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Review

The role of the ZEB family of transcription factors in development and disease

C. Vandewalle[a,b], F. Van Roy[b,c] and G. Berx[a,b,*]

[a] Unit of Molecular and Cellular Oncology, Department for Molecular Biomedical Research, Ghent University, Technologiepark 927, VIB, 9052 Ghent (Belgium), Fax: +32 9 331 36 09, e-mail: Geert.Berx@dmbr.UGent.be
[b] Department of Molecular Biology, Ghent University, Technologiepark 927, 9052 Ghent (Belgium)
[c] Molecular Cell Biology Unit, Department for Molecular Biomedical Research, VIB, Ghent University, Technologiepark 927, 9052 Ghent (Belgium)

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Abstract. The ZEB family of zinc finger transcription factors are essential players during normal embryonic development. One characteristic is that they induce epithelial to mesenchymal transition (EMT), a process that reorganizes epithelial cells to become migratory mesenchymal cells. E-cadherin is a major target gene of these transcriptional repressors, and this downregulation is considered a hallmark of EMT. In recent years, the involvement of the ZEB proteins in pathological contexts has been documented as well. Mutations in ZEB encoding genes cause severe syndromic malformations and evidence is mounting that links these factors to malignant tumor progression. In this review, we describe what is currently known on the molecular pathways these transcription factors are implicated in, and we highlight their roles in development and human diseases, with a focus on tumor malignancy.

Keywords. EMT, ZEB, development, cancer, invasion, transcription, migration.

Introduction

Epithelial mesenchymal transition (EMT) is considered a critical feature of normal development. This process, in which cells undergo a molecular switch from a polarized, epithelial phenotype to a highly motile, non-polarized mesenchymal phenotype, is essential for developmental processes such as gastrulation, neural crest formation, heart morphogenesis and formation of the musculoskeletal system and craniofacial structures. It has recently become clear that similar transitions can occur in epithelial tumors, giving rise to a population of highly motile and invasive cancer cells. One key feature of EMT is the downregulation of E-cadherin, a cell adhesion molecule present in the plasma membrane of normal epithelial cells. E-cadherin is considered a tumor and invasion suppressor and it is repressed by several mechanisms during malignant transformation. Mutations of the E-cadherin gene are found in diffuse gastric and infiltrative lobular breast carcinomas [1, 2]. However, in cancers at large, E-cadherin mutations seem to be quite rare, and focus has recently shifted to its transcriptional repression. Candidate E-cadherin repressors are members of the Snail family of transcription factors [3–5], bHLH factors such as E12/E47 [6], Twist [7] and the recently identified factors CBF-A (CArG box-binding factor-A) [8], FOXC2 (forkhead 1) [9], HOXB7 (homeobox gene B7) [10] and

* Corresponding author.

KLF8 (Krüppel-like factor 8) [11], as well as the ZEB family of transcription factors, including ZEB1 (also known as δEF1) [12] and ZEB2 (also known as SIP1) [13]. Over the past few years, the critical role of some of these EMT-inducing transcription factors both during normal development and in physiopathological situations has been well-documented. A model in which cancer cells acquire invasive and metastatic properties by exploiting EMT-inducing transcription factors is becoming plausible. This review will focus mainly on the ZEB family members and their roles and mechanisms during development and in human disease, in particular cancer progression.

The ZEB family of transcription factors: structural properties

Zinc fingers are among the most common DNA-binding motifs in eukaryotes. ZEB/δEF1/ZFH-1 zinc-finger-homeodomain proteins are complex transcription factors that have several functional domains. They are featured by two separate arrays of C_2H_2-type Zn-finger domains and a centrally located homeodomain (Fig. 1). This homeodomain is POU-like and does not bind DNA, so it might be mainly involved in protein-protein interactions. There is only one orthologue in *Drosophila melanogaster* and *Caenorhabditis elegans*, which is named, respectively, Zfh1 and Zag-1 [14, 15]. Vertebrates, on the other hand, have two homologous ZEB proteins. ZEB1 (also known as δEF1, Nil-2-a, Tcf8, Bzp, Areb6, Meb1, Zfhx1a and Zfhep) has been identified as a nuclear factor that specifically binds to and represses the lens-specific δ1-crystallin enhancer in chicken [16]. The second member, ZEB2 (also known as SIP1 and Zfhx1b) was isolated as a mouse cDNA encoding a protein that binds the MH2 domain of *Xenopus* Smad1 (XSmad1) heterologously expressed in yeast [17]. It is a DNA-binding transcriptional repressor that interacts in a ligand-dependent fashion with receptor-activated Smads involved in mediating TGF-β signaling [17]. Like ZEB1/δEF1, ZEB2/SIP1 contains two widely separated Zn-finger clusters (Fig. 1). The N-terminal cluster (NZF) contains four Zn-fingers (three CCHH fingers and one CCHC finger), while the other cluster (CZF), located in the C-terminal part of the protein, contains three CCHH zinc fingers. A high degree of sequence identity exists within the NZF (88%) and CZF (93%) clusters between ZEB2/SIP1 and ZEB1/δEF1, whereas the regions outside the Zn-fingers are considerably less conserved [17] (Fig. 1). This suggests that both proteins have similar DNA-binding specificities. Each Zn-finger cluster can bind independently to 5'-CACCT(G) sequences within the transcriptional regulatory regions of target genes. The integrity of the two Zn-finger clusters of ZEB2/SIP1 is necessary for its binding as a monomer to the target promoter sequences [18]. The high degree of similarity between ZEB1/δEF1 and ZEB2/SIP1 proteins is also reflected at the genomic level. The structure of the mouse ZEB1/δEF1 gene is strongly homologous to the ZEB2/SIP1 gene structure, although *Zeb1* has a transcriptional initiation site close to the start codon, whereas the mouse *Zeb2* 5'UTR exhibits a highly complex organization. Indeed, nine untranslated exons (U1 to U9) were identified upstream of the first translated exon, giving rise to multiple transcripts originating from different splice events. Furthermore, three alternative promoters can be distinguished (P1 to P3) of which P2, with a major transcription initiation site more than 2.7 kb upstream of the start codon, appears to be the most active *in vitro* [19].

