. In the MCF-7 ZEB1 cells, activation of phospho-Smad-5 can be observed upon induction of ZEB1 expression (Figure 48C).

This is furthermore reflected in an activation of the BMP responsive promoter, BRE-Luc, in response to an induction of ZEB1 expression (Fig. 31d). The BRE-luc reporter is based on a tandem repeat of a BMP responsive sequence in the Id1 promoter and has been shown to be activated by BMP2, 4, 6, 7, 8 and (David et al, 2007; Korchynskyi & ten Dijke, 2002; Lin et al, 2003; Maeda et al, 2004). Due to the diverse response in relation to EMT upon stimulation with the different BMPs, the BRE-Luc reporter is thus not discriminative towards BMP mediated induction or repression of the EMT phenotype. For this reason, we investigated whether BMP4, one of the BMPs that is known to enhance the EMT phenotype, was modulated due to ZEB1 expression (Figure 48d). An upregulation of BMP4 could indeed be observed in the ZEB1 induced cells. Knocking down Elk4 could partly inhibit this effect, thus showing an additional mechanism by which Elk4 is implicated in the ZEB1 induced EMT phenotype.
Figure 48: **Effect of Elk4 knock down on the TGFbeta pathway in MDA-MB231 cells.**  

**a.** TGFbeta reporter assay. MDA-MB231 shCon and MDA-MB231 shElk4 cells were transfected with the pCAGA reporter plasmid and subsequently treated with recombinant TGFbeta. Luciferase activity was measured 72 post transfection. Inh.: TGFbeta inhibitor  

**b.** Western blot analysis of the inhibitory Smad-6 and the effector Smad-2, showing that induction of ZEB1 leads to an activation of these critical TGFbeta effector proteins. p-Smad-2: phospho-Smad-2.  

**c.** Silencing Elk4 can disturb the ZEB1 mediated upregulation and/or activation of different Smad proteins. p-Smad-5: phospho-Smad-5.  

**d.** BMP reporter assay in MCF-7 ZEB1 cells, showing that ZEB1 induction leads to activation of the BMP responsive promoter.

### 3.3.10 Overexpression of Elk4 in parental MCF-7 cells does not lead to an EMT

Given the fact that Elk4 is a crucial functional interactor of ZEB1, we wanted to investigate whether overexpression of Elk4 alone could induce an EMT. For this purpose, parental MCF-7 epithelial breast cancer cells were transduced with an Elk4 overexpression vector. In extension, also the same was done for Elk3, a close homologue of Elk4 that also was identified as a hit in our siRNA screening. Both the Elk3 and Elk4 sequence were equipped with a C-terminal V5His tag. Transduction of parental MCF-7 cells resulted in the expression of both Elk3 and Elk4 proteins as is shown in Figure 49. In order to assess the impact of both Elk3 and Elk4, we investigated to what extent the expression level of several key genes that are implicated in EMT are altered upon Elk3/4 overexpression (Figure 49). Only modest changes could be observed for vimentin, Col5a2 and E-cadherin. Both for vimentin and Col5a2, an mRNA upregulation could be observed when overexpressing Elk3/4. For E-cadherin, this results in a small downregulation. Strikingly, a stronger upregulation could be observed for ZEB1 mRNA upon Elk3/4 overexpression. However, ZEB1 levels do not seem to be high enough in order to bring the MCF-7 cells into a mesenchymal state. The modest changes that are observed for vimentin, Col5a2 and E-cadherin are thus possibly not due to a direct effect of Elk3/4, but due to the upregulation of ZEB1 itself. Whether the upregulation of ZEB1 under influence of Elk3/4 is a direct effect remains to be investigated. However, one of the main
target genes of Elk4, namely Fos, has been shown to be able to drive ZEB1 expression in mouse mammary epithelial cells, suggesting that an indirect upregulation of ZEB1 by Elk4 is possible (Eger et al., 2005). For Elk3, these findings further suggest that a positive feedback loop with ZEB1 exists. Indeed, figure 16 shows that induction of ZEB1 in MCF-7 leads to a significant upregulation of Elk3. This is not the case for Elk4, of which mRNA expression levels remain constant upon ZEB1 overexpression. As described in the previous chapter, elaborate micro-array studies have showed that there is a significant correlation of Elk3 and ZEB1 in cell lines that are derived from the basal B subtype of breast cancer (Blick et al., 2008). The fact that a strong correlation between ZEB1 and Elk4 could not be detected, further suggests Elk4 is a relatively general transcription factor.
Figure 49: Overexpression of Elk3 and Elk4 in parental MCF-7 cells. Transduction of parental MCF-7 cells with lentiviral constructs expressing Elk3 and Elk4 with a C-terminal V5His tag. Gene expression could be confirmed both at the protein and mRNA level. Overexpression of Elk3 and Elk4 results in modest changes of vimentin, Col5a2 and E-cadherin expression in the direction of a more mesenchymal state.
3.3.11 Elk4 is able to form a multimeric complex with ZEB1

Given the fact that SRF has been shown to directly interact with ZEB1 during vascular smooth muscle cell differentiation and that Elk4 is a member of the TCF family that forms a complex with SRF, we wanted to investigate whether Elk4 and ZEB1 can be found in the same protein complex. In addition we also found SRF as a hit in our EMT screen. As a first experiment, we performed an overexpression study, whereby ZEB1, Elk4 and by extension Elk3 expressing plasmids were transfected in HEK293T cells (Figure 50a). Here ZEB1 is C-terminally myc tagged, while both Elk3 and Elk4 carry the V5His tag. The conditions where ZEB1 is transfected in combination with Elk3/4 reveals that via a pull down of the myc tag, the V5His tag could be detected in a subsequent western blot. Inversely, in V5His pulled down lysates, the presence of the myc tag could be shown. These results strongly suggest that ZEB1 and Elk3/4 can be found together in the same protein complex.

To exclude any aspecific effects that are inherent to the overexpression approach, we wanted to investigate whether the ZEB1/Elk4 complex can also be detected in the MDA-MB231 mesenchymal like breast cancer cell line. In an initial attempt, the MDA-MB231 cells were lentivirally transduced separately with the myc-tagged ZEB1 expression plasmid or with the V5His tagged Elk4 vector. When in the former case a pull down of the myc tag was done, a clear band could be detected with the Elk4 antibody. This indicates that ectopically expressed ZEB1 is able to form an complex with the endogenously expressed Elk4. The same conclusion can be made for the inverse setup. Pull down of the V5His tag in cells transduced with the Elk4 overexpression vector resulted in the detection of endogenous ZEB1 in a subsequent western blot (Figure 50b). This semi-endogenous approach greatly reduces the chances of detecting an aspecific interaction due to the nature of strong overexpression, but it cannot be excluded that the above findings are still a result from an artefact. To further eliminate these effects, a fully endogenous co-IP was performed in MDA-MB231 cells (Figure 50c). Also here, ZEB1 could be detected in protein lysates after performing a pull down for Elk4 and vice versa Elk4 could be stained after a ZEB1 pull down (Figure 50c).
Figure 50: **co-IP experiments indicating Elk4 and ZEB1 can be found in the same protein complex.**

**a.:** ZEB1, Elk3 and Elk4 overexpressing plasmids were transfected in HEK293T cells. A pull down was performed on both the myc tagged ZEB1 as well on the V5 tagged Elk3/4. Both approaches could confirm an interaction between ZEB1 and the Elk proteins.

**b.:** Semi-endogenous approach whereby a ZEB1 and Elk4 overexpression plasmid were transduced separately in MDA-MB231 cells. Pull down via the myc and V5 tag reveals that both overexpressed ZEB1 and Elk4 can interact with endogenous Elk4 and ZEB1, respectively.

**c.:** Endogenous interaction of Elk4 and ZEB1 in MDA-MB231 cells. Lysates with pulled down Elk4 were positive for ZEB1 and vice versa.

