Oncogene (2007) 26, 6979–6988
© 2007 Nature Publishing Group All rights reserved 0950-9232/07 $30.00
www.nature.com/onc

ORIGINAL ARTICLE

The transcription factor ZEB1 (δEF1) promotes tumour cell dedifferentiation by repressing master regulators of epithelial polarity

K Aigner[1], B Dampier[2,9], L Descovich[3,9], M Mikula[3,9], A Sultan[1,9], M Schreiber[2], W Mikulits[4], T Brabletz[5], D Strand[6], P Obrist[7], W Sommergruber[8], N Schweifer[8], A Wernitznig[8], H Beug[3], R Foisner[1] and A Eger[1]

[1]Max F Perutz Laboratories, Department of Medical Biochemistry, Medical University Vienna, Vienna, Austria; [2]Department of Obstetrics and Gynecology, Medical University Vienna, Vienna, Austria; [3]Research Institute of Molecular Pathology, Vienna, Austria; [4]Department of Medicine I, Institute of Cancer Research, Medical University Vienna, Vienna, Austria; [5]Department of Visceral and General Surgery, Albert-Ludwigs-University, Freiburg, Germany; [6]First Department of Internal Medicine, Johannes Gutenberg University, Mainz, Germany; [7]Pathology Laboratories Obrist and Brunhuber OEG, Zams, Austria and [8]Boehringer-Ingelheim Austria, Vienna, Austria

Epithelial to mesenchymal transition (EMT) is implicated in the progression of primary tumours towards metastasis and is likely caused by a pathological activation of transcription factors regulating EMT in embryonic development. To analyse EMT-causing pathways in tumourigenesis, we identified transcriptional targets of the E-cadherin repressor ZEB1 in invasive human cancer cells. We show that ZEB1 repressed multiple key determinants of epithelial differentiation and cell–cell adhesion, including the cell polarity genes Crumbs3, HUGL2 and Pals1-associated tight junction protein. ZEB1 associated with their endogenous promoters *in vivo*, and strongly repressed promotor activities in reporter assays. ZEB1 downregulation in undifferentiated cancer cells by RNA interference was sufficient to upregulate expression of these cell polarity genes on the RNA and protein level, to re-establish epithelial features and to impair cell motility *in vitro*. In human colorectal cancer, ZEB1 expression was limited to the tumour–host interface and was accompanied by loss of intercellular adhesion and tumour cell invasion. In invasive ductal and lobular breast cancer, upregulation of ZEB1 was stringently coupled to cancer cell dedifferentiation. Our data show that ZEB1 represents a key player in pathologic EMTs associated with tumour progression.
Oncogene (2007) **26,** 6979–6988; doi:10.1038/sj.onc.1210508; published online 7 May 2007

Keywords: epithelial to mesenchymal transition; invasion; transcription; epithelial polarity; cell adhesion

Correspondence: Professor R Foisner, Max F Perutz Laboratories, Department of Medical Biochemistry, Medical University Vienna, Dr Bohrgasse 9, A-1030 Vienna, Austria and Dr A Eger, Max F Perutz Laboratories, Department of Medical Biochemistry, Medical University Vienna, Dr Bohrgasse 9, A-1030 Vienna, Austria.
E-mails: roland.foisner@meduniwien.ac.at and andreas.eger@meduniwien.ac.at
[9]These authors contributed equally to this work.
Received 16 November 2006; revised 26 March 2007; accepted 1 April 2007; published online 7 May 2007

Introduction

Invasion and metastasis are the primary causes of morbidity and mortality in human cancer. Despite their central role in disease progression the molecular mechanisms driving tumour cell invasion and metastasis are poorly understood. The escape of cancer cells from the primary tumour is mostly associated with epithelial dedifferentiation, loss of intercellular adhesion and enhanced migratory potential, collectively termed epithelial to mesenchymal transition (EMT). While EMT is an essential process during embryogenesis, it is a major pathological event in cancer progression (Thiery, 2002; Eger and Mikulits, 2005; Huber *et al.*, 2005).

EMT-inducing pathways, such as transforming growth factor-β (TGFβ) and Wnt signalling trigger epithelial dedifferentiation by impairing the expression/function of the epithelial adhesion protein E-cadherin (De Craene *et al.*, 2005b; Thiery and Sleeman, 2006). Hence, loss of E-cadherin is one of the hallmarks of EMT and late-stage cancer progression, while re-expression of E-cadherin in tumour cells inhibits invasion and metastasis (Peinado *et al.*, 2004).

Repression of E-cadherin is mediated by distinct transcription factors, including Snail1 (Snail) (Batlle *et al.*, 2000; Cano *et al.*, 2000), Snail2 (Slug) (Hajra *et al.*, 2002), SIP1 (ZEB2) (Comijn *et al.*, 2001) and E47 (Perez-Moreno *et al.*, 2001). These repressors may regulate developmental transcriptional programmes of EMT in tumour cells predisposing them to invasion and metastasis (Gupta *et al.*, 2005). Therefore, the comprehensive identification of their *bona fide* targets is of prime importance for understanding tumour cell invasion at the molecular level.

The zinc finger (C2H2-type) and homeobox pit1, Oct 1/2, Unc 86 homology region (POU domain) containing protein ZEB1 (δEF1, TCF8 and AREB6) has recently been identified as a potent transcriptional repressor of E-cadherin (Grooteclaes and Frisch, 2000; Guaita *et al.*, 2002; Eger *et al.*, 2005). However, in contrast to other

E-cadherin repressors, comprehensive studies on ZEB1's function in cancer cell plasticity are missing. Here, we show that ZEB1 inhibits the expression of epithelial genes central to adhesion and epithelial polarity. In human colon and breast tumours, ZEB1-expressing tumour cells show impaired adhesion and invaded host tissues. We propose that ZEB1 functions as a master regulator of epithelial plasticity in cancer cell invasion and human tumour progression *in vivo*.

Results

ZEB1 represses major regulators of epithelial differentiation

To identify ZEB1 target genes in cancer cells we examined the genome-wide transcriptional repertoire of ZEB1 in the highly metastatic MDA-MB-231 breast cancer cell line, which has been widely used as an invasion and metastasis cell model (Lacroix and Leclercq, 2004). Knockdown of ZEB1 by small interfering RNAs (siRNAs) has previously been shown to induce E-cadherin expression and to re-establish epithelial features (Eger *et al*., 2005). To determine global gene expression changes in MDA-MB-231 cells following a 3 days knockdown of ZEB1, which reduced ZEB1 mRNA levels by 80–90% (Figure 1a), we performed Affymetrix GeneChip analyses (Human Genome U133 Plus 2.0). ZEB1 knockdown caused upregulation of ~200 genes (repressed by ZEB1) and downregulation of ~30 genes (activated by ZEB1) (complete microarray data will be published elsewhere). ZEB1 mRNA level was strongly reduced, confirming the efficiency of the knockdown, while other E-cadherin repressors were not affected (data not shown).