The dual role of ZEB proteins in transcriptional repression and activation

Molecular mechanisms of action. ZEB1/δEF1 and ZEB2/SIP1 can repress transcription by directly binding to 5'-CACCT sequences located in various gene promoters. The list of ZEB target genes is growing fast but the mechanism of action by ZEB proteins remains elusive. The structural complexity of these proteins, combining several binding sites for co-repressors with potential posttranslational modifications, points to intricate modes of action. CtBP was originally identified as a protein interacting with a PLDS sequence in the C-terminal segment of the adenovirus E1A oncoprotein [20]. The identification of PXDLS motifs in both ZEB1/δEF1 and ZEB2/SIP1 led to the assumption that CtBP can act as a co-repressor for either protein. Recently, a CtBP co-repressor core complex was identified. This complex contains both ZEB1/δEF1 and ZEB2/SIP1, together with histone modifying enzymes (histone deacetylases and histone methyltransferases), chromodomain-containing proteins, coREST and coREST related proteins, thereby combining all essential elements for promoter targeting, transcriptional repression and chromatin remodelling [21]. Furthermore, interaction between endogenous ZEB2/SIP1 and endogenous CtBP was shown to depend on the PXDLS motifs in the ZEB2/SIP1 protein, designated the CtBP interaction domain (CID) (Fig. 1). The CID of ZEB2/SIP1 alone can repress transcription in a CtBP-dependent manner when recruited to the E2-boxes of the E-cadherin promoter. Controversially, in overexpression experiments, CtBP does not seem to be required for repression of E-cadherin transcription by full-length

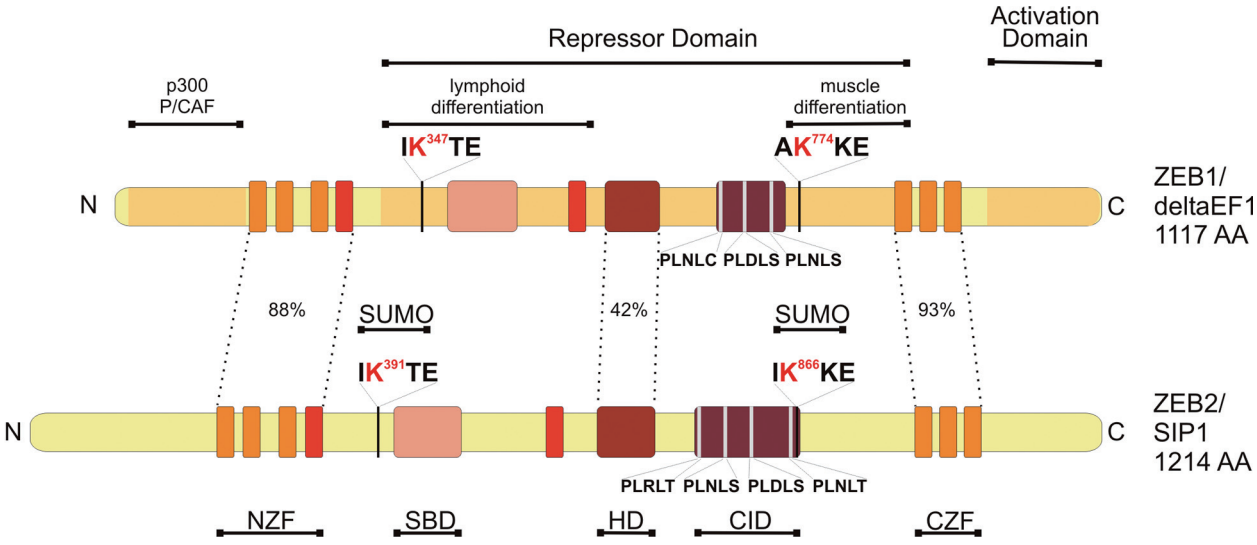

Figure 1. Schematic representation of the two members of the ZEB family of transcription factors. The ZEB family contains two members, ZEB1/δEF1 and ZEB2/SIP1. They are characterized by the presence of two zinc finger clusters, one at each end (NZF and CZF) and a centrally located homeodomain (HD). Other domains are the Smad binding domain (SBD) and the CtBP interaction domain (CID). ZEB1/δEF1 and ZEB2/SIP1 act mainly as transcriptional repressors by high-affinity binding of the two zinc finger domains to specific DNA binding sites, called z-boxes (CACCT(G)). An alternative role as transcriptional activator, however, cannot be excluded. In the ZEB1/δEF1 protein a transcriptional activation domain and a binding site for the co-activators p300 and P/CAF were identified. Both ZEB proteins are posttranslationally modified by SUMOylation (SUMO), which affects their repressor activity.

ZEB2/SIP1 or ZEB1/δEF1 [22]. Adenovirus protein E1A can relieve repression of a similar E-cadherin promoter construct in a CtBP-dependent manner, presumably by sequestering CtBP [12]. Direct knockdown of CtBP1 induces E-cadherin promoter activity in osteosarcoma U2OS cells expressing ZEB2/SIP1 and ZEB1/δEF1 [21]. In addition, pinin (Pnn/DRS), a CtBP1-interacting factor, can relieve CtBP1-dependent E-cadherin repression [23]. This indicates that CtBP is indeed involved in E-cadherin repression, although it is doubtful that this occurs by direct interaction with ZEB2/SIP1 and/or ZEB1/δEF1. This does not exclude the possibility that CtBP contributes as a ZEB-binding co-repressor for other target genes besides E-cadherin.

It has been suggested that ZEB1/δEF1 represses *in vitro* transcription of the immunoglobulin heavy chain enhancer by competing with activators of the basic helix-loop-helix family [24]. In support of this view, ectopic expression of ZEB1/δEF1 was shown to counteract MyoD/Myf5- or MyoD/Myf6-mediated transcriptional activation of p73. During muscle differentiation, the expression of p73 is then controlled by the coordinated action of these muscle regulatory transcriptional activators and the transcriptional repressor ZEB1/δEF1 [25, 26]. Recent studies have indicated that binding of ZEB1/δEF1 to the intronic regulatory sequence of p73 is impaired in Fanconi anemia cells (FA-A), which appears to be caused by methylation of this region [27]. Alpha7 integrin is another gene product important during skeletal myogenesis and myodifferentiation. ZEB1/δEF1 controls α7 integrin expression in myoblasts by competing with MyoD for binding to the negative regulatory region in the α7 integrin promoter [28]. The mechanism of action seems more complex than passive displacement of MyoD. Rather, the ability of ZEB1/δEF1 to compete for limiting amounts of the co-activator p300/CBP is probably responsible for repression of α7 integrin in myoblasts. Alpha4 integrin expression in hematopoietic cells, on the other hand, is regulated by a mechanism in which c-Myb and Ets hematopoietic transcription factors synergize to resist repression by ZEB1/δEF1 [29].