The fact that Elk4 and ZEB1 can be found in the same protein complex suggests that Elk4 acts as a cofactor of ZEB1. Removing Elk4 from this complex severely compromises the functionality of ZEB1, thus adding Elk4 to the list of proteins that have been shown to interact with ZEB1 and are necessary for its proper function (see introduction). Whether the presence of Elk4 in this complex is able to alter the other constituents that interact with ZEB1 and thereby modulating ZEB1 functionality remains to be investigated. Even so, the fact that knocking down Elk4 results in a dual response whereby critical EMT genes can be both up- or downregulated indeed suggests a differential interactome of ZEB1 might exist at the same moment. Interestingly, as is depicted in figure 18, members of the Id protein family are able to directly interact with both Elk3 and Elk4. It has however been shown that this interaction is able to disturb the association of Elk4 with SRF. In the frame of the ZEB1-Elk4 complex, this could imply that overexpression of Id
members could disrupt this interaction and thus disturb the functionality of the complex (Yates et al, 1999). The micro-array experiment described above learns us that knock down of Elk4 in MDA-MB231 cells results in the upregulation of Id2 in comparison to the control cells. It is tempting to speculate that Id2 expression is able to disturb the ZEB1-Elk4 complex and as such compromise its EMT inducing capabilities. This also opens up the possibility that the ZEB1-Elk4 complex or Elk4 alone is a negative modulator of Id2 itself. Since Id2 is also an inhibitor of TGFbeta signaling, a possible inhibiting role for Elk4 on Id2 expression would thus maintain the integrity of the ZEB1-Elk4 complex, while TGFbeta signaling is preserved. Alternatively, one has to keep in mind that Elk4 is a transcription factor that is strongly implicated in ERK and MAPK signaling pathways (Price et al., 1995). It has to be taken into account that these differential effects on ZEB1 functionality might also be partly explained because of the pleiotropic effects Elk4 knock down would inflict on these cellular pathways.

These results further supplement the experimental findings that have been described in the literature. Indeed, Elk4 has been identified as a binding partner of the transcription factor SRF which has been shown to be necessary for the functionality of the latter, for instance for the induction of early growth response genes (EGR) (Watson et al, 1997). In addition, an interaction has been reported between ZEB1 and SRF, providing a complex that is implicated in the differentiation of smooth muscle cells in vivo (Nishimura et al., 2006). It is thus tempting to speculate that the ZEB1 interaction with Elk4 occurs via SRF. Furthermore, the fact that SRF is also able to bind other members of the Elk family, such as Elk1 and Elk3, might provide us with an additional explanation for the apparent simultaneous activating and repressing effects of ZEB1.
3.3.12 Discussion

We identified the transcription factor Elk4 in our siRNA screening as a critical protein in the process of ZEB1 mediated EMT. These findings were confirmed in a secondary, deconvolution screening. In this chapter we further investigated the potential of Elk4 during the process of EMT. In a first experiment, we assessed the effect of Elk4 knock down on induced MCF-7 ZEB1 cells. At the morphological level, a striking difference could be observed between the knock down condition and the control. Silencing of Elk4 resulted in the maintenance of the epithelial morphology, while the control cells had a distinct altered morphology resembling the spindle shape that can be found back in fibroblasts. This differential outcome was also reflected in the expression profile of key EMT proteins, whereby the levels of vimentin were clearly reduced, while E-cadherin was maintained at a high level.

These findings prompted us to investigate whether the ZEB1-Elk4 axis is also a necessary mechanism for the establishment of the EMT phenotype in highly aggressive carcinoma cell lines. We decided to opt for the cell lines MDA-MB435S, MDA-MB231 and Mia-PaCa2. All three cell lines are derived from epithelial tissue, but are all characterized by a mesenchymal like morphology, indicating that at a certain time point during the development of the tumor they are derived from, they underwent an EMT. Interestingly, at least for MDA-MB231 and Mia-PaCa2 a clear connection between their EMT phenotype and ZEB1 expression has been established, thus being an additional criterion that adds to the possible functional interaction of ZEB1 with Elk4 (Eger et al., 2005; Wellner et al., 2009). Similar to what can be observed in the induced MCF-7 ZEB1 model system, knocking down Elk4 in all three of the above mentioned human malignant cancer cell lines resulted in a dramatic mesenchymal to epithelial transition. This was not only reflected at the morphological and protein level of key EMT related proteins, but was also translated into a functional effect. Indeed, when performing a slow aggregation assay in order to investigate the capability of the cells to form rigid cell cell contacts, it could be observed that knocking down Elk4 severely enhanced the functional establishment of intercellular contacts in all three cell lines. These findings correspond to the upregulation of several junction associated proteins such as E-cadherin, ZO1 and occludin under influence of Elk4 silencing. Furthermore, *in vitro* migration and invasion assays reveal
that the knock down of Elk4 results in a dramatic drop in the mobility of MDA-MB435S and MDA-MB231 cells both on a plastic substrate and in a three dimensional matrix environment. Interestingly, it has to be noted that apart from ZEB1 expression in these cell lines, additional EMT inducing transcription factors can be found back. For example, ZEB2 and Snail are also found in MDA-MB435S, Slug and ZEB2 in MDA-MB231 and Snail in Mia-Paca2. Due to the initial setup of the *in vitro* siRNA screening, Elk4 was selected for its potential to modulate the functionality of ZEB1. It is striking that interfering with ZEB1 via Elk4 silencing is able to completely disturb the mesenchymal like phenotype of these three cell lines while additional EMT transcription factors are still present. One could speculate that for all three cell lines, ZEB1 is the dominant player and is thus fully responsible for the establishment of the EMT phenotype. This could also include the possibility that expression of the additional EMT inducing transcription factors is dependent on the functionality of ZEB1. A second possibility could be that Elk4 is able to disturb crucial cellular pathways, which are necessary for the correct function of ZEB1, ZEB2, Slug and Snail. One observation that seems to oppose this latter argumentation is the fact that knocking down Elk4 and ZEB1 in Mia-PaCa2 does not result in upregulation of E-cadherin expression, while this seems to be the case when silencing Snail.

An unexpected observation was the fact that Elk4 knock down in MDA-MB231 resulted in a marked repression of vinculin. Vinculin is part of the multimeric focal adhesion complex comprising talin, paxillin and alpha actinin. Its role has been described to mechanically link the actin cytoskeleton with the integrin receptors. These specific properties allow vinculin to be responsible for the generation of contractile forces within the cell and thus its role in migratory and invasive behavior of the cell (Mierke, 2009). This view has recently been confirmed in a study where wild type embryonic fibroblasts (MEFs) were compared to vinculin knockout MEFs. The invasive capabilities of the vinculin mutant MEFs in a three dimensional collagen matrix were threefold lower in comparison to the control (Mierke et al, 2008). As such, vinculin can be regarded as an effector of invasion and reduced expression thus further points to an enhanced differentiation state of the MDA-MB231 cells upon Elk4 knock down.

At the functional level, we have demonstrated that Elk4 is necessary for *in vitro* clustering (slow aggregation assay) and migration/ invasion. To provide data with an *in vivo*
background, we performed a tail vein injection experiment, where we observed that Elk4 is necessary for the formation of metastatic lung nodules of MDA-MB 231 cells. However, it has to be noted that with this assay, we only assessed a fraction of the different steps that are necessary for the complete process of metastasis formation. Indeed, with this experiment, only the mechanisms from extravasation on till outgrowth of full metastatic nodules are considered. The steps that lead to intravasation and long term survival in the blood stream are omitted from this experiment. Nevertheless, the fact that we were unable to detect lung nodules in the mice injected with MDA-MB231 shElk4 cells in contrast to mice treated with the control cells, strongly indicates that Elk4 is a key factor in the process of metastatic formation.

Elk4 was first discovered as a transcriptional activator of the c-fos serum response element and is a direct substrate of ERK signaling (Janknecht et al., 1995). Together with Elk1 and Elk3, Elk4 is a member of the ternary complex factors (TCF), which physically interact with the Serum Response Factor (SRF). This protein complex is as such able to induce the transactivation of several target genes, including immediate early genes such as c-fos (Posern & Treisman, 2006). It is thought that the specificity of the SRF-Elk complex lies in the fact that the Elk factors are preferentially phosphorylated by different MAP kinase pathways. For instance, while Elk1 has been shown to be a target for both ERK and JNK signaling, Elk4 only seems to be efficiently phosphorylated by ERK (Strahl et al, 1996). TCFs bind DNA via their Ets motif. However, one of existing differences is the fact that the DNA binding motif of Elk1 seems to be more strict than that of Elk4. As such, it has been postulated that Elk4 has more target genes than Elk1 (Shore & Sharrocks, 1995).

The involvement of Elk4 in MAPK signaling and c-fos transcription is in line with the observations that MAP kinases are known inducing pathways of EMT (see introduction). Interestingly, c-fos forms a complex with c-jun, giving rise to the AP-1 transcription factor complex. The participation of AP-1 in the onset of aggressive carcinomas in vivo has been well documented. Furthermore, given the fact that c-jun is part of the AP-1 complex, implicates that the JNK pathway becomes an additional EMT modulator. As such it becomes clear that Elk4 is able to cover a broad spectrum of cellular responses.