In this study, we focused on potential ZEB1 targets involved in epithelial differentiation. Besides E-cadherin, ZEB1 depletion also induced re-expression of a multitude of genes crucial for epithelial cell–cell adhesion and differentiation (Table 1). Potential ZEB1 targets include the cell polarity genes Crumbs3, Pals1-associated tight junction protein (PATJ) and human lethal giant larvae homologue 2 (LLGL2; HUGL2); members of the classical cadherin superfamily (placental (P-) and retinal (R-) cadherin); components of tight

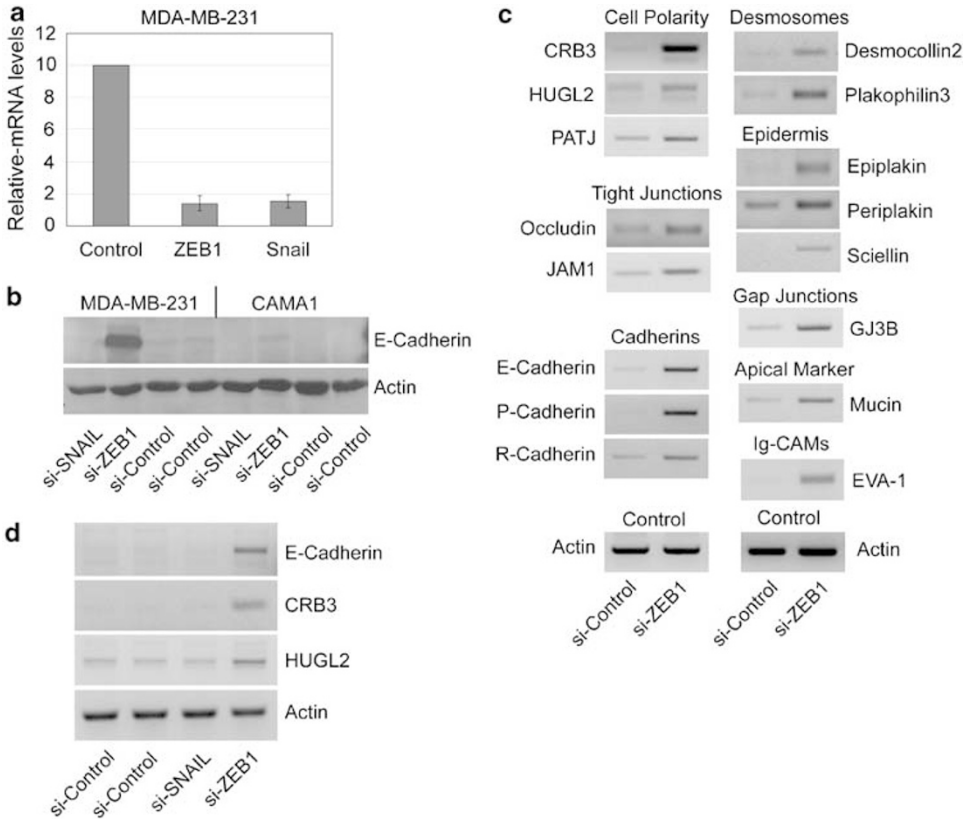

Figure 1 RNAi-mediated knockdown of ZEB1 reactivates epithelial gene expression. (**a**) RNAi-mediated knockdown of ZEB1 and Snail1 in MDA-MB-231 cells. Transcript levels of ZEB1 and Snail1 were determined by real-time PCR using the TaqMan system with assay-on-demand (Applied Biosystems, Foster City, CA, USA). The relative expression of ZEB1 and Snail1 was normalized to β-actin using the standard curve method described by the manufacturer. Mean values of the relative transcript levels of three independent experiments are shown. Bars represent standard error. (**b**) E-cadherin protein expression levels after knockdown of ZEB1 (si-ZEB1) and Snail1 (si-Snail) in MDA-MB-231 and CAMA1 cells. si-Control, unspecific scrambled siRNA. Actin was included as loading control. (**c**) Knockdown of ZEB1 (si-ZEB1) causes transcriptional upregulation of epithelial polarity genes (see also Table 1). Actin was included as loading control. (**d**) E-cadherin, Crumbs3 (CRB3) and HUGL2 mRNA levels upon knockdown of ZEB1 (si-ZEB1) and Snail1 (si-Snail). Actin was included as loading control.

Table 1 Functional classification of epithelial-specific genes repressed by ZEB1. Fold-change indicates relative increase in transcript levels after ZEB1-specific compared to unspecific (scrambled) siRNA treatments

Symbol	*Name*	*Fold-change*	P-*value*
	Cadherins		
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	14.8	4.34E-08
CDH3	Cadherin 3, type 1, P-cadherin (placental)	2.7	0.00032
PCDH7	Brain–heart (BH) protocadherin	2.6	0.00407
CDH11	Cadherin 11, type 2, OB-cadherin (osteoblast)	1.9	0.01544
CDH4	Cadherin 4, type 1, R-cadherin (retinal)	1.7	0.00228
	Cell polarity genes		
CRB3	Crumbs homologue 3 (*Drosophila*)	2.6	0.00011
PATJ	Pals1-associated tight junction protein	2.3	0.00907
HUGL2	Lethal giant larvae homologue 2 (*Drosophila*)	1.6	0.01616
	Tight junctions		
F11R	F11 receptor (Junctional adhesion molecule 1, JAM1)	1.9	1.62E-05
MARVELD2	MARVEL domain containing 2, Tricellulin	1.8	3.90E-04
OCLN	Occludin	1.7	0.00023
CLDN7	Claudin 7	1.5	0.02286
SHRM	Shroom	1.5	0.01073
	Desmosomes and epidermis		
SCEL	Sciellin	3.4	4.20E-07
SH3YL1	SH3 domain containing, Ysc84-like	2.9	2.10E-04
PKP3	Plakophilin 3	2.7	5.73E-06
ZD52F10	Dermokine	2.2	0.00025
EPPK1	Epiplakin 1	2.2	0.00044
DSC2	Desmocollin 2	1.8	0.03886
SFN	Stratifin	1.8	0.00046
PPL	Periplakin	1.6	0.00413
DSP	Desmoplakin	1.5	0.00051
SG2	Desmoglein 2	1.5	0.00323
	Gap junctions		
GJB3	Gap junction protein, β3, 31 kDa (connexin 31)	1.6	0.00578
GJB2	Gap junction protein, β2, 26 kDa (connexin 26)	1.5	0.04312
	Apical proteins		
MUC1	Mucin 1, transmembrane	1.9	0.00269
	Cell surface receptors		
EVA1	Epithelial V-like antigen 1	7.1	2.09E-05
TACSTD1	Tumor-associated calcium signal transducer 1 (EpCAM)	5.2	9.82E-08
TSPAN1	Tetraspanin 1	2.6	1.01E-05
CXADR	Coxsackie virus and adenovirus receptor	2.3	6.61E-05
CD24	CD24 antigen	2.3	0.008
TMEPAI	Transmembrane, prostate androgen induced	2.2	0.00705
TSPAN15	Tetraspanin 15	2	0.0013
TACSTD2	Tumor-associated calcium signal transducer 2	2	0.00145
	Vesicle transport		
TMEM30B	Transmembrane protein 30B, yeast homologue Cdc50p	3.3	5.18E-06
MAL2	Mal, T-cell differentiation protein 2	2.8	4.12E-02
SYTL1	Synaptotagmin-like 1	2	7.64E-06

junctions (occludin, JAM1, claudin 7, tricellulin and shroom), desmosomes (desmoplakin, plakophilin 3, desmocollin 2 and desmoglein 2) and gap junctions (connexin 26 and 31); epithelial-specific adhesion molecules with Ig-like domains (epithelial V-like antigen 1); the apically localized protein Mucin 1 and various genes involved in vesicular trafficking and transcytosis in epithelial cells, including the yeast homologue CDC50p implicated in the polarized establishment of actin patches at bud sites (Table 1). Transcript levels were confirmed by reverse transcription (RT)–PCR in three independent siRNA experiments (Figure 1c, one representative experiment is shown).