Evidence that ZEB1/δEF1 is an active repressor has been proposed because a repression domain close to the N-terminus is necessary for repression of the δ-crystallin enhancer [30]. Postigo et al. [31], postulated that ZEB1/δEF1 contains two independent repressor domains, with one domain, close to the C-terminus, regulating muscle differentiation and specifically blocking the activity of the myogenic transcription factor MEF2C (Fig. 1). The other domain, near the N-terminus, is postulated to function in lymphocytes to regulate the activity of hematopoietic factors such as c-Myb and Ets family members.

The Tat-interacting protein TIP60 of the human immunodeficiency virus type 1 has been suggested as a co-repressor for ZEB1/δEF1 in repressing CD4-enhancer/promoter activity [32]. Overexpression studies, however, excluded TIP60 as a potential co-repressor for ZEB2/SIP1 (Michiels and van Grunsv-

en, personal communication). Interaction between ZEB1/δEF1 and the ubiquitous negative cofactor NC2 has also been shown, providing another possible mechanism of transcriptional repression [33].

Though most research has focused on the ability of ZEB1/δEF1 and ZEB2/SIP1 to repress gene expression, their ability to activate transcription has also been reported. Regulation of vitamin D_3 receptor (VDR), a steroid-thyroid receptor determining developmental differentiation processes as well as the differentiation status of several malignant cell lines, is at least partly mediated by ZEB1/δEF1. ZEB1/δEF1 binds to two sites within the VDR promoter *in vitro* and therefore presumably activates transcription of this receptor directly [34]. Recruitment of co-activators like p300 or P/CAF and displacement of CtBP may also be part of this mechanism. Indeed coexpression analysis of CtBP, p300, ZEB1/δEF1, VDR and CDH1 in a series of colon carcinomas indicates that the expression level of the co-regulator determines the repressor or activator status of ZEB1/δEF1 [35]. Nevertheless, the upregulation of VDR by ZEB1/δEF1 remains puzzling because VDR activates E-cadherin expression and stimulates differentiation of colon carcinoma cells upon ligand binding [36]. Recently, functional cooperation between FOXO transcription factors and ZEB1/δEF1 in B lymphocytes has been revealed [37]. ZEB1/δEF1 binds to and activates the promoters of two FOXO target genes, Ccng2 (cyclin G2) and Rbl2 (retinoblastoma-like protein p130/Rb2), both of which are implicated in cell cycle arrest and FOXO-dependent quiescence in fibroblasts. ZEB1/δEF1 activates transcription of these two genes and strongly synergizes with FOXO proteins.

The molecular mechanisms underlying the choice between repression and activation by ZEB1/δEF1 or ZEB2/SIP1 are currently unknown, but may include cell type specific differences in post-translational modification. Both hyperphosphorylated and hypophosphorylated forms of ZEB1/δEF1 are expressed in cell lines. Differential expression of these two forms may contribute to cell type specific activities of ZEB1/δEF1 [38]. Phosphorylation may also modify the ability of ZEB1/δEF1 to interact with certain co-repressors or co-activators, providing an additional mechanism for regulating transcriptional activity by ZEB1/δEF1.

SUMOylation is an important posttranslational modification that can regulate multiple functional aspects of target proteins. Recently, it was shown that ZEB2/SIP1 and ZEB1/δEF1 are both SUMOylated and that, at least for ZEB2/SIP1, this is mediated by polycomb protein Pc2, which acts as a SUMO E3-ligase [39] (Fig. 1). This covalent modification does not affect the subcellular localization of ZEB2/SIP1, but attenuates its transcriptional activity in a manner that depends on the promoter context, resulting in less efficient repression of E-cadherin.

ZEB target genes. Correct spatio-temporal gene regulation is essential for the successful execution of developmental and differentiation programs. Numerous reports have identified ZEB proteins as transcriptional regulators of crucial steps in cartilage, bone and muscle formation as well as in the development of hematopoietic cells (Fig. 2). ZEB proteins are thus at the crossroads of multiple developmental pathways, which assigns them an essential role in the development of normal architecture of the whole organism. An inverse correlation exists between ZEB1/δEF1 mRNA expression and the differentiated phenotype of chondrocytes [40]. Indeed, ZEB1/δEF1 acts as a repressor of type II collagen gene expression during chondrogenesis by binding to an E2-box (CACCTG sequence) in the proximal promoter of the *Col2a1* gene. These data, together with the expression pattern of ZEB1/δEF1 during embryogenesis [41] suggests a role for ZEB1/δEF1 in the suppression of chondrocyte-specific genes in limb bud mesenchyme before the onset of chondrogenesis. In addition, ZEB1/δEF1 also affects type I collagen expression in osteoblasts. A 123 bp repressor element, named COIN-1, was identified in the mouse pro-α1(I) collagen promoter. This element consists of an almost perfect three-fold repeat of a 41 bp motif containing an E2-box. It is able to bind ZEB1/δEF1 protein and point mutations in these E2-boxes not only abolish the repressor effect of COIN-1 but also abrogate the binding of ZEB1/δEF1 [42]. Within the pro-*Col1a2* gene, an enhancer region located about -17 kb from the transcription start site, contains a specific vascular smooth muscle cell (vSMC) element. Transcriptional regulation by this element is achieved by a finely tuned repression-activation mechanism in which the repressing transcription factor ZEB1/δEF1 and the activating homeodomain factor Nkx2.5 compete for an overlapping site [43]. The onset of *in vitro* osteogenic differentiation is also associated with the upregulation of the expression of the Liver/Bone/Kidney Alkaline Phosphatase (LBK-ALP) gene. ZEB2/SIP1 was shown to repress LBK-ALK promoter activity by virtue of its binding to the CACCT/CACCTG sites in the latter promoter [44].