Since these experiments uncover the importance of Elk4 as a seemingly crucial mediator of the malignant phenotype, we wanted to investigate the extent of the altered expression
profile under influence of Elk4 knock down. For this purpose, we carried out a micro-array experiment where we compared MDA-MB231 shElk4 cells with their control counterparts MDA-MB231 shCon. A pathway analysis on the attained results reveals that several key pathways that have been brought into association with EMT before are affected. Most striking is the fact that several components that are able to modulate the TGFbeta pathway are altered in such a way that the functionality of TGFbeta signaling is strongly inhibited upon Elk4 knock down. Examples are the upregulation of the TGFbeta signaling inhibitors Id2 and FKBP1A and the downregulation of GDF15, which is identical to placental TGFbeta. Given the fact that these changes do not necessarily prove that the functionality of the TGFbeta pathway is affected, we tested this with a TGFbeta sensitive reporter. Indeed, when knocking down Elk4 in MDA-MB231 we could observe a clear reduction in the TGFbeta functionality. In addition, we repeated the experiment in MCF-7 ZEB1 cells where we could indeed show that ZEB1 is capable of inducing an activation of the TGFbeta pathway. Strikingly, knocking down Elk4 in the induced MCF-7 ZEB1 cells could completely inhibit the ZEB1 mediated activation of the pCAGA promoter, indicating that also in this case Elk4 is necessary for the functionality of ZEB1. These observations are furthermore supported by the regulation of several Smads at the protein level. Indeed, induction of ZEB1 leads to upregulation of Smad-2, phospho-Smad-2 and phospho-Smad-5. In contrast, the inhibitory Smad-6 is shown to be downregulated at the protein level under influence of ZEB1. Knocking down Elk4 could largely undo these alterations by resulting in a negative modulation of Smad-2, phospho-Smad-2, Smad-5 and phospho-Smad-5. Additional major alterations that could be observed in the micro-array data, was the downregulation of several matrix metalloproteases, of which it has been shown that they are implicated in the degradation of extracellular matrix components during malignant invasion (Kessenbrock et al, 2010). Furthermore, the expression of two key components of the Notch signaling pathway, namely Jag1 and Hes1, are negatively influenced by Elk4 knockdown in MDA-MB231 cells. This is an interesting observation because of the fact that a connection between Notch signaling and ZEB1 has been established recently (Brabletz et al., 2010). The fact that also Elk4 can influence Notch signaling further emphasizes the functional entanglement of both Elk4 and ZEB1. Also of notable interest is the fact that silencing of Elk4 has its consequences on the breast cancer stem cell markers CD24 and CD44. The first report that connected EMT with stem cell properties came in 2008, whereby it was
shown that the induction of EMT via ectopic expression of Snail and Twist, resulted in an EMT phenotype accompanied by a gain in cancer stem cell like properties. Inversely, mammary breast cancer cells that were selected on the basis of their stem cell like capabilities, showed an increase in expression of several EMT inducing transcription factors (Mani et al., 2008). In general, cells with a CD24 low/CD44 high profile are considered to be enriched with stem cell like properties (Al-Hajj et al., 2003). Knocking down Elk4 in MDA-MB231 resulted in a downregulation of CD44, while CD24 was upregulated at the mRNA level. This suggests that the Elk4 knockdown cells are losing their stem cell like capabilities, next to the fact that a general epithelialization trend is occurring. Again, these findings further stipulate the importance of Elk4 in the process of EMT. A connection between stemness and ZEB1 has been made through the fact that ZEB1 is a negative regulator of the miR200 microRNAs. Indeed, it has been shown that several targets of the miR200 family are critical for maintaining the stem cell phenotype. These include Sox2, p63, Bmi1 and Klf1 (Wellner et al., 2009). Via a qPCR analysis, we investigated the expression levels of several miR200 family members which showed that indeed knock down of Elk4 in MDA-MB231 and MDA-MB435S cells resulted in an upregulation of several of these microRNAs. Together with the fact that these microRNAs are also known to be modulated by ZEB1, this again indicates the cooperation of ZEB1 and Elk4. What still remains to be investigated is whether the change of the CD24/CD44 profile under influence of Elk4 silencing actually results in a decrease of the stem cell like capabilities at the functional level in comparison to the control cells.

Since all these experiments point to the close relation between ZEB1 and Elk4, we further investigated whether a physical interaction between these two transcription factors could exist. This assumption was based on the fact that both Elk4 and ZEB1 have been shown to physically interact with the transcription factor SRF. Via overexpression experiments and endogenous co-IPs we were indeed able to show that a physical interaction between Elk4 and ZEB1 is possible. However, it remains to be shown to what extent this interaction is necessary for the functionality of ZEB1 as an EMT inducing transcription factor. Indeed, it has to be noted that Elk4 is implicated in several MAPK signaling cascades and it can therefore not be excluded that the epithelialization effect we observe upon ELK4 knock down is not due to interference with these general pathways. Furthermore, a extensive list of additional transcription factors and cofactors have been found to interact with ZEB1. An interesting question is thus whether the interaction of
Elk4 with ZEB1 is thus able to alter the interactome of ZEB1 itself, thereby enhancing the EMT inducing capacities of the latter. Removing Elk4 from this complex could thus imply that an alteration of this interactome takes place, thus preventing the EMT inducing functionality of ZEB1. In addition, it might very well occur that Elk4 on its own has transcriptional activating or inhibiting capacities that alter the function of ZEB1. A graphical depiction of the above findings can be found in Figure 51.

Figure 51: Schematic overview of the functionality of the ZEB1-Elk4 complex. Knocking down Elk4 in mesenchymal like cell lines resulted in a perturbation of the Notch, BMP and TGFbeta pathway. Furthermore, several members of the miR200 family of microRNAs seem to be affected by an Elk4 knock down. In addition, silencing of Elk4 leads to a shift towards a non stem cell like profile based on the CD24/CD44 profile. Also, we were able to show a physical interaction between ZEB1 and Elk4. Whether the influence of Elk4 on the ZEB1 functionality in relation to EMT is based on the complex formation of these two transcription factors or whether this is due to autonomous effects of Elk4, which is implicated in several MAPK pathways, remains to be investigated.
3.3.13 Materials and methods

Viral transduction protocol

For the production of lentiviral particles, 1E6 HEK293T cells were seeded in a 25 cm$^2$ flask. Twenty four hours later, 3 µg of the shElk4 and shCon lentiviral plasmid, together with 3 µg of the packaging plasmid pCMVdR8.91 and 1.5 µg of the envelope plasmid pMD2G-VSVG (both provided by Didier Trono) were precipitated and subsequently transfected into the HEK293T cells using the calcium phosphate method. The medium was refreshed after 6h and the cells were incubated for 48h in new medium in order to produce the viral particles in the medium. Subsequently, the medium containing the viral particles was filtered through a low protein binding 0.45 µm filter (Millipore, Billerica, MA).

The transduction was done in a 24 well plate on 40 000 cells (MCF7, MDA-MB231 and MDA-MB435S) suspended in a total volume of 100 µl. Immediately after the seeding, 900 µl of lentivirus containing medium was added to each well. The plates were centrifuged for 90 min at 1500 rpm at a temperature of 32 °C and were subsequently incubated for 24h at 37 °C. Hereafter, the medium was refreshed and the cells were placed under puromycin selection in order to obtain a homogenous cell population containing the transgene of interest, in casu the shRNA expression cassette for Elk4.

Slow aggregation assay

Agar well substrate was made by adding 300 mg of bacto-agar to 15 ml of Ringers salt solution (for 1l: 8.6 g NaCl; 330 mg CaCl$_2$.2H$_2$O; 300 mg Kcl, put at pH 7.4 with NaOH). After autoclaving, 150 µl of the solution was added per well of a 96 well plate. After cooling down and solidifying of the agar substrate, 10000 cells per well were added. Pictures were taken after 24h.
Micro-array experiment

mRNA lysates of MDA-MB231 shCon and MDA-MB231 shElk4 were made by lysing the cells with Trizol according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO). In order to assess degradation and purity, the RNA was analyzed on the Agilent 2100 Bioanalyser (Agilent Technologies, Waldbronn, Germany). The micro-array was carried out at the VIB micro-array facility (MAF) on an Affymetrix GeneChip Human Genome U133A 2.0 Array (Affymetrix, Santa Clara, CA). This specific type of gene chip represents 14500 human genes identified by 22000 probes.

Luciferase reporter assay

Luciferase was used as a reporter for the activity of the pCAGA promoter which is responsive for TGFbeta signaling. MDA-MB231 and MCF7 ZEB1 cells (2E5) were seeded in a 6 well plate and transfected with 1 µg of the pCAGA reporter plasmid with Fufene 6 (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol. The cells were refreshed 24 later and the luciferase activity was measured 96h post transfection. The luciferase activity was measured with a Tropix Galacto-Star kit (Applied Biosystems, Bedford, MA). Normalization was done by quantification of the protein content of the wells with the Bio-rad DC protein assay (Biorad Laboratories, Hercules, CA).