Knockdown of Snail1 is not sufficient to establish epithelial features

We have recently demonstrated that, unlike ZEB1, the expression of Snail1 did not tightly correlate with repression of E-cadherin in a panel of 20 breast cancer cell lines (Eger *et al*., 2005). To compare directly the functions of ZEB1 and Snail1 in MDA-MB-231 cells, we abrogated expression of Snail1 by siRNA treatment. Although transcript levels of Snail1 were reduced by ~90% (Figure 1a), the downregulation of Snail1 was not sufficient to activate expression of E-cadherin Crumbs3 and HUGL2 (Figure 1b and d). Consequently, MDA-MB-231 cells remained fibroblastoid (data not

shown). Thus, ZEB1 rather than Snail1 controls epithelial plasticity of MDA-MB-231 cells.

Knockdown of ZEB1 partially restores epithelial polarity
De-repression of Crumbs3, HUGL2 and PATJ upon ZEB1 knockdown also induced strong upregulation of respective proteins (Figure 2a and b). Upregulated Crumbs3 and HUGL2 accumulated in the cytoplasm and at the plasma membrane in MDA-MB-231 cells (Figure 2a). PATJ accumulated in the nucleus but failed to localize to the membrane (Figure 2a). As PATJ has been implicated in the biogenesis of tight junctions (Michel *et al*., 2005; Shin *et al*., 2005), we analysed tight junction formation after ZEB1 knockdown. The tight junction marker ZO1 was detected at cell–cell contact sites (Figure 2a), indicating that PATJ may be dispensable for initial stages of tight junction formation. Altogether, ZEB1 depletion caused *de novo* expression of cell polarity proteins, their partial translocation to the plasma membrane and formation of rudimentary peripheral tight junction complexes.

Ectopic E-cadherin expression is not sufficient to induce epithelial genes
Expression of E-cadherin in undifferentiated cancer cells rescues epithelial architecture and affects several signalling pathways (Wheelock and Johnson, 2003). Since E-cadherin upregulation is a hallmark of ZEB1 downregulation in MDA-MB-231 cells (Eger *et al*., 2005) (Table 1, Figure 1b and d), the observed changes in gene expression upon ZEB1 depletion may be indirectly caused by increased E-cadherin levels. To address this issue, we stably expressed E-cadherin in MDA-MB-231 cells. In 90% of the cells, E-cadherin was detected in the cytoplasm (Figure 2c, E-cadherin-1) and cells were unable to develop pronounced epithelial features (Figure 2c, E-cadherin-1). Only 10% of cells formed 'epitheloid' cell clusters in which E-cadherin accumulated at cell–cell contact sites (Figure 2c, E-cadherin-2). RT–PCR analyses revealed that transcription of the ZEB1 target genes (Crumbs3, HUGL2 and PATJ) was unaffected upon expression of E-cadherin (Figure 2d). Crumbs3 and HUGL2 proteins were neither upregulated nor redistributed, even in the 10% of E-cadherin expressing 'epitheloid' cells (Figure 2c).

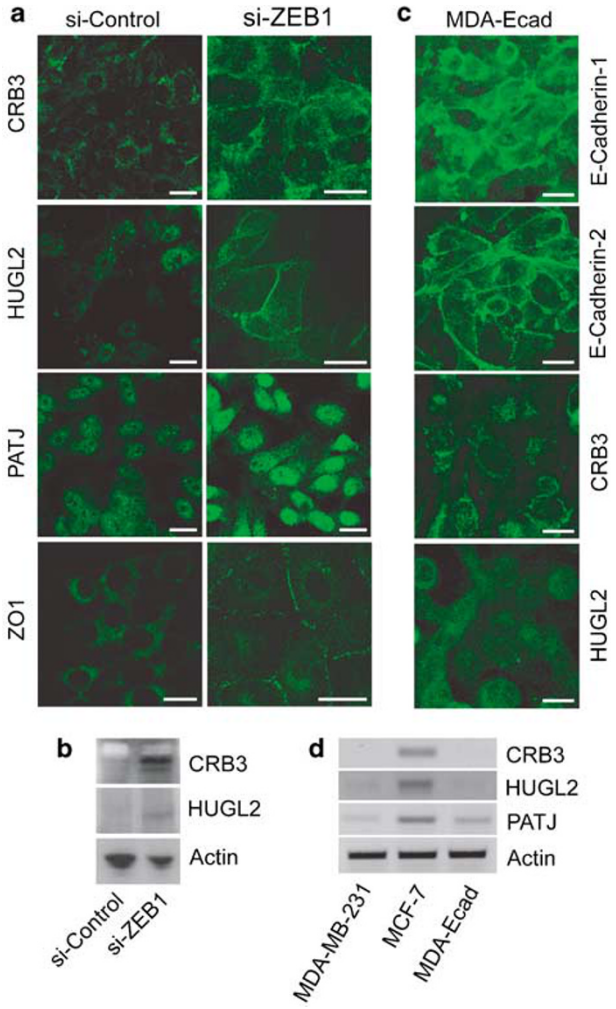

Figure 2 Cell polarity proteins are upregulated and partially redistributed to the plasma membrane upon ZEB1 depletion. (**a**) Immunolocalization of Crumbs3 (CRB3), HUGL2, PATJ and ZO1 in MDA-MB-231 cells treated with unspecific (si-Control) or ZEB1-specific (si-ZEB1) siRNA. (**b**) Crumbs3 (CRB3) and HUGL2 protein levels are upregulated upon knockdown of ZEB1 (si-ZEB1). si-Control, unspecific scrambled si-RNA. (**c**) Immunolocalization of Crumbs3 (CRB3) and HUGL2 in E-cadherin-expressing MDA-MB-231 cells (MDA-Ecad). (**d**) Transcript levels of Crumbs3 (CRB3), HUGL2 and PATJ in mock- and E-cadherin-transfected MDA-MB-231 cells. Transcript levels were determined by RT–PCR. Transcript levels in MCF7 cells were included as positive control, actin as loading control. Bars in (**a**) and (**c**), 10 μm.