ZEB1/δEF1 plays another important regulatory role during T cell development. Targeted disruption of ZEB1/δEF1 in mice results in a large reduction of thymocytes and the few cells that reach maturity are predominantly CD4+ [45]. Brabletz et al. [46] showed that ZEB1/δEF1 negatively regulates CD4 gene

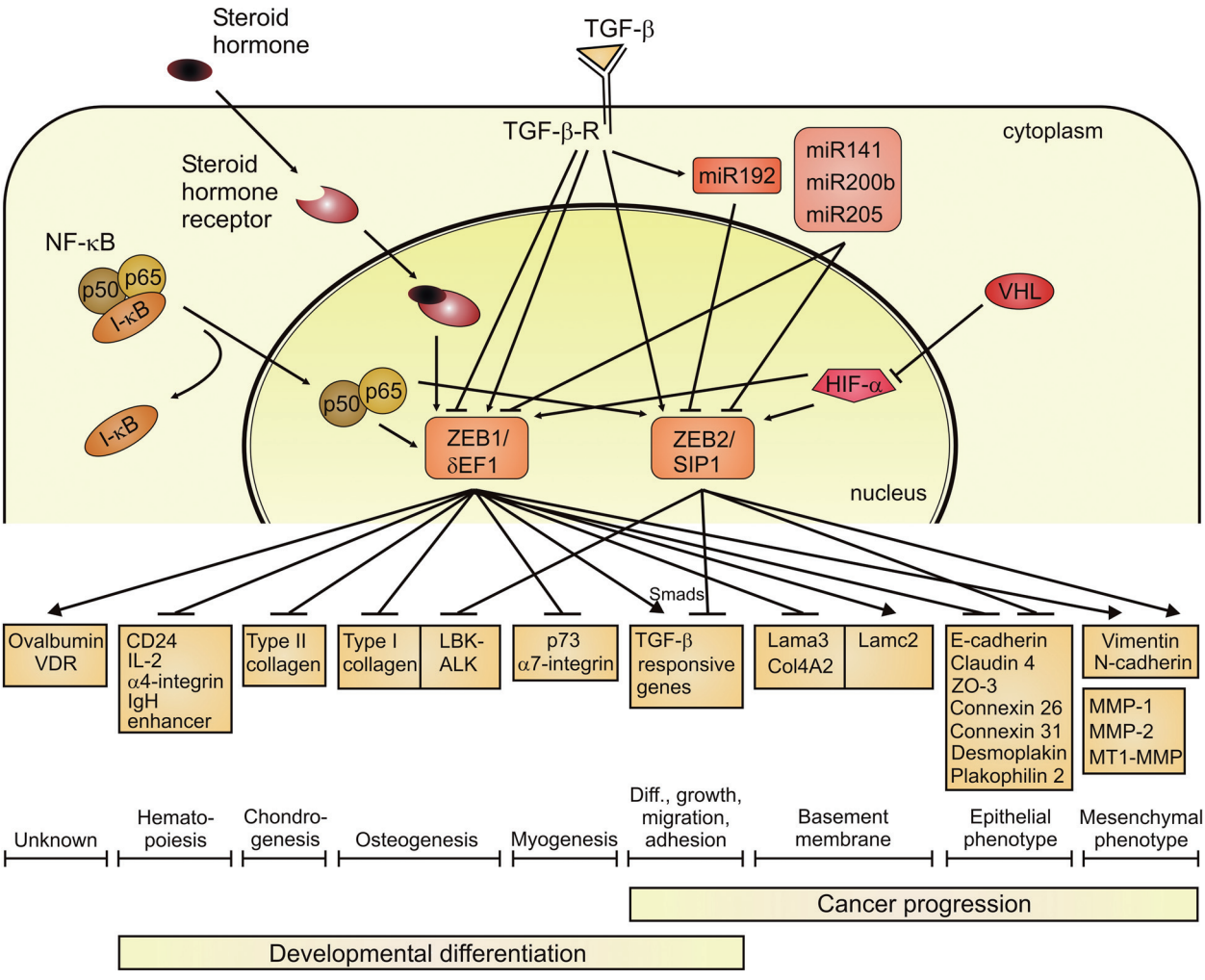

Figure 2. Upstream signaling events and downstream targets of the ZEB family of transcription factors. ZEB1/δEF1 and ZEB2/SIP1 are both downstream effectors of TGF-β-mediated EMT but can also be downregulated by TGF-β. For ZEB2/SIP1, this occurs by the action of microRNA miR192. In addition miR141, miR200 family and miR205 target both ZEB1/δEF1 and ZEB2/SIP1. Other pathways leading to ZEB1/δEF1 and ZEB2/SIP1 expression involve NF-κB- and HIF-α-dependent mechanisms, and ZEB1/δEF1 has been implicated in steroid hormone signaling as well. An array of genes is modulated by ZEB1/δEF1 and ZEB2/SIP1 during developmental processes (bottom left) or during cancer progression (bottom right). The functional implications of induced expression of ovalbumin and the vitamin D3 receptor (VDR) by ZEB1/δEF1 are unknown.

expression by binding to the 5' E-box within the proximal CD4 enhancer thereby competing with the transcriptional activators E12 and HEB. Furthermore, ZEB1/δEF1 represses IL-2 gene expression in terminally differentiated Th2 cells by binding the negative regulatory element, NRE-A, in the IL-2 promoter [47].

The life cycle of Epstein-Barr virus (EBV), a human herpes virus capable of establishing a latent state in B lymphocytes, might also be regulated by ZEB1/δEF1. A negative cis-acting element within the promoter of the immediate-early *BZLF1* viral gene was found to bind to ZEB1/δEF1 leading to repressed BZLF1 promoter activity [48].

ZEB proteins and signaling

As mentioned before, ZEB2/SIP1 was identified as a protein capable of interacting with receptor activated Smads [17]. More recently, also ZEB1/δEF1 was shown to bind, although less efficiently, to activated R-Smads-1, -2 and -3, indicating a role for both ZEB proteins in both BMP and TGF-β signaling [49]. A conserved region downstream of the N-terminal zinc finger cluster was recognized in both proteins as the Smad interacting domain (SBD in Fig. 1) [17, 49]. TGF-β family members exert a variety of effects on cell proliferation, differentiation, migration, adhesion and apoptosis [50]. Binding of TGF-β or BMP factors to their respective receptors leads to translocation of Smad proteins to the nucleus, where they activate