Immunocytochemistry

Cells (4E5) were seeded on coverslips in a 24 well plate and induced with doxycyclin. Ninety six hours post induction, cells were fixed in 2% paraformaldehyde (PAF) for 20 min. Next, cells were washed three times with PBS and permeabilized for 5 min in 50 mM NH₄Cl. Subsequently, the cells were again washed three times for 5 minutes with PBS, treated with 2% Triton X100 and finally washed with PBS. Primary antibodies were dissolved in a 0,5 gelatine solution in PBS and put on the cells for 1-2 h. After washing, the secondary antibody was put on the cells for one hour. Cells were washed a final time, mounted and examined under the fluorescent microscope.
qPCR analysis

Cells were lysed with the Trizol reagent (Sigma-Aldrich, St. Louis, MO) and processed according to the manufacturers protocol. Subsequently, the obtained mRNA was reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). QPCR analysis was performed using the Lightcycler 480 SYBR green I master mix (Roche, Rotkreuz, Switzerland). The sequence of the used primers can be found in the materials and method section of chapter 2.

Western blot analysis

For immunoblotting, the cells were rinsed with phosphate-buffered saline (PBS) and lysed with 1X Laemmli buffer. Total protein lysates were loaded and separated on a 8% SDS-polyacrylamide gel. The separated proteins were transferred to an Immobilon-P membrane (Millipore Corp). The membrane was blocked with 5% non-fat dry milk in Tris buffered saline (TBS, pH 7.4) containing 0.01% Tween-20 and was incubated with primary antibody. Following three washing steps, the membranes were incubated with secondary horseradish peroxidase (HRP) conjugated antibodies with a dilution of 1:3000 (Amersham Pharmacia Biotech). Staining was performed with the enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech). The following primary antibodies were used:

<table>
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<th>Antibody</th>
<th>Type</th>
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<tr>
<td>Occludin</td>
<td>Mouse monoclonal</td>
<td>Zymed</td>
<td>33-1500</td>
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<tr>
<td>Vinculin</td>
<td>Mouse monoclonal</td>
<td>Sigma</td>
<td>V9131</td>
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<tr>
<td>Desmoglein2</td>
<td>Mouse monoclonal</td>
<td>Progen</td>
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Immunoprecipitation

HEK293T cells were seeded with a density of $10^6$ cells per $25\, \text{cm}^2$ one day before transfection and transiently transfected with the different expression plasmids using the calcium phosphate transfection method. After 48h the culture flasks were put on ice and washed with ice cold phosphate buffered saline (PBS). For $10^6$ cells 200 $\mu\text{l}$ NP40 lysis buffer was added containing 20 mM Tris HCl pH 8, 137 mM NaCl, 10% glycerol, 1% NP40, 2mM EDTA and supplemented with a 1x complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). The cells were scraped with a cold plastic scraper and transferred into a pre-cooled microfuge tube. After putting the lysates under constant agitation for 30 minutes at 4°C, a centrifugation was performed for 20 minutes at 12000 rpm and the supernatant was kept. To prepare the immunoprecipitates, 500 $\mu\text{g}$ of cell lysates were incubated with mouse monoclonal V5 antibody (Invitrogen, Carlsbad, CA) or the mouse monoclonal myc antibody (Home made) and kept for agitation at 4°C. After 16h, 80 $\mu\text{l}$ of Magnetic Dynabeads M-280 Sheep anti-mouse IgG (Invitrogen, Carlsbad, CA) were added and incubated for 4h at 4°C under agitation. After this, the beads were pulled down using a magnet and washed five times with lysis buffer. Finally, 50 $\mu\text{l}$ of SDS loading buffer was added and the absorbed proteins were solubilized by boiling the samples for five minutes. By using the magnet, the supernatant was separated from the Dynabeads and used for SDS page and immunoblotting as described above.

**In vitro migration assay**

The Oris cell migration assay (Platypus Technologies, Madison, WI) was used in order to assess the migratory potential of the MCF-7 ZEB1 cells. The assay was carried out in a 96 well plate. The cells were detached and seeded at a density of 30 000 cells per well in the presence of the stopper mechanism that is provided in the kit. After attachment of the cells, the cells were transfected with the individual siRNAs as explained above. Ten hours post transfection, the cells were refreshed by medium containing 1% fetal calf serum (FCS) and doxicyclin to induce the cells and the stoppers were removed. At the
measuring time point, the cells were visualised with calcein AM provided by the kit. Briefly, the medium was replaced by calcein AM solution (0.5 mM) in Ca\(^{2+}\) containing PBS and incubated at 37°C for 30 minutes. Subsequently, the cells could be visualized fluorescently using the green filter. After measuring, the calcein Am solution was replaced by normal cell medium with 1% FCS and doxicyclin until the next measuring time point.

With the image processing software Image J a migration index was calculated (Abramov et al., 2004). At time point zero, a picture of the cells was taken and the non invaded area was measured (A0). The same was done at a specific measuring timepoint (A1). As such, the migration index was calculated by using the formula (A0-A1)/A0.
4 SUMMARY AND FUTURE PERSPECTIVES

4.1 Summary

The aim of this project was to identify novel functional interactors of the transcription factor ZEB1 in the context of EMT. As described in the introduction, a substantial amount of transcription factors and cofactors have been shown to interact with ZEB1, as such potentiating its function in varying cellular and molecular backgrounds. However, most of these studies have focused on a restricted subset of functionalities of ZEB1, whereby only specific molecular aspects were taken into account. These discovered interactions have thus not been tested for their significance during EMT. To accommodate for these limitations, we conducted an in vitro siRNA screening with a library targeting all known human transcription factors in an EMT model.

For the construction of the screening system, we decided to choose for A431 and MCF-7 epithelial cancer cell lines originating from non invasive skin and breast cancer, respectively. This selection is of particular interest concerning the MCF-7 cell line, since strong indications exist that a specific subtype of breast cancer, namely the basal B group, displays a multitude of characteristics that are associated with EMT. Both cell lines were equipped with inducible ZEB1 expression, which resulted for both cases in a mesenchymal transition upon treatment with doxicyclin. The next step was to determine a suitable readout, which could act as a robust and unambiguous EMT sensor. For this purpose, several gene promoters which have been described to be EMT sensitive were placed in front of a the luciferase reporter gene and subsequently tested for their responsiveness to ZEB1 induction in both the A431 and MCF-7 cell line. While for the MCF-7 cell line, the vimentin promoter gave an optimal result, for A431 none of the tested constructs were able to deliver a satisfactory outcome, hence our decision to only continue with the MCF-7 cell line to carry out the screening. Since the vimentin promoter was chosen as our readout, a hit would be determined by a reduction of the promoter activity. To exclude that this decrease is the result of an siRNA that targets crucial
components essential for cell survival, we incorporated an additional reporter cassette that is based on the EF1alpha promoter followed by the secreted alkaline phosphatase reporter (SEAP) gene. As such we have an additional filter to distinguish a particular group of false positive results. Both reporters were stably integrated into the ZEB1 inducible MCF-7 cells and yielded results that were optimal enough to allow for screening.

The screening was performed in a 96 well plate and was carried out in triplicate. A total of 1040 genes were silenced, yielding 27 primary hits which were able to counteract the vimentin promoter activation under influence of ZEB1 induction. However, due to the risk of aspecific effects that are inherently associated with siRNA mediated gene silencing, we conducted a secondary screening with four independent siRNAs for each identified target. As such, we were able to confirm 22 hits of which at least two out of four independent siRNAs were able to counteract the ZEB1 induced upregulation of the vimentin promoter activity.

Since our primary aim was to identify functional interactors of ZEB1 that are able to modulate at least a significant part of the EMT driven transcriptional program, the information provided by the vimentin promoter activity is not sufficient. In order to accommodate for these shortcomings, we performed an expression analysis of a predefined EMT mRNA expression profile that was chosen on the basis of a micro-array experiment where the induced MCF-7 ZEB1 cells were compared with their non induced counterparts. As such, a total of eight genes were checked for changes in their expression pattern under the influence of the selected hits. Clustering of the obtained profiles revealed the existence of two groups of hits, namely one group that had little or no influence on the EMT profile besides having an effect on vimentin and a second group of nine hits that was able to elicit a profound effect on the complete profile. Although our EMT profile only contained eight genes, these results suggest that only these nine genes can be regarded as true positive hits as was supposed at the beginning of the screening. On the basis of this group, a gene interaction map was created that was indeed able to connect several of the discovered hits in a broader functional network. Two main nodes can be observed in our network where on one hand ZEB1 and on the other hand Elk4 are central players. Interestingly, these two nodes are connected with each other via the transcription factor SRF. Investigation of the literature revealed that both Elk4 and ZEB1 are able to physically bind to SRF. The Elk4-SRF complex has been found to be
necessary for the induction of early growth response genes, while the ZEB1-SRF interaction is responsible for the differentiation of vascular smooth muscle cells under influence of TGFbeta signaling. Together with the fact that only silencing of the hit Elk4 in the primary screening was able to maintain the cells in an epithelial morphology in the induced MCF-7 ZEB1 cells, these findings prompted us to further investigate the role of Elk4 in ZEB1 induced EMT.