ZEB1 directly repressed promoter activity of cell polarity genes
Next, we examined whether ZEB1 can directly repress the promoters of Crumbs3, HUGL2 and PATJ. Since ZEB1 represses transcription via binding to E-box elements (5′-CACCTG-3′) (Grooteclaes and Frisch, 2000; Eger *et al*., 2005), we screened the proximal promoter regions of Crumbs3 and HUGL2 for the presence of E-box consensus sites and cloned relevant fragments into reporter constructs. For human Crumbs3, we generated three reporter constructs spanning different, albeit overlapping promoter regions (CRB3-prom 1–3, Figure 3a), which cover $\sim$3.5 kbp (-3421 to $+74$) and contain 20 E-box consensus sites mostly arranged in three clusters (Figure 3a). For HUGL2, a reporter construct containing a $\sim$900 bp fragment with four E-box consensus sites was generated (-743 to $+135$) (Figure 3c).

Epithelial MCF7 cells expressing endogenous Crumbs3 and HUGL2 were transfected with the reporter plasmids together with a ZEB1 expression vector or empty control vector and a constitutive β-galactosidase expression plasmid for normalization of transfection efficiency. Unlike the control, ZEB1 repressed the activity of the two proximally located Crumbs3 promoter fragments CRB3-prom1 ($-1402/+74$) and

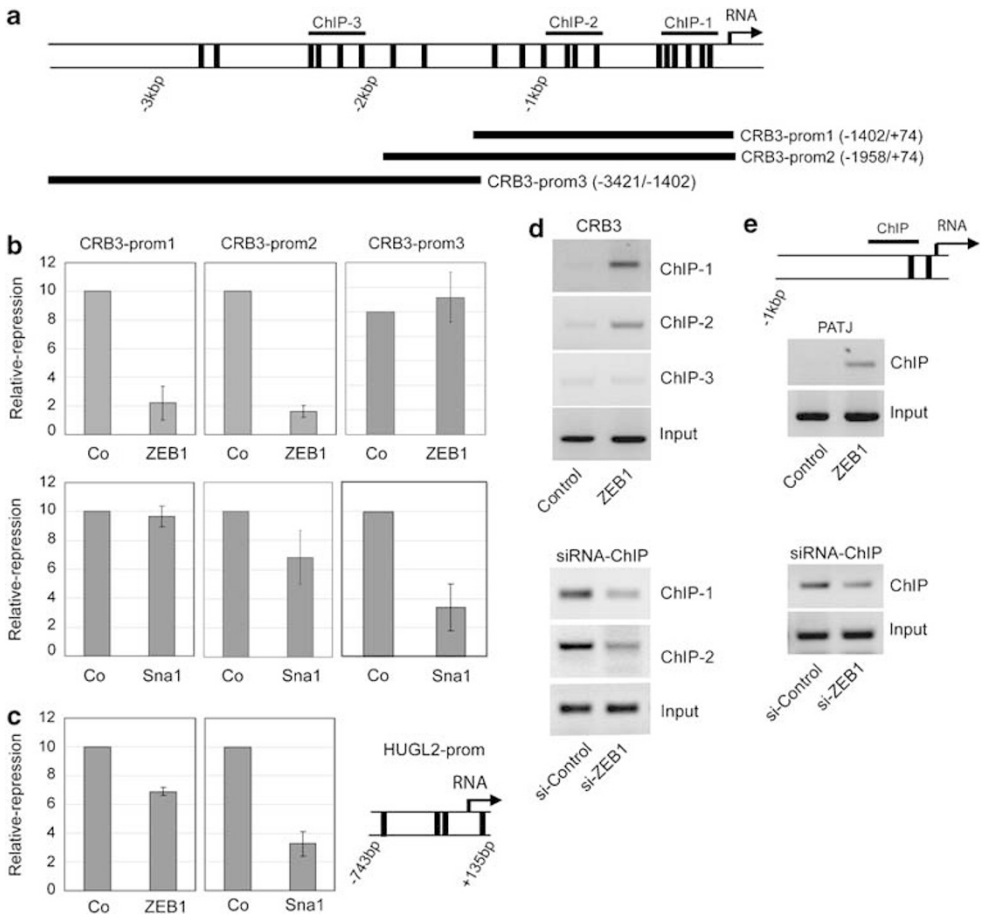

Figure 3 ZEB1 directly represses the proximal promoter of cell polarity genes. (**a**) Scheme of the Crumbs3 promoter. E-boxes are indicated as vertical black bars. (**b**) ZEB1 and Snail repress the human Crumbs3 promoter. The relative repression of promoter fragments (see scheme in (**a**)) by ZEB1 and Snail1 compared to empty control vectors are shown. Mean values are derived from four independent experiments. Bars represent standard error. (**c**) ZEB1 and Snail1 repress the proximal HUGL2 promoter (−743/+135). Black vertical bars in the promoter scheme denote the relative positions of the E-boxes. (**d**) Upper panel: ZEB1 associates with the proximal Crumbs3 promoter at the chromatin level. ZEB1-specific (ZEB1) or unrelated goat Abs (Control) were used in ChIP experiments. Amplified human Crumbs3 promoter fragments are shown (ChIP1, −282/−29; ChIP2, −924/−646; ChIP3, −2284/−2037. Illustration in (**a**) shows the relative position of the amplified Crumbs3 promoter fragments. Amount of Crumbs3 promoter in input confirms equal loading of chromatin. Lower panel: knockdown of ZEB1 (si-ZEB1) reduces the interaction of ZEB1 with Crumbs3 promoter fragments. si-Control, unspecific scrambled si-RNA (**e**). Upper panel: ZEB1 associates with the PATJ promoter at the chromatin level. Illustration denotes the relative position of the two E-boxes (black vertical bars) as well as the amplified DNA fragment in ChIP (−335/−127). Lower panel: depletion of ZEB1 by siRNA (si-ZEB1) results in reduced precipitation of PATJ promoter fragments compared to control experiments (scrambled si-Control).

CRB3-prom2 (−1958/+74) as well as the HUGL2 promoter fragment HUGL2-prom (−743/+135) (Figure 3b and c). ZEB1 expression had no effect on the more distally located Crumbs3 promoter region (CRB3-prom3; −3421/−1402) (Figure 3b). Ectopic expression of Snail1 also repressed promoter activities of both genes (Figure 3b and c), but unlike ZEB1, Snail1 preferentially repressed the activity of the most upstream promoter fragment (CRB3-prom3; −3421/−1402).

To test whether ZEB1 can directly interact with the endogenous promoters *in vivo*, we performed chromatin immunoprecipitations (ChIP). In line with the reporter analyses, chromatin fragments containing the first or the second proximal E-box clusters of the Crumbs3 promoter were efficiently pulled down by ZEB1 antibodies (Abs) (Figure 3d, ChIP1 and ChIP2), whereas fragments located at the most distal E-box cluster were barely detectable above background levels (Figure 3d, ChIP3). Similarly, ZEB1 physically interacted with the proximal PATJ promoter, which contained two E-box consensus sites (Figure 3e). To demonstrate specificity, we knocked down ZEB1 via siRNA before ChIP. Reduction of ZEB1 significantly decreased coprecipitated Crumbs3 and PATJ promoter fragment levels (Figure 3d and e; lower panels, siRNA-ChIP). Thus, ZEB1 can directly repress Crumbs3 and PATJ by binding to defined proximal promoter segments.