transcription of target genes. What roles ZEB1/δEF1 and ZEB2/SIP1 play in TGF-β or BMP signaling, remains vague. Postigo et al. [49] postulated ZEB1/δEF1 and ZEB2/SIP1 as possible regulators of this pathway with opposing effects on TGF-β/BMP-mediated transcription. While ZEB1/δEF1 would synergize with Smad proteins to activate transcription of TGF-β responsive reporter constructs, the structurally very similar ZEB2/SIP1 would inhibit activation by TGF-β (Fig. 2). These antagonistic effects were reported to result from the differential recruitment of transcriptional co-activators (p300 and P/CAF) and co-repressors (CtBP) to Smads by ZEB1/δEF1 and ZEB2/SIP1, respectively [51]. Consensus binding sites for ZEB1/δEF1 and ZEB2/SIP1 are present in the promoters of several TGF-β/BMP target genes, which could be crucial for concentrating endogenous ZEB and Smad proteins at target sites. ZEB2/SIP1 and ZEB1/δEF1 would then target only a subset of TGF-β/BMP-dependent genes according to the distribution of their binding sites. Recently, these data were extended with the finding that ZEB1/δEF1, ZEB2/SIP1 and Snail are induced in NmuMG cells upon treatment with TGF-β. This results in a spectacular EMT with loss of E-cadherin expression that is dependent on both ZEB2/SIP1 or ZEB1/δEF1 but not on Snail. Interestingly, a direct target of the TGF-β-Smad pathway is the Ets1 transcription factor which is known to stimulate expression of ZEB1/δEF1 and ZEB2/SIP1. Ets1 activity is negatively controlled by Id2, a nuclear factor that is repressed by TGF-β [52]. In a mouse model of diabetic kidney failure, TGF-β was recently also shown to downmodulate the expression levels of both ZEB1/δEF1 and ZEB2/SIP1 [53]. TGF-β1-mediated induction of the collagen gene *Col1a2* in mouse mesangial cells was found to result from a derepression at the E-box elements located in the *Col1a2* gene, caused by ZEB2/SIP1 and ZEB1/δEF1 depletion. Moreover, the authors provided evidence for a novel mechanism in which ZEB2/SIP1 expression is targeted by a microRNA, miR192, which is upregulated by TGF-β1 in the kidney (Fig. 2). In addition, recent reports indicated that the miR141, miR200 family and miR205 directly target ZEB1/δEF1 and ZEB2/SIP1 resulting in repression of ZEB1/δEF1 and ZEB2/SIP1 protein expression [54–56, 58, 59]. Expression analysis of miRNAs in normal human tissues made clear that the miRNAs targeting ZEB family members are particularly abundant in epithelial tissues [57]. Interestingly, these different miRNAs are repressed by TGF-β treatment or via overexpression of the tyrosine phosphatase Pez, which results in EMT with loss of E-cadherin expression [58]. Furthermore, ZEB1/δEF1 potently represses transcription of miR141 and miR200c. The EMT activator TGF-β2 is also strongly downregulated by these miRNAs, indicating that ZEB1/δEF1 induces a microRNA-mediated feedforward loop [59].

Estrogen is a lipophilic hormone that diffuses into cells, binds the estrogen receptor and subsequently regulates genes called primary estrogen response genes. A primary response gene often encodes a transcription factor capable of regulating downstream genes, including secondary response genes. ZEB1/δEF1 has been shown to be a key player in estrogen-signaling cascades [60]. In this pathway, estrogen induces the expression of ZEB1/δEF1, which concomitantly activates transcription of downstream targets including ovalbumin (Fig. 2). Two cis-acting DNA elements in the 5' flanking region of the chick ovalbumin gene were identified as essential for specific induction by estrogen and found to be bound by ZEB1/δEF1 [61]. Mechanistically, ZEB1/δEF1 and the ubiquitous basic helix-loop-helix transcription factor USF (Upstream Stimulatory Factor) have been demonstrated to form a functionally relevant protein complex whereby USF would be guided by ZEB1/δEF1 to the 5' regulatory regions of the ovalbumin gene [62]. Furthermore, deletion of the C-terminal acidic domain of ZEB1/δEF1, a polyglutamic acid tract, was shown to be detrimental for the ovalbumin activation ability of ZEB1/δEF1, suggesting that the latter can activate by itself [63]. These observations again highlight the multifunctionality of ZEB1/δEF1 as a transcription factor capable of activating as well as repressing transcription, the choice of which is most likely determined by DNA-context and cell type. A global examination of progesterone receptor (PR) regulated genes in a breast cancer model determined that ZEB1/δEF1 is selectively upregulated by the PR B isoform, implicating ZEB1/δEF1 in progesterone signaling as well [64].

NF-κB, initially discovered as a major activator of immune and inflammatory functions as it induces expression of genes encoding cytokines, cytokine receptors and cell adhesion molecules, has more recently been implicated in the control of cell proliferation and oncogenesis [65]. The precise role of activated NF-κB in tumor progression is, however, unknown. A recent study, making use of a breast cancer model overexpressing the constitutively active p65 subunit of NF-κB, demonstrated that NF-κB induces an epithelial to mesenchymal transition that coincides with elevated expression of ZEB1/δEF1 and ZEB2/SIP1 and concomitant loss of epithelial-specific genes, such as E-cadherin and desmoplakin [66]. These data point to a role for ZEB1/δEF1 and ZEB2/SIP1 in the NF-κB signaling pathway, at least during breast cancer progression (Fig. 2).

Key roles of ZEB family members in development

Expression and function during embryogenesis. The first studies on ZEB2/SIP1 mRNA expression were performed in Xenopus embryos and indicated an important role in early embryogenesis. XSIP1 mRNA is first detected at stage 10.5 (early gastrula) in Xenopus embryos, primarily in dorsal mesoderm and ectoderm [67]. During gastrula and neurula stages, expression persists in neuroectoderm, the neural folds, neural tube, migrating neural crest and lateral plate mesoderm. Whereas XSIP1 is expressed early in the development of the nervous system, XδEF1 transcription is activated only during neurulation and is restricted to the paraxial mesoderm [68]. From early tail bud stage on, XδEF1 and XSIP1 are coexpressed in the migratory cranial neural crest, retina and neural tube. In the adult, XSIP1 mRNA is detected in organs derived from ectodermal, mesodermal and endodermal origin, including brain, spinal cord, eye, skin, heart, liver and lung [67]. XSIP1 is known to play an important role in the regulation of Xbra (Xenopus Brachyury), a member of the T-box family of transcription factors and essential in mesoderm formation during early development. Whereas Xbra and XSIP1 are coexpressed at the onset of gastrulation, their expression patterns become mutually exclusive in that Xbra is present in prospective mesoderm and XSIP1 in anterior neuroectoderm. This is due in part to the ability of XSIP1 to directly repress Xbra expression by interacting with the SIP1-binding sequences present in the 5' regulatory region of the *Xbra* gene [17, 69]. This is illustrated by the fact that Xbra reporter constructs carrying a point mutation in the XSIP1-binding site show widespread misexpression in ectoderm at the early gastrula stage [70]. Proof for the importance of XSIP1 comes not only from its expression pattern but also from overexpression studies versus knock-down experiments in Xenopus embryos [71, 72]. Further, it was shown that XSIP1 acts as a direct repressor of BMP4 and that efficient repression depends on the interaction of XSIP1 with the co-repressor CtBP, thus limiting BMP signaling and subsequent epidermal differentiation. Nevertheless, downregulation of certain epidermal genes by XSIP1 occurs independently of BMP repression [72].