For this purpose, we transduced the MCF-7 ZEB1 cells with a lentiviral short hairpin construct targeting Elk4 (shElk4) and compared them with cells transduced with a control vector. We were able to confirm the findings from both the primary and the secondary screening in the fact that the epithelial morphology was maintained in the shElk4 condition even in the presence of ZEB1 expression, which was not the case in the control cell line. This was furthermore reflected in the protein level of E-cadherin, ZO-1 and vimentin, which all suggested the conservation of the epithelial program. Since the inducible MCF-7 ZEB1 cell line is an constructed EMT model, we wanted to elucidate whether knock down of Elk4 has a similar influence on dedifferentiated cancer cells with high endogenous ZEB1 expression. For this goal, we stably transduced the breast cancer cell lines MDA-MB435S, MDA-MB231 and pancreatic Mia-Paca2 cells with both the lentiviral shElk4 and control vector. Moreover, all three cell lines have a spindle shaped morphology in vitro, reflecting their EMT status. Spectacularly, knocking down Elk4 in all three cell lines resulted in a reversal of their mesenchymal morphology towards an epithelial appearance. In addition, this was reflected at the protein level for several key epithelial and mesenchymal genes in all three cell lines. Besides the effect on the EMT expression signature, knocking down Elk4 also resulted in an inhibition of mesenchymal characteristics at the functional level. Indeed, when assessing the intercellular adherence capacities by means of a slow aggregation assay, a significant increase of adhesion could be observed upon silencing of Elk4 in MDA-MB435S and MDA-MB231 cells. This gain of adhesive functionality is also reflected by the fact that both ZO-1 and E-cadherin are relocated to the membrane in respectively MDA-MB435S and MDA-MB231 as was shown by immunocytochemistry. For the Mia-Paca2 cell line, only ZO-1 was relocated to the membrane, while this could not be observed for E-cadherin. This is consistent with the findings that by knocking down ZEB1 in Mia-Paca2 cells, only ZO-1 was upregulated and not E-cadherin and thus further indicates that Elk4 is a true functional interactor of
One of the main characteristics of cancer cells that have undergone EMT is the acquisition of invasive and migratory capacities, which provides these cells with the possibility to leave their original tissue boundaries, to reach neighboring blood and lymph vessels and finally metastasize. In order to assess whether Elk4 is a necessary component for these driving forces behind the malignant phenotype, we performed an *in vitro* migration and invasion assay. In accordance with the findings that a shift occurs towards an epithelial phenotype, we could observe a dramatic loss of migratory and invasive capacities of MDA-MB435S and MDA-MB231 cancer cells upon knock down of Elk4. In addition, the mesenchymal like morphology of both cell lines is strongly shifted towards a more epithelial appearance in the three dimensional matrix of the *in vitro* invasion experiment under influence of Elk4 silencing, suggesting that extracellular signals originating from the complex stromal micro-environment are not able to compensate for the loss of Elk4 in order to maintain the mesenchymal like status. Taken a step further, MDA-MD231 shElk4 cells were injected in the tail vein of immunocompromised mice and were not able to metastasize to the lungs when compared to control cells. This suggests that Elk4 is, next to the establishment of the EMT phenotype, also crucial for the later steps in the metastatic process.

The above described results all demonstrate that Elk4 is a critical factor for the establishment and maintenance of a mesenchymal like state that contributes to the malignant behaviour of cancer cells. However, the molecular mechanisms that lay behind these Elk4 dependent effects are still unclear. In order to get a more detailed view on these aspects, we performed a micro-array mRNA expression analysis whereby the MDA-MB231 cell line with Elk4 knock down was compared with its control. Gene ontology analysis revealed that several key pathways were affected by the knock down, mainly being the TGFbeta and Notch pathway. In addition, several members of the MMP family, which are known to be implicated in invasion and migration are downregulated upon silencing of Elk4. Examples are MMP1 and MMP16, which have both been shown to be implicated in the progression of malignant tumor formation. This is of particular interest for MMP1, because it has been shown that MMP1 is directly regulated by ZEB1 in MDA-MB231 cells, providing a direct link with the effects Elk4 knockdown has in the same cell line. Furthermore, also the putative stem cell markers CD44 and CD24 are up-
and downregulated, respectively, upon Elk4 knock down, suggesting that the cells are losing their cancer stemcellness that has been associated with EMT. To further prove this statement, additional experiments are however necessary, including mammosphere and colony forming assay and assessing the tumorigenic potential of these cells to be tumorigenic in vivo. To verify in part the results that were obtained by the above described micro-array experiment, we determined which functional consequences Elk4 knock down has on TGFbeta signaling. For this purpose, we transfected the MDA-MB231 shElk4 cell line with a TGFbeta responsive promoter and compared this with the control cells. As could be deduced from the micro-array data, we observed a marked reduction of the TGFbeta activity in the cells with the Elk4 knock down. Western blot analysis revealed that this effect was mediated due to a significant upregulation of the inhibitory Smad6. Extrapolating these findings to our inducible MCF-7 ZEB1 cell line confirmed these results. While induction of ZEB1 leads to an increase of TGFbeta activation, this effect can be counteracted by silencing Elk4. At the protein level, ZEB1 is responsible for the upregulation of phospho-Smad2, while a knock down of Elk4 can inhibit this increased expression. There are also indications that BMP signaling is affected by Elk4 silencing, since similar observations were made for Smad1 and Smad5.

The connection between ZEB1 and the microRNAs of the miR200 family has recently been described, whereby an inverse correlation between both ZEB1 and the miR200s can be observed. As such, it has become clear that the miR200 family can be considered as stabilizers of the epithelial phenotype and thus play a pivotal role in the establishment and maintenance of the epithelial homeostasis. Due to the existence of a negative feed back loop with ZEB1, a minor disruption of the miR200 levels might be sufficient to drive the system towards a mesenchymal fate (Brabletz and Brabletz, 2010). Since the expression levels of the miR200's are generally low in mesenchymal like cell lines, we wanted to investigate whether these low levels could be modulated as consequence of Elk4 knock down. Therefore, we analyzed both the MDA-MB231 and MDA-MB435S cell lines for expression of miR200a, b and c. In both cases, silencing of Elk4 resulted in upregulation of the different miR200's to a variable extent. This was more outspoken for MDA-MB435S, where reduced levels of Elk4 resulted in the upregulation of miR200a and b. For MDA-MB231, the strongest effect could be observed for miR200b. These findings further stipulate the fact that upon Elk4 knock down, these initial mesenchymal like cells

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are driven back towards their original epithelial state and thus implies that also the ZEB1 mediated downregulation of these miRs is Elk4 dependent. Whether Elk4 binds together with ZEB1 to the promoters of the miR200 clusters remains to be investigated.

The above described findings strongly suggest that without the presence of Elk4, ZEB1 is unable to exert its EMT inducing capacities. As a next step, we wanted to assess whether this functional interaction could be due to a physical association between these two transcription factors. For this purpose, we transiently overexpressed both ZEB1 and Elk4 in HEK293T cells and subsequently performed an immunoprecipitation in both directions. This revealed that ZEB1 and Elk4 are able to physically interact with each other in the context of an overexpression experiment. In addition, we also discovered that a close family member of Elk4, namely Elk3, was able to bind to ZEB1 under these conditions. Strikingly, also Elk3 was identified as a hit from both the primary and secondary screening and could be found back in the same group of Elk4 which is able to extensively alter the ZEB1 induced EMT expression profile towards a more epithelial outcome. The ZEB1-Elk4 interaction could also be observed endogenously in MDA-MB231 cells These findings thus strongly suggest the existence of a ZEB1-Elk4 complex in vivo, providing an extension to interactions that have been described in the literature. Indeed, Elk4 has been identified as an interaction partner of the transcription factor SRF and is necessary to mediate the function of the latter, for instance for the induction of the early growth response genes (EGR) (Watson et al., 1997). In addition, ZEB1 has been shown to directly interact with SRF, forming a complex that is necessary for the differentiation of aortic smooth muscle cells in vivo and thus makes it tempting to speculate that the interaction between ZEB1 and Elk4 occurs via SRF (Nishimura et al., 2006). Future experiments will clarify this interesting hypothesis.