ZEB1 enhances the migratory potential of cells

Next, we performed transpore migration assays to determine whether knockdown of ZEB1 also affects cell

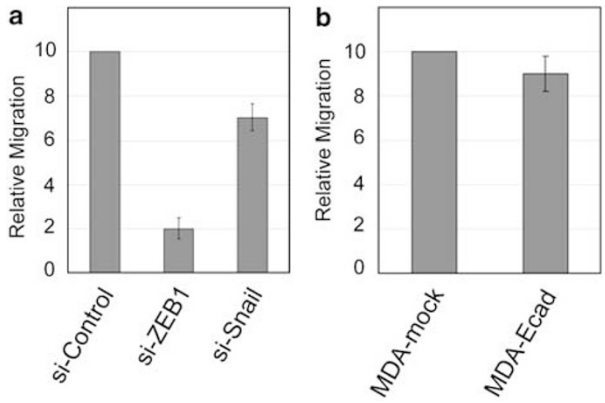

Figure 4 ZEB1 and Snail depletion reduced the motility of MDA-MB-231 cells *in vitro*. MDA-MB-231, treated with control or ZEB1 and Snail-specific siRNAs for 3 days, and MDA-MB-231 cells stably transfected with empty or E-cadherin-expressing plasmids, were seeded on Transwell filter inserts and migrating cells quantified after 24 h by Hoechst staining and fluorescence microscopy.

motility *in vitro*. MDA-MB-231 cells treated with ZEB1-specific siRNA for 3 days were seeded onto Transwell filter inserts and cell migration through pores was analysed 24 h later. ZEB1 depletion impaired cell motility by 80% (Figure 4a). The reduced motility is not only a result of E-cadherin, upregulation, since ectopic expression of E-cadherin in MDA-MB-231 cells barely affected motility (Figure 4b). Interestingly, also Snail1 depletion affected transmigration of MDA-MB-231 cells through filters (Figure 4a), although they retained a fibroblastoid morphology.

Expression of ZEB1 in human tumours correlated with tumour cell dedifferentiation and invasion

Our findings suggested that aberrant upregulation of ZEB1 in human tumours may induce cancer cell dissemination. To test this hypothesis, we screened paraffin-embedded human colon and breast neoplasm specimens immunohistochemically for ZEB1, E-cadherin, cytokeratin and HUGL2 expression.

In colon cancer samples, the bulk tumour area stained positively for E-cadherin, cytokeratin and HUGL2 (Figure 5a and Supplementary Figure 1, arrows). ZEB1 expression was not detected in differentiated tumour cells but in many stroma cells adjacent to the tumour areas (Figure 5a and Supplementary Figure 1, arrowheads). In normal colon tissue, ZEB1-positive stroma cells were mostly absent (Supplementary Figure 1, arrowheads). Unspecific goat IgG controls did not reveal specific signals (Supplementary Figure 2, cytokeratin/goat IgG double stainings).

As EMT-like phenotypic conversions at the tumour–host interface are prominent features of colorectal cancer progression (Brabletz *et al*., 2001, 2005), we screened tumour–host interfaces for ZEB1 expression. In eight out of ten colon tumours, we found strong ZEB1 expression at tumour margins exhibiting tumour cell dedifferentiation. ZEB1-positive cells expressed low levels of cytokeratin, formed loosely attached clusters or invaded the tumour stroma as single cells (Figure 5b and Supplementary Figure 3, arrows). ZEB1-positive cancer cells were clearly distinguishable from tumour-associated stroma cells by perinuclear and/or cytoplasmic cytokeratin remnants and by nuclear morphology (Figure 5b and Supplementary Figure 1, ZEB1/cytokeratin, arrows). In addition, we found co-expression of ZEB1 and β-catenin in a significant number of invasive tumour cells (Supplementary Figure 4, lower panel, arrows).

We also examined ZEB1 expression in eight ductal and five lobular breast cancer specimens. In all ductal carcinomas, ZEB1 expression inversely correlated with cancer cell differentiation. Nuclear accumulation of ZEB1 was always associated with low expression of cytokeratin (Figure 5c, Ductal-Dediff., arrows) and weakened intercellular adhesion, while differentiated tumour areas were strongly positive for cytokeratin and lacked ZEB1 (Figure 5c, Ductal-Diff., arrow). In three ductal carcinomas, ZEB1 was highly expressed in tumour cells invading host tissue as loosely associated group or single cells, reminiscent of infiltrating tumour cells in invasive lobular breast cancer (Figure 5c, Mixed, arrows). Accordingly, the most striking upregulation of ZEB1 was found in breast cancer specimens exhibiting all histological criteria of invasive lobular carcinomas (Figure 5c, Lobular).

Discussion

ZEB1 represses epithelial determinants in cancer cells

Here, we show that, ZEB1 affects expression of several genes critically involved in epithelial polarity, supporting ZEB1's regulatory role in EMT. Our approach for the identification of ZEB1 target genes was to selectively knockdown endogenous ZEB1, thus avoiding potential overexpression artefacts. Genes affected by ZEB1 include constituents of all junctional complexes located along the lateral membrane of epithelial cells and apical membrane proteins and epithelial Ig-domain adhesion molecules. Most interestingly, we identified ZEB1 as potent direct transcriptional repressor of the cell polarity genes Crumbs3, PATJ and HUGL2, as shown by ChIP and reporter assays. Loss of epithelial cell polarity proteins causes severe defects in epithelial polarity, which is a primary diagnostic mark for malignant carcinomas. Their functional loss also enhances tumour cell proliferation and invasion (Bilder, 2004; Brumby and Richardson, 2005). Hence, ZEB1-mediated repression of Crumbs3, PATJ and HUGL2 in cancer cells may affect multiple aspects of normal epithelial physiology favouring cancer progression, invasion and metastasis.

The transcription programmes of different E-cadherin repressors

Like ZEB1 the E-cadherin repressors Snail1, Snail2, SIP1 and E47 also modulate epithelial architecture and induce EMT (Peinado *et al*., 2004; De Craene *et al*.,