Northern blot analysis and *in situ* hybridization experiments demonstrated strong ZEB2/SIP1 mRNA expression at early stages in the developing peripheral and central nervous systems of both mice and humans [73]. Furthermore, ZEB2/SIP1 could be detected in all neuronal regions of the brains in adult mice and 25-week old human fetuses and at varying levels in numerous non-neuronal tissues including thymus, heart, liver, skeletal muscle, kidney and bladder. The early and prominent expression of ZEB2/SIP1 in developing and adult neural tissues implicates a potentially important role for ZEB2/SIP1 in the control of diverse neuronal cell functions.

In the developing mouse embryo, ZEB1/δEF1 is expressed in the notochord, somites, limb, neural crest derivatives and restricted sites of the brain and spinal cord [41, 74].

To investigate the *in vivo* function of ZEB1/δEF1 during mouse development, knock-out mice were generated. Homozygous ZEB1/δEF1-deficient mice die perinatally due to respiratory failure and exhibit multiple skeletal defects including craniofacial abnormalities, limb and sternum defects, malformed ribs and hypoplasia of intervertebral discs, in addition to severe T cell deficiency of the thymus [41]. This is in accordance with the previously mentioned role for ZEB1/δEF1 as a repressor of collagen type-I and -II genes, indicating that ZEB1/δEF1 might be a direct modifier of chondrogenesis. Although ZEB1/δEF1 expression was shown in developing neural tissues, no distinctive phenotypic change was seen in the central nervous system of the ZEB1/δEF1-deficient mice. It has been suggested that ZEB2/SIP1, with its comparable DNA-binding specificities, might fulfil a compensating role for the loss of ZEB1/δEF1 in these tissues. Therefore, mice deficient in ZEB2/SIP1 were also generated [75]. Homozygous mutant embryos exhibit defects from E8.5 onwards. The neural tube fails to close, a sharp boundary between the neural plate and the rest of the ectoderm is absent, the somites are short and the first branchial arch is missing. The mice are severely retarded in their growth by E9.5, do not undergo embryonic turning and die [76]. Compared to normal E8.5 embryos, homozygous $Zeb2^{-/-}$ mutants show an early arrest in cranial neural crest cell migration and absence of neural crest cells at the postotic vagal level. Furthermore, specific knock-out of ZEB2/SIP1 in neural crest cells results in particular abnormalities in craniofacial, melanocyte and heart development, as well as defects in the peripheral nervous system [77]. E-cadherin expression, which is normally downregulated *in vivo* when the neuroepithelium differentiates from the ectoderm, persists in the homozygous mutant neuroepithelium [76]. This is consistent with the role assigned to ZEB2/SIP1 as a transcriptional repressor of E-cadherin *in vitro* [13]. Altogether, as the transient embryonic formation of neural crest cells is one of the best known EMT processes during development, the various abnormalities in neural crest cell formation, migration and differentiation in the ZEB2/SIP1 mutant mice suggests an essential role for ZEB proteins during EMT. Compound $Zeb1^{-/-};Zeb2^{-/-}$

homozygotes were recently analyzed and found to be similar to *Zeb2*$^{-/-}$ embryos in overall morphology and developmental evolution [78]. Compound *Zeb1*$^{-/-}$; *Zeb2*$^{-/-}$ embryos, however, showed a wider opening of the neural tube, and marked thinning of the portion that normally forms the dorsal half of the neural tube [78]. *Zeb2*$^{+/-}$ heterozygous mice were healthy, except for the frequent occurrence of vaginal orifice closure in females and, as mentioned above, *Zeb1*$^{-/-}$ embryos develop to term but die perinatally. Embryos with the compound genotype *Zeb1*$^{-/-}$;*Zeb2*$^{+/-}$ die at about E13.5, indicating a genetic interaction between the genotypes. From E10 on, these embryos develop various morphological defects mainly affecting maxillary tissues and the neural tube. Such defects are not seen in *Zeb1*$^{-/-}$ embryos [78]. Taken together, these data indicate that the activities of ZEB2/SIP1 and ZEB1/δEF1 are functionally additive, although ZEB2/SIP1 and ZEB1/δEF1 are expressed in areas of the central nervous system with only limited overlap.

ZEB1/δEF1 and ZEB2/SIP1 mutations cause syndromic malformations. Mowat-Wilson Syndrome (MWS) is a delineated, congenital mental retardation anomaly syndrome, characterized by a distinctive facial appearance, severe mental retardation and variable congenital malformations including genital abnormalities, agenesis of the corpus callosum, poor hippocampal formation, congenital heart disease and Hirschsprung disease (HSCR) [79]. HSCR, or aganglionic megacolon, is characterized by abdominal distension, constipation and severe vomiting due to the absence of ganglion cells along a part of the intestine. HSCR results from a defect in normal development of neural crest cells at the vagal level. The genetic etiology of this neurocristopathy is complex and multifactorial, with contributions from at least eight genes and possibly also environmental factors [80]. In 2001, *de novo* heterozygous mutations within the *ZEB2* gene were reported to correlate with the Hirschsprung disease-mental retardation syndrome [81–84]. Although most patients were ascertained on the basis of HSCR, several reports showing ZEB2/SIP1 mutations also in patients without HSCR have now been made [85–87]. Moreover, not all patients suffering from syndromic HSCR have a mutated *ZEB2* allele, suggesting that some *ZEB2* abnormalities are undetected so far or that mutations affecting other genes but active in the same developmental pathways may be involved [84, 85]. A few patients were reported to have a missense mutation in *ZEB2*, but all other ZEB2/SIP1 mutations described to date – deletions, insertions, nonsense and frame shift mutations – cause early truncation of the encoded protein leading to a loss of function [87, 88]. As a WT allele was also present, haploinsufficiency at the *ZEB2* locus is the most likely disease-causing mechanism. Heterozygous mutant mice, however, do not develop aganglionic phenotypes similar to those seen in human patients with Hirschsprung disease-mental retardation syndrome. Nonetheless, the embryonic phenotype of homozygous ZEB2/SIP1 mutant mice can be useful to clarify many aspects of the syndrome. Complete lack of vagal neural crest precursor cells in the mutant embryos reflects the dependence of these precursors on ZEB2/SIP1 activity for their normal development. The specific importance of ZEB2/SIP1 in neural development was recently further demonstrated using conditionally deficient mice, lacking ZEB2/SIP1 expression in the dorsal telencephalon. These mice survive to juvenile age but lack the entire hippocampus and corpus callosum, defects with variable penetrance, which are also present in human patients diagnosed with Mowat-Wilson Syndrome. A combination of decreased proliferation of neuronal progenitors and increased apoptosis of postmitotic cells was shown to be at the basis of the neural abnormalities in the ZEB2/SIP1 mutant mice. The Wnt inhibitor SFRP1 (secreted Frizzled related protein 1) was shown to be a direct ZEB2/SIP1 target gene in the developing hippocampus. Its upregulation in the ZEB2/SIP1 mutant mice might account for at least part of the phenotype by blocking of the JNK-dependent non-canonical Wnt signaling pathway [89]. Posterior polymorphous corneal dystrophy (PPCD) is a rare corneal disease often inherited in an autosomal dominant manner. Abnormalities in PPCD include metaplasia and overgrowth of corneal endothelial cells with an epithelial morphology and gene expression pattern, besides an aberrant corneal endothelial cell basement membrane (Descemet membrane) [90]. Several genes have been implicated in PPCD, including *VSX1* (visual system homeobox gene 1) and *COL8A2* (collagen VIII alpha-2 chain). Recently, in roughly half of the PPCD patients, examined heterozygous frameshift and nonsense mutations were found in the *ZEB1* gene [91, 92]. ZEB1/δEF1 mutations could be at the root of the PPCD phenotype because of lack of correct transcriptional regulation of multiple genes. One example is dysregulation of basement membrane collagen synthesis, which contributes to aberrant formation of the Descemet membrane. Ectopic expression of collagen type IV alpha 3 was shown in the presence of heterozygous ZEB1/δEF1 mutations, which implies that the *COL4A3* gene might be a ZEB1/δEF1 target gene [91].