As a general conclusion, we identified a series of novel ZEB1 functional interactors using a reversed genetic screening approach. It is clear that the transcription factor Elk4 is a functional modulator of ZEB1 as knockdown of Elk4 results in several mesenchymal like cell lines in an epithelial transition. This is demonstrated at both the protein as the functional level. Since Elk4 is discovered by means of an in vitro siRNA screening on the basis of a ZEB1 mediated EMT model, this suggests that our screening is capable of delivering robust results and suggests that other high confidence hits might provide us with additional transcription factors that are able to modulate the ZEB1 functionality in
several mesenchymal like cell lines. A general overview can be seen in Figure 52.

4.2 Future perspectives

It is becoming increasingly clear that epigenetic modifications can contribute to both the onset and further progression of cancer (Berdasco and Esteller, 2010). For instance, hypermethylation of CpG islands in promoters of key tumor suppressor genes, such as Rb (Retinoblastoma), VHL (Von Hippel Lindau), p16INK4A and BRCA1 have been detected in several cancers (Herman and Baylin, 2003). Furthermore, in basal like breast cancers, promoter hypermethylation of E-cadherin has been reported, resulting in a dysfunctionality of the adherence junctions (Dumont et al., 2008). In addition, methylation also seems implicated in the miR200-ZEB feed back loop, whereby sustained treatment of TGFbeta has been shown to both induce ZEB1 and results in hypermethylation of the promoters of the miR200 clusters (Gregory et al., 2011). Together with the fact that Elk4 is a necessary factor for the establishment of a mesenchymal phenotype, it would thus be interesting to investigate whether Elk4 could influence the general DNA methylation pattern of the mesenchymal like cell lines we have studied. This can be done via Methylated DNA immunoprecipitation (MeDIP) although this would only provide us with a limited view on the whole genome. This can be overcome by performing a MeDIP followed by high throughput sequencing. As such, this would allow us to get a detailed genome wide view on the epigenetic impact of Elk4 functionality in the context of EMT. Complementary to this experiment would be the comparison of DNA methylation changes of ZEB1 modulation, which would allow us to discriminate between Elk4 dependent and independent effects of methylation.

A second aspect that has to be clarified, is to find the precise molecular function of the ZEB1-Elk4 protein complex. Indeed, although we have strong evidence for the existence of this complex, we currently do not know exactly how this is implicated in the establishment of a mesenchymal phenotype. At this stage, we cannot exclude that besides being in a complex with ZEB1, Elk4 influences the EMT phenotype in a ZEB1 independent way. There are several routes to elucidate the function of this complex.
Knowing that ELK4 is known to be phosphorylated by kinases as MAPK1 and MAPK8 it is highly intriguing to find out if specific phosphorylation is essential for the interaction with ZEB1 and/or its function in the induction and maintenance of the EMT status. Therefore it would be most interesting to map the specific phosphorylation sites of ELK4 during EMT using mass spectrometric methods and analyse the particular function of these phosphorylation sites in EMT and their potential role in the binding of ELK4 with ZEB1. An additional approach that could be taken, is by trying to locate the domains of Elk4 and ZEB1 that are necessary for the complex formation. As is suggested earlier, the formation of this complex might depend on the transcription factor SRF and SRF-homeodomain interactions have been extensively described (Grueneberg et al., 1995). It can thus be speculated that the ZEB1 homeodomain is crucial for the ZEB1-Elk4 complex formation. Mutating or (partial) deletion of these discovered interaction sites could thus reveal the necessity of complex formation in the development of the mesenchymal phenotype. As such, we could identify EMT related genes of which their modulation is dependent on this complex, allowing us to investigate whether specific motifs in the gene promoter exists that preferentially bind the complex. Although it has been extensively described that the ZEB transcription factors bind the E-box motif, it can not be excluded that nearby motifs are necessary for the complex to bind to specific promoters. This would furthermore provide us with information whether the Ets binding site and the CarG box motif, which have been shown to be necessary for the SRF-Elk4 complex to bind to specific promoters, are implicated or necessary for the DNA binding of the ZEB1-Elk4 complex. To furthermore pinpoint the importance of the ZEB1-Elk4 complex for the modulation of specific promoter activity, a ChIP analysis could be performed on promoters that were identified by following the above described approach.

Apart from studying the effect of Elk4 on EMT, it would be of particular interest to examine the influence of Elk4 on stem cell derived characteristics. Indeed, the connection between cancer stemness, EMT, ZEB1 and the miR200 family of microRNAs is becoming increasingly clear. Furthermore, we have observed an up- and downregulation of the stem cell related markers CD44 and CD24, respectively, upon Elk4 knock down in MDA-MB231 cells, indicating Elk4 is indeed capable of influencing the stem cell like properties of cells. Via an in vitro approach, we could assess the influence of Elk4 on the colony forming properties of the above studied mesenchymal like cell lines. In addition,
FACS profiling can be done on putative stem cell markers to give us a clearer picture of the penetrance of Elk4 on the stem cell phenotype. In addition to these *in vitro* aspects, we also could assess the tumor forming capacities of for instance MDA-MB231 shElk4 cells when orthotopically injected at low cell concentrations (limited dilution injection).

Very robust proof that Elk4 is implicated in the establishment of ZEB1 induced EMT phenotype *in vivo* could be delivered by making use of transgenic mice. Indeed, by combining conditional ZEB1 expression with a conditional Elk4 knock out in a p53 deficient background, it would in theory be possible to investigate whether the assumptions that are shown by the *in vitro* work can be maintained *in vivo*. Given the fact that p53 deficient mice are prone to develop mammary neoplasms, it could be of particular interest to conditionally overexpress and knockout ZEB1 and Elk4, respectively, in the breast epithelium. Finally, it would be of particular interest to examine a panel of human (breast) tumors for expression of the Elk4 protein and whether a correlation could be made with disease progression. Having in mind the fact that Elk4 is a substrate for several MAPK proteins, it is tempting to speculate whether an altered phosphorylation status of Elk4 occurs in advanced stages of cancer and more in particular, at the invasive front of the tumors.
Figure 52: **General overview of the obtained results concerning the hit Elk4.** Silencing of Elk4 in dedifferentiated cancer cells leads to a general redifferentiation, which is characterized by a shift towards an epithelial morphology, loss of in vitro invasion, gain of aggregation and the loss of metastatic forming capability in tail vein experiments of immunocompromised mice. Furthermore, a severe disturbance of TGFbeta dependent transcription has been observed, as well as the upregulation of the epithelial stabilizing microRNAs of the miR200 family.
5 NEDERLANDSE SAMENVATTING

Dit project had als doel het identificeren van nieuwe functionele interactoren van ZEB1 in de context van EMT. Zoals reeds beschreven in de introductie, zijn reeds een veelvoud van transcriptiefactoren en cofactoren bekend die in staat zijn zowel fysiek als functioneel met ZEB1 te interageren. Echter, voor deze factoren werd slechts een beperkt deelaspect van de ZEB1 functionaliteit onder de loep genomen, waarbij enkel gefocust werd op specifieke signalisatiewegen. De studies van deze functionele associaties werden dus nog niet in verband gebracht met het proces van EMT. Om hieraan tegemoet te komen, is een in vitro siRNA screening uitgevoerd in een cellulair EMT model aan de hand van een bibliotheek die als doelwit alle gekende humane transcriptiefactoren heeft.