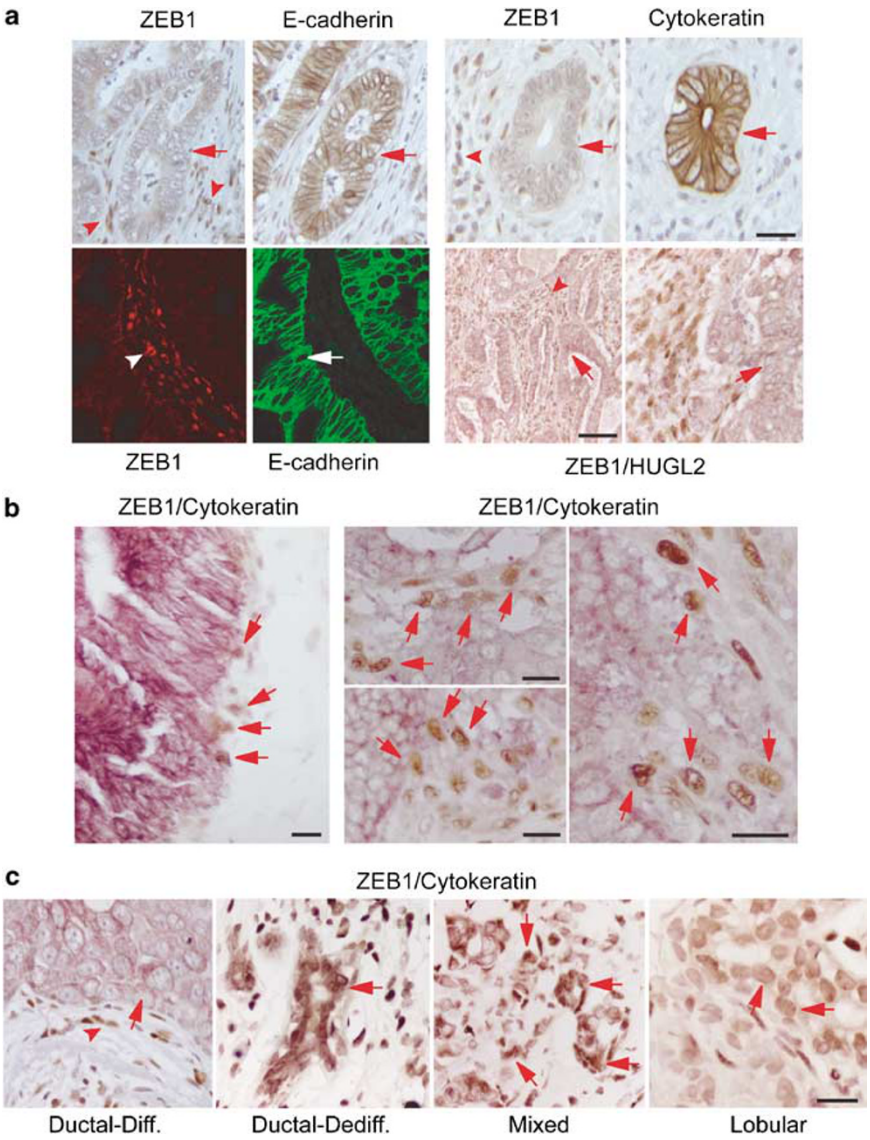

Figure 5 ZEB1 expression in colon and breast tumours correlates with cancer cell dedifferentiation. (**a**) Serial tumour sections were immunohistochemically stained for ZEB1, E-cadherin or cytokeratin (brown). In addition, single sections were double stained for ZEB1 and E-cadherin and subjected to immunofluorescence analysis (ZEB1 in red, E-cadherin in green). ZEB1 and HUGL2 represent immunohistochemical double stainings (ZEB1, brown; HUGL2, violet; two different magnifications of adjacent regions are shown). Note that differentiated areas (arrows) stain strongly for E-cadherin, cytokeratin and HUGL2 but do not express significant levels of ZEB1. Tumour-associated stroma cells express high amounts of nuclear ZEB1 (arrowheads). (**b**) ZEB1 induces EMT-like cell conversions at the invasive front of colorectal adenocarcinomas. Sections were double stained for ZEB1 (brown) and cytokeratin (violet). At the tumour–host interface ZEB1-positive tumour cells invade the host stroma but retain residual cytokeratin expression (see arrows in left panel: ZEB1, brown; cytokeratin, violet). Small cancer cell cluster or single isolated tumour cells express ZEB1 and often contain perinuclear and/or cytoplasmic cytokeratin remnants (see arrows in right panel: ZEB1, brown; cytokeratin, violet). (**c**) Expression of ZEB1 in breast tumours correlates with impaired cancer cell differentiation. Invasive ductal and lobular breast cancer specimens were double stained for ZEB1 (brown) and cytokeratin (violet). In differentiated areas of ductal tumours, ZEB1 is expressed in many tumour-associated stroma cells (Ductal-Diff., arrowhead), whereas the tumour lacks ZEB1-specific staining (Ductal-Diff., arrow). Dedifferentiated ductal compartments strongly express ZEB1 (Ductal-Dediff. and Mixed, arrows). In lobular cancer ZEB1 is highly expressed in large areas of the tumour (Lobular). Bars 20 and 80 μm.

2005b). However, the specific contributions of these repressors to EMT in development and tumour progression are poorly understood. First insights have been gained from recent microarray studies, in which specific transcription programmes were determined by overexpression of these proteins in human colon cancer or Madin–Darby canine kidney cells (De Craene *et al*., 2005a; Vandewalle *et al*., 2005; Moreno-Bueno *et al*., 2006). Despite the different cell lines and experimental set-ups used, a few important conclusions can be drawn from the synopsis of the four data sets. First, among the E-cadherin repressors ZEB1 showed the strongest negative effect on epithelial-specific transcription. Second, none of the other repressors was capable of repressing

cell polarity genes. Third, the related repressors ZEB1 and SIP1 may regulate different sets of junctional genes.

Interestingly, Snail1 is not always sufficient to repress E-cadherin, as demonstrated in DLD-1 colon cancer cells overexpressing Snail1 (De Craene *et al*., 2005a), and in our previous studies, revealing that Snail1 expression did not correlate with E-cadherin repression in human breast cancer cell lines (Eger *et al*., 2005). In this study, we show that knockdown of Snail1 was not sufficient to activate expression of E-cadherin, Crumbs3 or HUGL2 in MDA-MB-231 cells. Yet, numerous studies have shown a negative impact of Snail1 on E-cadherin expression and epithelial differentiation (Batlle *et al*., 2000; Cano *et al*., 2000; Palmer *et al*., 2004; Peinado *et al*. 2004; Pena *et al*., 2005, 2006). In addition, we found that overexpressed Snail1 also affected Crumbs3 and HUGL2 promoter activity, indicating Snail1's intrinsic capability to affect these genes in breast cancer cells. Therefore, overall expression levels of the E-cadherin repressors and the availability of cofactors may determine target gene specificity and their final impact on EMT, tumour progression and metastasis (Pena *et al*., 2006).

ZEB1 and human cancer progression in vivo

To analyse the role of ZEB1 in human cancer *in vivo* we tested ZEB1 expression in colon and breast cancer specimens. In colon cancer, ZEB1 was upregulated at the tumour–host interface and was accompanied by epithelial dedifferentiation and tumour cell invasion. In invasive ductal carcinomas of the breast, ZEB1 upregulation correlated with epithelial dedifferentiation and undifferentiated lobular breast tumours expressed ZEB1 in a large proportion of cells. ZEB1 was also highly expressed in tumour-associated stroma cells. It is unclear whether ZEB1-positive stromal fibroblasts may be derived from epithelial cancer cells through a *bona fide* ZEB1-dependent EMT *in vivo*.

Several recent reports provide supporting evidence for a role of ZEB1 in the malignant progression of different cancer types. (1) In non-small cell lung cancer and in renal cell carcinomas ZEB1 repressed E-cadherin expression in response to cyclooxygenase-2 activation or hypoxia-inducible factor-1 mediated signalling, respectively (Dohadwala *et al*., 2006; Krishnamachary *et al*., 2006). (2) In high-grade endometrioid adenocarcinomas and other aggressive types of uterine cancers, ZEB1 was strongly expressed in E-cadherin negative carcinoma cells (Spoelstra *et al*., 2006). (3) In colon tumours, ZEB1 repressed the expression of specific laminin genes and this transient basement membrane loss correlated with increased distant metastasis and poor patient survival (Spaderna *et al*., 2006). Likewise, an inverse correlation of ZEB1 and E-cadherin levels was observed in colon tumours lacking Snail1 (Pena *et al*., 2005).