ZEB family members as inducers of epithelial dedifferentiation during cancer progression

In epithelial cells, E-cadherin is located in the adherens junctions, where it acts as a major cell-cell adhesion molecule responsible for strong intercellular interactions and distinct epithelial cell polarity. E-cadherin downregulation is regarded as a central event in tumor metastasis, as reduction of cell adhesion between tumor cells facilitates their ability to migrate individually and invade. Although several genetic and epigenetic mechanisms have been proposed, loss of E-cadherin can often be attributed to transcriptional dysregulation. Over the past few years, several transcription factors were uncovered as repressors of E-cadherin transcription. Among them, ZEB1/δEF1 and ZEB2/SIP1 are increasingly considered important contributors to the process of malignant cancer progression. Comijn et al. [13] showed that conditional ZEB2/SIP1 expression in epithelial cells results in the specific loss of E-cadherin expression and strongly correlates with the loss of cell aggregation and with induction of invasion *in vitro*. Likewise, ectopic expression of ZEB1/δEF1 is sufficient to downregulate E-cadherin and to induce EMT in a breast cancer model [93]. Both ZEB1/δEF1 and ZEB2/SIP1 downregulate E-cadherin transcription, by binding to the conserved E2-boxes in the minimal E-cadherin promoter. A recent study implicated ZEB1/δEF1 and Snail in the cyclooxygenase-2-dependent downregulation of E-cadherin in non-small cell lung cancer (NSCLC) [94]. In human lung adenocarcinoma biopsies, an inverse correlation between E-cadherin and ZEB1/δEF1 and a direct relationship between COX-2 and ZEB1/δEF1 was demonstrated. Spoelstra et al. [95] showed elevated ZEB1/δEF1 expression in tumor-associated stromal cells of low-grade type-I uterine cancers while aggressive type-II endometrial carcinomas showed strong expression of ZEB1/δEF1 in both stroma and epithelial-derived cancer cells. Emphasizing the role of the microenvironment, it is likely that malignant tumor cells growing uncontrollably eventually outstrip their blood supply and experience hypoxia. These hypoxic conditions in turn may stimulate cell detachment and invasiveness. It has been proposed that ZEB1/δEF1 and ZEB2/SIP1 are involved in a mechanism of HIF-α-dependent E-cadherin downregulation in von Hippel-Lindau (VHL)-negative renal clear cell carcinoma (RCC) cells, partly explaining the aggressive nature of these tumors [96, 97] (Fig. 2).

Adherens junctions are not the only cell-cell junctions nullified during EMT processes. We revealed that ZEB2/SIP1 simultaneously and directly downregulates a panel of cell junctional genes encoding proteins of the adherens junctions, tight junctions, desmosomes and gap junctions [98]. Furthermore, ZEB2/SIP1 is effectively involved in the upregulation of mesenchymal markers including vimentin and N-cadherin [98, 99]. Increased expression of matrix metalloproteinase family members has been associated with dedifferentiation, invasion, metastasis and tumor recurrence. ZEB2/SIP1-dependent upregulation of MMP-1, MMP-2 and MT1-MMP mRNA was shown in cell lines derived from hepatocellular carcinoma (HCC) [100]. A similar transcriptional signature was recently shown for ZEB1/δEF1 in the dedifferentiated breast cancer cell line MDAMB231 in which RNAi-mediated knock-down of ZEB1/δEF1 led to the upregulation of a set of cell junctional genes as well as cell polarity genes [101, 102]. These data suggest that both ZEB1/δEF1 and ZEB2/SIP1 can transcriptionally reprogram the epithelial cell signature towards a more mesenchymal type (Fig. 2). Elevated ZEB2/SIP1 expression has been reported in biopsies of several human cancer types including breast, ovarian, gastric and oral squamous cell carcinomas [103–105]. That is indicative of the physiopathological relevance of ZEB2/SIP1 in these cancers.

Surprisingly, recent evidence associates ZEB2/SIP1 with replicative senescence in breast cancer and hepatocellular carcinoma cell lines [106, 107]. In that context ZEB2/SIP1 would act, in a TGF-β-dependent fashion, as a negative regulator of hTERT expression, and thereby exercise a tumor suppressive activity instead of its more commonly accepted role in epithelial tumor invasion and malignancy. Diverging from this, from the above it was shown that knock-out of ZEB1/δEF1 in embryonic fibroblasts results in accumulation of the cell-cycle-inhibitory protein $p21^{Cip1}$ and induces premature senescence [108]. In further support of a role of EMT in senescence regulation is the finding that the EMT inducing transcription factor Twist is also able to repress cellular senescence and, as such, allowing oncogenic ras transformation together with EMT, resulting in a full malignant phenotype [109]. Therefore by deregulating EMT and cellular senescence, EMT inducing transcription factors like ZEB1/δEF1 and ZEB2/SIP1 can have a double effect, resulting in a strong contribution to the malignant phenotype.