Bij de constructie van ons EMT model hebben we ons initieel gebaseerd op de cellijnen A431 en MCF-7, twee epitheliale kankercellijnen respectievelijk afkomstig van niet invasieve huid en borstkanker tumoren. Deze keuze is bijzonder interessant in het geval van MCF-7 omdat er sterke evidenties bestaan die wijzen op het bestaan van een EMT gerelateerd fenotype in een specifieke subgroep van borstkanker tumoren, namelijk de basale B groep. Beide cellijnen zijn vervolgens uitgerust met induceerbare ZEB1 expressie, waarbij inductie in de twee gevallen leidt tot een mesenchymaal fenotype. Vervolgens werd een selectie gestart voor een geschikt EMT rapporteersysteem. Hiervoor zijn verschillende EMT gerelateerde genpromoters voor een luciferase rapporteer cassette geplaatst en getest naar hun respons op ZEB1 inductie in beide induceerbare systemen. Op basis hiervan is besloten om in het MCF-7 systeem te opteren voor de vimentinepromoter als robuuste EMT sensor. Echter, voor A431 kwam geen enkel rapporteerconstruct in aanmerking om een ondubbelzinnige meting te leveren voor het al dan niet aanwezig zijn van een EMT. Hierdoor is besloten om alleen verder te werken met de MCF-7 cellijn voor het uitvoeren van de screening. De keuze voor de vimentinepromoter brengt echter een additioneel probleem met zich mee. Een positief resultaat betekent immers dat de activiteit van de vimentinepromoter dient naar beneden te gaan. Echter, het valt niet uit te sluiten dat dit effect een aspecifieke oorzaak heeft, bijvoorbeeld door het affecteren van de viobiliteitsmechanismen van de cel. Hierdoor zal de activiteit van de vimentinepromoter dalen, maar dit effect is niet EMT gerelateerd. Om
hieraan te verhelpen, is een additionele rapporteer cassette in het MCF-7 systeem gebracht die gebaseerd is op de EF1alpha promoter, gevolgd door het “gesecreteerd alkaline fosfatase” (SEAP) rapporteergen. Stabiele integratie van beide rapporteerconstructen in de ZEB1 induceerbare MCF-7 cellen leverde resultaten die optimaal zijn voor het uitvoeren van de screening.

De screening is in drievoud uitgevoerd in een 96 well plaat, waarbij in totaal 1040 gekende transcriptiefactoren onderdrukt werden. Dit heeft geresulteerd in de identificatie van 27 doelwitgenen die in staat zijn de opregulatie van de vimentinepromoteractiviteit onder invloed van ZEB1 tegen te gaan. Om aspecifieke effecten verder uit te sluiten, is een secundaire screening uitgevoerd, waarbij gebruik gemaakt is van vier onafhankelijke siRNA oligo's per nieuw geïdentificeerd doelwitgen. Aan de hand van deze deconvolutiescreening zijn aldus 22 van de 27 genen bevestigd als potentiële functionele interactoren van ZEB1 gemedieerde EMT.

Echter, het feit dat in de screening enkel is gekeken naar de activiteit van de vimentinepromoter betekent dat we niet beschikken over een volledige kennis inzake de EMT verstoringe effecten van onze doelwitgenen. Om hieraan te tegemoet te komen, is een expressie analyse uitgevoerd waarbij gekeken is naar centrale EMT genen waarvan geweten is dat deze gemoduleerd worden door ZEB1. Dit EMT profiel is gekozen aan de hand van een micro-array experiment waarbij geïnduceerde MCF-7 ZEB1 cellen vergeleken zijn met hun controle. De op deze manier verkregen gegevens stelden ons in staat een clusteranalyse uit te voeren, waarin twee hoofdgroepen van doelwitgenen te onderscheiden waren. De eerste groep bestaat uit doelwitgenen die slechts een minimale impact op het door ons geselecteerde EMT profiel hebben. Dit betekent dus dat hoofdzakelijk de expressie van vimentine geaffecteerd wordt en dat deze doelwitgenen dus geen algemeen effect hebben op EMT. In de tweede groep daarentegen, worden negen genen teruggevonden die een significante invloed hebben op het volledige EMT profiel. Hoewel dit profiel slechts uit acht door ons geselecteerde genen gaat en dus slechts een beperkt beeld geeft, gaan we ervan uit dat de doelwitgenen in deze groep kunnen beschowd worden als ware positieve kandidaten. Op basis van deze overgebleven genen, is een functionele interactiemap gecreëerd waarbij verscheidene kandidaten met elkaar gelinkt werden. Dit netwerk wordt hoofdzakelijk gekenmerkt door de centrale rol van de twee transcriptiefactoren ZEB1 en Elk4. Behalve het feit dat deze twee genen
direct met elkaar verbonden zijn (zoals later zal blijken), lijkt ook de transcriptiefactor SRF betrokken te zijn in deze interactie. Het is inderdaad aangetoond dat Elk4 en SRF directe interactiepartners zijn en op deze manier zorgen voor de inductie van “early growth response” genen. Anderzijds is een ZEB1-SRF interactie reeds aangetoond die noodzakelijk is voor de \textit{in vivo} differentiatie voor endotheelcellen uit de aorta (Nishimura et al., 2006). Samen met het feit dat enkel bij het onderdrukken van Elk4 het behoud van een epitheliale morfologie in de aanwezigheid van ZEB1 werd waargenomen, hebben de bovenstaande bevindingen er ons toe aangezet de rol van Elk4 in ZEB1 gemedieerde EMT verder te onderzoeken.

Hiervoor is in eerste instantie de MCF-7 ZEB1 cellijn getransduceerd met een lentiviraal “short hairpin” construct dat Elk4 als doelwit heeft (shElk4). Aan de hand van deze cellijn zijn in eerste instantie bovenstaande bevindingen bevestigd. Het behoud van het epitheliale fenotype van de geïnduceerde MCF-7 ZEB1 cellen door toedoen van Elk4 onderdrukking komt verder tot uiting door het behoud van E-cadherine en ZO-1 op proteineniveau, terwijl vimentine significant minder tot expressie komt. De MCF-7 ZEB1 cellijn is echter een geconstrueerd EMT model waarin niet noodzakelijk dezelfde processen zich afspelen als in een kankercel die een EMT ondergaat tijdens kwaadaardige tumorprogressie. Hierdoor is besloten om Elk4 te onderdrukken in kankercellijn met een hoge endogene ZEB1 expressie en beschikken over mesenchymale eigenschappen. Hiertoe zijn de MDA-MB435, MDA-MB231 en Mia-PaCa2 cellijnen, respectievelijk afkomstig van borst- en pancreascarcinoma, stabiel getransduceerd met de lentivirale shElk4 vector. Alle drie deze cellijn hebben een mesenchymale morfologie en zijn reeds uitvoerig gekarakteriseerd als kankercellijn die een \textit{in vivo} EMT ondergaan hebben. Onderdrukking van Elk4 in in alle drie de gevallen resulteert in een reversie van het mesenchymale fenotype naar cellen met een epitheliale morfologie. Dit wordt tevens gereflecteerd op het proteineniveau, waar een opregulering van verschillende epitheliale merkers wordt waargenomen ten kost van het verlies van mesenchymale eiwitten. Naast deze bevindingen is ook nagegaan of Elk4 onderdrukking ook een effect heeft op functioneel gebied. Hiertoe is in eerste instantie een slow aggregatioin assay uitgevoerd, waarbij een significante toename van de cel-cel adhesie kon waargenomen worden in de MDA-MB231 en MDA-MB435S cellen waar Elk4 onderdrukt is ten opzichte van de controlecellijn. Dit wordt bovendien ondersteund.
door de relocatie van E-cadherine en ZO-1 naar de celmembrana. Voor de Mia-PaCa2
cellijn kon dit enkel bevestigd worden voor ZO-1, waar Elk4 onderdrukking verder niet
voor de opregulatie van E-cadherine zorgt. Deze bevinding is gelijkaardig aan het effect
dat waargenomen wordt wanneer ZEB1 in deze cellijn onderdrukt wordt. Ook hier wordt
geen opregulatie van E-cadherine waargenomen, alhoewel de cellen een duidelijke
verschuiving naar een epitheliaal fenotype vertonen. Deze parallelle effecten die optreden
bij zowel Elk4 als ZEB1 onderdrukking duiden op een nauwe samenwerking van beide
transcriptiefactoren op het gebied van EMT. Bovendien hebben we via injecties van
MDA-MB231 shElk4 cellen in de staartader van immunodeficiënte muizen aangetoond
dat Elk4 noodzakelijk is voor metastasevorming in de longen in vergelijking met controle
cellen. Dit betekent dat, naast het belang in de initiatie van het EMT fenotype, Elk4 ook
noodzakelijk blijkt te zijn voor latere stappen naar de vorming van metastasen toe.

Een belangrijk aspect van kankercellen die een EMT hebben ondergaan, is het verkrijgen
van migratorische en invasieve eigenschappen die aldus toelaten deze kwaadaardige
cellen nabijgelegen bloed- of lymfevaten te bereiken. Om na te gaan of Elk4 betrokken is
bij deze processen, is voor MDA-MB231 en MDA-MB435S een *in vitro* migratie en
invasie assay uitgevoerd. In overeenstemming met wat de hierboven beschreven
bevindingen suggereren, zorgt de onderdrukking van Elk4 in beide cellijnen voor een
verminderde migratie en invasie. Dit ging verder gepaard met het verkrijgen van een
epitheliaal morfologie van de cellen in de drie dimensionele matrix van de *in vitro*
invasietest. Bovendien kon een duidelijke clustering van de cellen waargenomen worden
in de conditie met de Elk4 onderdrukking, terwijl dit niet het geval was voor de
controlecellijnen. Deze waarnemingen suggereren verder dat extracellulaire stimuli die
eventueel aanwezig zijn in de drie dimensionele matrix niet in staat zijn het effect van
Elk4 onderdrukking op te heffen.