The molecular cues that induce ZEB1 expression in particular cancer settings or in particular tumour areas are still elusive. Candidates for ZEB1 inducers are TGFβ, tumour necrosis factor-α (TNF-α) and Wnts, which are often excessively produced by different tumour-associated stroma cells (Ohira *et al*., 2003; Chua *et al*., 2006; Nishimura *et al*., 2006). One attractive hypothesis is that tumour infiltrating macrophages, which are abundantly detected at the tumour–host interface, express cytokines that may induce ZEB1 expression (Condeelis and Pollard, 2006).

In summary, the data of this study provide strong evidence for a key function of ZEB1 in late-stage cancer progression. Large-scale tumour studies with well-documented clinical records will be critical to determine whether ZEB1 can be used as a prognostic parameter to predict metastatic burden and patient survival.

Materials and methods

Cell culture and Affymetrix GeneChip analyses

MDA-MB-231 and CAMA1 were cultivated as reported (Eger *et al*., 2005). For generation of E-cadherin-expressing cells, MDA-MB-231 cells were transfected with E-cadherin-green fluorescent protein vector and selected in 800 μg/ml Neomycin (Invitrogen, Carlsbad, CA, USA). RNA isolation, quality control, labelling and hybridization on Affymetrix GeneChip (Human Genome U133 Plus 2.0) and data acquisition were done as described in Pacher *et al*. (2006).

Reporter assays, RT–PCR

Proximal human Crumbs3 promoter fragments $-1402/+74$ and $-1958/+74$ were cloned into *Nhe*I/*Sma*I and *Sac*I/*Sma*I sites of the pGL3-basic reporter vector (Promega, Madison, WI, USA), respectively, the human Crumbs3 promoter fragment $-3421/-1402$ into *Kpn*I/*Nhe*I sites and the human $-743/+135$ HUGL2 fragment into *Mlu*I/*Xho*I sites. Transient reporter assays were done as described (Eger *et al*., 2000).

Preparation of RNA and cDNA, and PCR analyses were done as described (Eger *et al*., 2005) For PCR primers see Supplementary Table 1. ZEB1 and Snail1 transcript levels were determined by real-time PCR as described in Eger *et al*. (2005).

Antibodies

The following Abs were used: mouse monoclonal Ab to E-cadherin and ZO1 (BD Biosciences, Franklin Lakes, NJ, USA); rabbit polyclonal Ab to Crumbs3, provided by Ben Margolis (University of Michigan Medical School, Ann Arbor, MI, USA); rabbit polyclonal Ab against HUGL2; goat polyclonal Ab to ZEB1 (ZEB-E20) and PATJ (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); anti-active-β-catenin Ab (Upstate Biotechnology, Lake Placid, NY, USA); mouse monoclonal Ab to cytokeratin 18 and rabbit polyclonal Ab against actin (Sigma, St Louis, MO, USA); secondary Abs coupled to Alexa Fluor 488 (Molecular Probes Inc., Eugene, OR, USA); Texas Red or peroxidase (Jackson Laboratories, West Grove, PA, USA).

Immunofluorescence microscopy, immunoblotting and immunohistochemistry

Cells were fixed in 2.5% formaldehyde (Merck Inc., Whitehouse Station, NJ, USA) and processed for immunofluorescence microscopy (Eger *et al*., 2000). Immunoblotting of total cell lysates is described elsewhere (Eger *et al*., 2000).

Formalin-fixed, paraffin-embedded colorectal adenocarcinomas or ductal and lobular breast tumours were obtained from surgical resection specimens. Tumour samples were obtained from the archive of the Institute for Pathology, University of Erlangen-Nürnberg and from the Pathology Laboratory Obrist and Brunhuber, Tyrolpath OEG. The age of colon cancer patients ranged from 45 to 65 years and breast cancer patients from 40 to 60 years. Immunohistochemistry was performed according to the instructions of the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA).

Chromatin immunoprecipitations
ChIP analyses were performed according to the instructions of the ChIP Assay Kit from Upstate Biotechnology (Eger *et al*., 2005). For PCR primers see Supplementary Table 1.

RNA inhibition experiments
ZEB1- and Snail1-specific siRNA experiments were performed as described (Eger *et al*., 2005). Snail1-specific siRNAs were purchased from Ambion (Austin, TX, USA) (Supplementary Table 1).

Transpore migration assay
Three days after ZEB1- and Snail1-siRNA treatment, 50 000 cells were transferred to 24-well Transwell filter inserts (Corning, NY, USA) (8 μm, Costar). After 24 h, cells were fixed in 2.5% formaldehyde and cells in the lower chamber were stained with Hoechst33258 (Invitrogen) and quantified by fluorescence microscopy.

Acknowledgements

We thank Gabriele Stengl and Peter Steinlein, Institute of Molecular Pathology, Vienna, for Flow Cytometry (FACS) experiments. Furthermore, we thank Jürgen Pollheimer and Martin Knofler for their technical expertise in transpore migration assays and Heidemarie Huber for support in immunohistochemistry. This study was supported by funds from the Hochschuljubiläumsstiftung of the city of Vienna to AE (H-703/2005), by grants from the Austrian Science Research Fund (FWF) No. SFB 006 to RF (603) and HB (612), SFB-F28 to WM and by funds of the Austrian Ministry of Education, Science, and the Arts (Austrian Genome Research Program GEN-AU) to MS, WS, NS, AW. AS is supported by the ÖAD-Pakistan Scholarship programme.

References

Batlle E, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J *et al*. (2000). The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat Cell Biol* **2**: 84–89.

Bilder D. (2004). Epithelial polarity and proliferation control: links from the *Drosophila* neoplastic tumor suppressors. *Genes Dev* **18**: 1909–1925.

Brabletz T, Jung A, Reu S, Porzner M, Hlubek F, Kunz-Schughart LA *et al*. (2001). Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. *Proc Natl Acad Sci USA* **98**: 10356–10361.

Brabletz T, Jung A, Spaderna S, Hlubek F, Kirchner T. (2005). Opinion: migrating cancer stem cells – an integrated concept of malignant tumour progression. *Nat Rev Cancer* **5**: 744–749.

Brumby AM, Richardson HE. (2005). Using *Drosophila melanogaster* to map human cancer pathways. *Nat Rev Cancer* **5**: 626–639.

Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG *et al*. (2000). The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* **2**: 76–83.

Chua HL, Bhat-Nakshatri P, Clare SE, Morimiya A, Badve S, Nakshatri H. (2006). NF-kappaB represses E-cadherin expression and enhances epithelial to mesenchymal transition of mammary epithelial cells: potential involvement of ZEB-1 and ZEB-2. *Oncogene* **26**: 711–724.