ZEB1/δEF1 was immunohistochemically detected at the tumor-host interface in colorectal cancer specimens, accompanying nuclear β-catenin and reduced cytokeratin, strongly indicating dedifferentiation and tumor cell invasion [102]. Furthermore, ZEB1/δEF1 is the main modulator of the basement membrane (BM) components encoded by genes *LAMA3*, *COL4A2* and *LAMC2*, and it generates a local,

transient loss of the BM at the invasive front [110]. ZEB1/δEF1 therefore seems to be a major constituent allowing tumor cell dissemination at these invasive fronts. Moreover, ZEB1/δEF1 is highly detectable in tumor-associated stromal fibroblasts, which may or may not be derived from epithelial cancer cells through a ZEB1/δEF1-dependent EMT program. In carcinomas of the breast, ZEB1/δEF1 expression is particularly upregulated in invasive lobular specimens [102].

Conclusions and future directions

Invasion and metastasis of epithelial tumors remains the primary cause of treatment failure and death of cancer patients. Acquiring further insights into the mechanisms leading to malignancy is a prerequisite for identifying new, clinically valuable prognostic markers and for creating new possibilities for development or optimization of alternative therapies. Over the last few years, substantial research has focused on the involvement of epithelial mesenchymal transitions in pathological conditions and malignant cancer progression. In this review, our attention has centred on the ZEB family of transcription factors, comprising two members, ZEB1/δEF1 and ZEB2/SIP1. Experimental evidence has made it clear that these factors take a central position in physiological as well as pathophysiological EMT. First, complete absence of ZEB2/SIP1, as shown in knock-out mice, is incompatible with life. Mutation of *ZEB2*, leading to haploinsufficiency of the ZEB2/SIP1 protein, causes Mowat-Wilson Sydrome, often accompanied by Hirschsprung disease-mental retardation syndrome. These patients carry, among other abnormalities, distinct facial characteristics, pointing to the importance of ZEB2/SIP1 in the migratory behavior of cranial neural crest cells and indicating its active role in processes triggering EMT. Second, ZEB1/δEF1 manifests itself, among many other things, as an important regulator of BM synthesis. Indeed, the structure of the Descemet membrane is abnormal in patients carrying ZEB1/δEF1 mutations. On the other hand, ZEB1/δEF1-mediated loss of the BM at the invasive front of cancer cells goes hand in hand with EMT and facilitates migration and local invasion of the tumor cells.

A milestone in cancer research has been the identification of several repressors of the tumor invasion suppressor E-cadherin: the ZEB/δEF1 family but also the Snail family, Twist, E12/E47 and the very recently identified Krüppel-like factor 8 (KLF8) [11]. All of these factors can trigger EMT and induce invasive and tumorigenic behavior. However, one remaining important question concerns the specific contributions of each of these repressors or their potential co-operation in specific cellular contexts or in different types of carcinomas. Several lines of evidence indicate that they may operate on different steps of the metastatic cascade. Organotypic culture assays and *in vivo* transplantation assays indicated that, while Snail is predominantly implicated in promoting initial invasion, E47 acts to maintain a dedifferentiated and migratory phenotype and plays an active role in tumor cell growth by promoting angiogenesis [111]. Furthermore, Twist expression appears to be essential for the entry of tumor cells into the bloodstream, an important early step towards metastasis [7]. Comparative gene expression profiling of epithelial cells expressing different E-cadherin repressors in the same genetic background has shown that only a subset of differentially expressed genes is commonly regulated [112]. Most of these genes are regulated by only one or by no more than two of the factors combined. This implies that the different E-cadherin repressors contribute to both general and specific aspects of EMT. The specific factors involved in the epithelial dedifferentiation program probably vary according to cell type and context. Moreover, it is not only the specific expression patterns of the E-cadherin repressors that are important, the presence of certain co-repressors and the affinity for them in different cell types might even be more critical in the cell's choice for the predominant transcriptional repressor. Additionally, the latter may act alone or in concert, and currently unidentified factors may also participate in the transcriptional silencing of E-cadherin and other epithelial-specific genes in cancer cells.

Expression studies of the different repressors in tumor biopsies shed further light on the specific role of each of the transcription factors in distinct tumor types and stages. For example, a study in primary human gastric cancers revealed elevated Snail and Twist expression in diffuse type gastric cancer, whereas ZEB2/SIP1 was primarily expressed in the intestinal type [104]. Another study reports on the differential expression of Snail, Slug and ZEB2/SIP1 in metastatic ovarian and breast carcinoma biopsies [103]. These mRNA expression studies, however, do not strictly exclude the contribution of contaminating fibroblasts to the expression status of the transcriptional repressors. An extended and useful immunohistochemical expression analysis of these transcription factors in large numbers of tumor samples is therefore required.

The reverse process of EMT, known as mesenchymal-epithelial transition or MET has also been reported. MET occurs during somitogenesis, kidney development and coelomic-cavity formation [113–115]. Spaderna et al. [110] recently observed an EMT-associated basement membrane loss at the invasive front of

colorectal adenocarcinomas which was rebuilt in the metastases, showing in most cases the same phenotype as the differentiated primary tumor. This indicates that EMT is a regulated reversible and dynamic process which means that, at least theoretically, interfering with the reversible negative regulation of the epithelial phenotype of cancer cells could lead to new therapeutic strategies. Knock-down of ZEB1/δEF1 in breast cancer and colorectal cancer models has indeed led to partial restoration of epithelial differentiation with re-expression of E-cadherin and other known epithelial-specific tumor suppressor genes [93, 102, 110].

The EMT field recently faced the identification of a series of novel EMT-inducing transcription factors including goosecoid and HOXB7, which are members of the homeobox family [10, 116], the forkhead transcription factor FOXC2 [9] and the fibroblast-specific protein 1-inducing transcription factor, CBF-A [8]. Further research into the specific functions of these different EMT modulators, how they are related to each other, where they are expressed, and what mechanisms they use, including identification and characterization of functional partners, will undoubtedly help to further solve the complexity of the EMT puzzle. Deeper understanding of the tumor invasion process may in time contribute to the development of new therapeutic strategies based on inhibition of the expression or function of EMT-inducing transcription factors in malignant carcinomas.

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