Bovenstaand beschreven experimenten duiden er dus op dat Elk4 een kritieke factor is in
ZEB1 gemedieerde EMT en EMT in het algemeen. Echter, op basis van deze
waarnemingen valt niet af te leiden welke moleculaire mechanismen die verantwoordelijk
zijn voor deze Elk4 afhankelijke effecten. Om hierover een meer gedetailleerd beeld te
verkrijgen, is een micro-array experiment uitgevoerd waarbij MDA-MB231 cellen met
een stabiele Elk4 onderdrukking vergeleken zijn met de controle. Hieruit is gebleken dat
verschillende cruciale signalisatiewegen en in het bijzonder de TGFbeta en Notch
pathway geaffecteerd zijn door Elk4 onderdrukking. Verder worden ook verschillende leden van de MMP familie, waarvan hun implicatie in tumorprogressie reeds is bewezen, onderdrukt door Elk4. Deze bevindingen zijn van bijzonder belang voor MMP1, dat op een directe manier door ZEB1 wordt opgereguleerd in MDA-MB231 en dus verder wijst op de connectie met Elk4. Additionele genen die beïnvloed worden door onderdrukking van Elk4 zijn de vermeende kanker tamcelmerkers CD44 en CD24 die respectievelijk open neergereguleerd worden. Dit suggereert dat Elk4 (partieel) verantwoordelijk is voor het in stand houden van stamceleigenschappen die recent werden gekoppeld aan het EMT fenotype. Echter, additioneel bewijs voor deze resultaten dient geleverd te worden waarbij 

in vitro de kolonievormende capaciteit wordt nagegaan en 

in vivo gecontroleerd worden op tumorigeniciteit door limiterende verdunningsexperimenten. Om het effect op de TGFbeta pathway te bevestigen, zijn MDA-MB231 shElk4 cellen getransfecteerd met een TGFbeta responsief rapporteerconstruct en vergeleken met de controle cellen. Hieruit blijkt dat de modulering van verschillende TGFbeta pathway componenten door Elk4 onderdrukking ook resulteert in een functionele inhibitie van de TGFbeta pathway. Op eiwitniveau uit dit zich in een opregulatie van de inhibitorische Smad6. Verder zijn deze resultaten tevens bevestigd in de induceerbare MCF-7 ZEB1 cellijn. Hier leidt ZEB1 expressie tot functionele opregulatie van TGFbeta, terwijl Elk4 onderdrukking dit ongedaan maakt. Western bot analyse toont aan dat Elk4 noodzakelijk is voor de fosforylatie van Smad2.

Recentelijk is de connectie tussen ZEB1 expressie en de microRNAs van de miR200 familie aangetoond, waarbij een inverse correlatie werd beschreven tussen ZEB1 en de microRNAs. Er wordt aangenomen dat de microRNAs van de miR200 familie stabiliserende factoren zijn voor het epitheliaal fenotype en dus een cruciale rol spelen bij het instellen en onderhouden van de epitheliaal homeostase. Door het bestaan van een negatieve “feed-back” regulatie met ZEB1, kan een relatief kleine daling in het niveau van de miR200 leden ervoor zorgen dat een epitheliaal systeem gedreven wordt naar een mesenchymaal lot (Brabletz and Brabletz, 2010). De expressie van miR200 leden is in gededifferentieerde kankercellijn, zoals MDA-MB435S en MDA-MB231, laag. Gezien de noodzakelijkheid van Elk4 expressie voor het mesenchymale fenotype, is nagegaan of Elk4 onderdrukking leidt tot opregulering van de miR200 familie. Aan de hand van een qPCR analyse, hebben we aangetoond dat Elk4 onderdrukking inderdaad in staat is tot het
verhogen van de expressieniveau's van miR200 leden in zowel MDA-MB231 als MDA-MN435S. Meer specifiek is onder invloed van Elk4 onderdrukking een opregulering van miR200 a en b in MDA-MB435S en miR200b in MDA-MB231. Deze bevindingen duiden op het feit dat Elk4 onderdrukking het systeem in een stabiele epitheliaal toestand brengt en suggereren verder dat Elk4 samen met ZEB1 strikt noodzakelijk is voor de onderdrukking van deze microRNAs. Verder onderzoek dient uit te maken of ook Elk4 kan binden op de promoters van de miR200 familie.

De hierboven beschreven bevindingen tonen dus aan dat in afwezigheid van Elk4, ZEB1 niet in staat is zijn EMT inducerende eigenschappen uit te oefenen. Ons hierop baserend, zijn we nagegaan of deze functionele interactie ook kan leiden tot een fysieke associatie tussen ZEB1 en Elk4. Dit is in eerste instantie nagegaan in HEK293T cellen waarbij beide factoren tot overexpressie gebracht zijn. Immunoprecipitatie in beide richtingen toont inderdaad aan dat een fysieke interactie mogelijk is tussen beide factoren. Bovendien is een interactie tussen Elk3, een homologe factor van Elk4, en ZEB1 hier eveneens aangetoond. Om deze bevindingen verder te verifiëren, is vervolgens een endogene immunoprecipitatie uitgevoerd in MDA-MB231 cellen. Ook hier is de fysieke interactie tussen ZEB1 en Elk4 aangetoond.

Als algemene conclusie kunnen we stellen dat we aan de hand van een reversed genetic screening verschillende nieuwe modulatoren hebben aangetoond voor de functionaliteit van ZEB1. Meer specifiek is besloten de transcriptiefactor Elk4 beter te karakteriseren. Elk4 onderdrukking in verschillende EMT modelsystemen leidt tot een reversie van het mesenchymale naar het epitheliaal fenotype. Elk4 is geïdentificeerd aan de hand van een \textit{in vitro} siRNA screening en bovenstaande bevindingen duiden aan dat onze screening in staat is om relevante data te genereren. Dit suggereert dat de alternatieve gevonden hits eveneens behoren tot een groep van transcriptiefactoren die een cruciale rol spelen in ZEB1 regulatie.
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7 CURRICULUM VITAE

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And then we have Mara! Coming from sunny Greece to the grey Belgium must have been quite a shock. To make things even worse, you decided to work in a lab with two guys who always think they are funny. Hopefully this has brought at least some benefit to you apart from the huge increase of “Belgian joke” knowledge. Besides that, I really enjoyed working with you during the past five years and I hope that everything goes well with your future scientific career. Also, I hope that you will be able to drive your Yaris for the next 20 years or so, because I cannot imagine a better car suited for you. First of all, it looks amazing. Second, the speeds you can attain with that little car are immense (even in reverse!). It can also easily fit 10 people plus an additional sofa. So as a conclusion, the combination of you and the Yaris provides so many thrills that you don’t even have to go to the fitness again.

Trui, je dagdagelijkse gedrevenheid lijkt onuitputtelijk. Waar komt dat toch vandaan? Misschien moeten wij hier allemaal eens wat meer exotisch beginnen eten of nu en dan eens een (heel) verre reis maken om het te kunnen begrijpen. Hoe dan ook, ik heb het je al eens verteld: ik ben geen held in het maken van verre reizen, dus zal ik me moeten beperken tot het eten. Na je reis in Uganda kan je ons misschien laten maken met de Afrikaanse keuken en eventueel op die manier ervoor zorgen dat we jouw tempo kunnen volgen. Of ligt het misschien aan het feit dat we nu niet bepaald getrainde salsadansers zijn? Misschien moet je begrijpen dat je aan een bijna onmogelijke opdracht begint (in mijn geval dan toch). Maar goed, een mens is nooit te oud om te leren. Misschien moet je beginnen om Joachim al even dansles te geven, dan kunnen de anderen toekijken of het wel iets voor hen is. Of misschien kan Kelly het ons ook leren. Je hebt nog de keuze Joachim.

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daaropvolgend doctoraat. Niet alleen heb je een talent om alles goed uit te kunnen leggen, ook je regisseurcapaciteiten mogen gezien worden. Hoewel dat laatste nu niet onmiddellijk iets is wat je nodig hebt om je experimenten te doen slagen, levert dat wel veel respect op bij je labcollega’s. Hou je misschien toch maar een beetje in voor mijn filmpjes. Kwestie van alles een beetje deftig te houden, het is zo al erg genoeg. Eén dingetje dat je misschien nog moet leren is dat je je bril niet afkuist met de zakdoek waar je net je neus in gesnoten hebt. Maar dat is een detail natuurlijk.

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Alexander