Comijn J, Berx G, Vermassen P, Verschueren K, van Grunsven L, Bruyneel E *et al*. (2001). The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. *Mol Cell* **7**: 1267–1278.

Condeelis J, Pollard JW. (2006). Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* **124**: 263–266.

De Craene B, Gilbert B, Stove C, Bruyneel E, van Roy F, Berx G. (2005a). The transcription factor snail induces tumor cell invasion through modulation of the epithelial cell differentiation program. *Cancer Res* **65**: 6237–6244.

De Craene B, van Roy F, Berx G. (2005b). Unraveling signalling cascades for the Snail family of transcription factors. *Cell Signal* **17**: 535–547.

Dohadwala M, Yang SC, Luo J, Sharma S, Batra RK, Huang M *et al*. (2006). Cyclooxygenase-2-dependent regulation of E-cadherin: prostaglandin E(2) induces transcriptional repressors ZEB1 and snail in non-small cell lung cancer. *Cancer Res* **66**: 5338–5345.

Eger A, Aigner K, Sonderegger S, Dampier B, Oehler S, Schreiber M *et al*. (2005). DeltaEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells. *Oncogene* **24**: 2375–2385.

Eger A, Mikulits W. (2005). Models of epithelial-mesenchymal transition. *Drug Discovery Today: Disease Models* **2**: 57–63.

Eger A, Stockinger A, Schaffhauser B, Beug H, Foisner R. (2000). Epithelial mesenchymal transition by c-Fos estrogen receptor activation involves nuclear translocation of beta-catenin and upregulation of beta-catenin/lymphoid enhancer binding factor-1 transcriptional activity. *J Cell Biol* **148**: 173–188.

Grooteclaes ML, Frisch SM. (2000). Evidence for a function of CtBP in epithelial gene regulation and anoikis. *Oncogene* **19**: 3823–3828.

Guaita S, Puig I, Franci C, Garrido M, Dominguez D, Batlle E *et al*. (2002). Snail induction of epithelial to mesenchymal transition in tumor cells is accompanied by MUC1 repression and ZEB1 expression. *J Biol Chem* **277**: 39209–39216.

Gupta PB, Kuperwasser C, Brunet JP, Ramaswamy S, Kuo WL, Gray JW *et al*. (2005). The melanocyte differentiation program predisposes to metastasis after neoplastic transformation. *Nat Genet* **37**: 1047–1054.

Hajra KM, Chen DY, Fearon ER. (2002). The SLUG zinc-finger protein represses E-cadherin in breast cancer. *Cancer Res* **62**: 1613–1618.

Huber MA, Kraut N, Beug H. (2005). Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr Opin Cell Biol* **17**: 548–558.

Krishnamachary B, Zagzag D, Nagasawa H, Rainey K, Okuyama H, Baek JH *et al*. (2006). Hypoxia-inducible factor-1-dependent repression of E-cadherin in von Hippel-Lindau tumor

suppressor-null renal cell carcinoma mediated by TCF3, ZFHX1A, and ZFHX1B. *Cancer Res* **66**: 2725–2731.

Lacroix M, Leclercq G. (2004). Relevance of breast cancer cell lines as models for breast tumours: an update. *Breast Cancer Res Treat* **83**: 249–289.

Michel D, Arsanto JP, Massey-Harroche D, Beclin C, Wijnholds J, Le Bivic A. (2005). PATJ connects and stabilizes apical and lateral components of tight junctions in human intestinal cells. *J Cell Sci* **118**: 4049–4057.

Moreno-Bueno G, Cubillo E, Sarrio D, Peinado H, Rodriguez-Pinilla SM, Villa S *et al*. (2006). Genetic profiling of epithelial cells expressing e-cadherin repressors reveals a distinct role for snail, slug, and e47 factors in epithelial-mesenchymal transition. *Cancer Res* **66**: 9543–9556.

Nishimura G, Manabe I, Tsushima K, Fujiu K, Oishi Y, Imai Y *et al*. (2006). DeltaEF1 mediates TGF-beta signaling in vascular smooth muscle cell differentiation. *Dev Cell* **11**: 93–104.

Ohira T, Gemmill RM, Ferguson K, Kusy S, Roche J, Brambilla E *et al*. (2003). WNT7a induces E-cadherin in lung cancer cells. *Proc Natl Acad Sci USA* **100**: 10429–10434.

Pacher M, Seewald MJ, Mikula M, Oehler S, Mogg M, Vinatzer U *et al*. (2006). Impact of constitutive IGF1/IGF2 stimulation on the transcriptional program of human breast cancer cells. *Carcinogenesis* **28**: 49–59.

Palmer HG, Larriba MJ, Garcia JM, Ordonez-Moran P, Pena C, Peiro S *et al*. (2004). The transcription factor SNAIL represses vitamin D receptor expression and responsiveness in human colon cancer. *Nat Med* **10**: 917–919.

Peinado H, Portillo F, Cano A. (2004). Transcriptional regulation of cadherins during development and carcinogenesis. *Int J Dev Biol* **48**: 365–375.

Pena C, Garcia JM, Garcia V, Silva J, Dominguez G, Rodriguez R *et al*. (2006). The expression levels of the transcriptional regulators p300 and CtBP modulate the correlations between SNAIL, ZEB1, E-cadherin and vitamin D receptor in human colon carcinomas. *Int J Cancer* **119**: 2098–2104.

Pena C, Garcia JM, Silva J, Garcia V, Rodriguez R, Alonso I *et al*. (2005). E-cadherin and vitamin D receptor regulation by SNAIL and ZEB1 in colon cancer: clinicopathological correlations. *Hum Mol Genet* **14**: 3361–3370.

Perez-Moreno MA, Locascio A, Rodrigo I, Dhondt G, Portillo F, Nieto MA *et al*. (2001). A new role for E12/E47 in the repression of E-cadherin expression and epithelial-mesenchymal transitions. *J Biol Chem* **276**: 27424–27431.

Shin K, Straight S, Margolis B. (2005). PATJ regulates tight junction formation and polarity in mammalian epithelial cells. *J Cell Biol* **168**: 705–711.

Spaderna S, Schmalhofer O, Hlubek F, Berx G, Eger A, Merkel S *et al*. (2006). A transient, EMT-linked loss of basement membranes indicates metastasis and poor survival in colorectal cancer. *Gastroenterology* **131**: 830–840.

Spoelstra NS, Manning NG, Higashi Y, Darling D, Singh M, Shroyer KR *et al*. (2006). The transcription factor ZEB1 is aberrantly expressed in aggressive uterine cancers. *Cancer Res* **66**: 3893–3902.

Thiery JP. (2002). Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* **2**: 442–454.

Thiery JP, Sleeman JP. (2006). Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol* **7**: 131–142.

Vandewalle C, Comijn J, De Craene B, Vermassen P, Bruyneel E, Andersen H *et al*. (2005). SIP1/ZEB2 induces EMT by repressing genes of different epithelial cell–cell junctions. *Nucleic Acids Res* **33**: 6566–6578.

Wheelock MJ, Johnson KR. (2003). Cadherins as modulators of cellular phenotype. *Annu Rev Cell Dev Biol* **19**: 207–235.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).