

SIP1/ZEB2 induces EMT by repressing genes of different epithelial cell–cell junctions

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ABSTRACT

SIP1/ZEB2 is a member of the δ EF-1 family of two-handed zinc finger nuclear factors. The expression of these transcription factors is associated with epithelial mesenchymal transitions (EMT) during development. SIP1 is also expressed in some breast cancer cell lines and was detected in intestinal gastric carcinomas, where its expression is inversely correlated with that of E-cadherin. Here, we show that expression of SIP1 in human epithelial cells results in a clear morphological change from an epithelial to a mesenchymal phenotype. Induction of this epithelial dedifferentiation was accompanied by repression of several cell junctional proteins, with concomitant repression of their mRNA levels. Besides E-cadherin, other genes coding for crucial proteins of tight junctions, desmosomes and gap junctions were found to be transcriptionally regulated by the transcriptional repressor SIP1. Moreover, study of the promoter regions of selected genes by luciferase reporter assays and chromatin immunoprecipitation shows that repression is directly mediated by SIP1. These data indicate that, during epithelial dedifferentiation, SIP1 represses in a coordinated manner the transcription of genes coding for junctional proteins contributing to the dedifferentiated state; this repression occurs by a general mechanism mediated by Smad Interacting Protein 1 (SIP1)-binding sites.

INTRODUCTION

Smad Interacting Protein 1 (SIP1; also known as ZEB2, for zinc finger E-box-binding protein 2 and *ZFHXB1*) belongs to the δ EF-1 or ZEB protein family. These proteins are characterized by a homeodomain flanked by two separated, highly conserved zinc finger clusters: an N-terminal and a C-terminal one, which contain four and three zinc fingers, respectively (1). Each zinc finger cluster can bind independently to CACCT(G) sequences present in promoter regions of genes involved in differentiation and development, such as the *Xenopus Xbra2* promoter, the human α 4-integrin promoter and the E-cadherin promoter (2). The integrity of the two zinc finger clusters of SIP1 is necessary for its binding as a monomer to the target promoter sequences (2). SIP1 acts as a transcriptional repressor and contains consensus binding sites for the corepressor CtBP (3,4). Gene repression by SIP1 has been reported to occur both dependent on and independent of a CtBP corepressor complex (4,5). Recently it was reported that the SIP1 protein can be sumoylated, which attenuates gene repression by disruption of CtBP recruitment (6).

We reported previously that binding of the E-cadherin promoter by SIP1 downregulates E-cadherin expression (7). In epithelial MDCK cells, this suppression of E-cadherin expression was accompanied by loss of aggregation and acquisition of invasive properties. An inverse correlation between SIP1 and E-cadherin expression levels was observed in several epithelial tumor cell lines, such as MDA-MB-435S1 and MDA-MB-231; high levels of SIP1 mRNA are observed in these cells while E-cadherin transcripts are not detectable. Vice versa, a transformed breast cancer cell line, MCF7/AZ, still expresses E-cadherin but lacks SIP1 expression (7). In the intestinal type of gastric carcinomas, the downregulation of

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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E-cadherin expression was again shown to be inversely correlated with SIP1 mRNA expression levels (8). SIP1 was also identified in a large-scale screen for cancer related genes, which demonstrates its putative role in oncogenic transformation (9). In addition, SIP1 expression is involved in neurogenesis of *Xenopus laevis* (10,11). SIP1 deletions as well as nonsense and frameshift mutations were demonstrated to play a role in Hirschsprung disease, a syndrome characterized by mental retardation and multiple congenital anomalies (12–15).

In the adherens junction, E-cadherin complexes contain several catenins, through which E-cadherin is linked to the actin cytoskeleton. Intercellular interactions between the E-cadherin proteins on adjacent cells result in strong cell–cell adhesion and explicit epithelial cell polarity. Abnormalities in epithelial cells are at the root of the majority of human cancers. In these cells, E-cadherin fulfills the role of a major cell–cell adhesion molecule and potently suppresses invasion. Epithelial mesenchymal transition (EMT) occurs in pathological situations, such as wound healing, fibrosis and the acquisition of an invasive phenotype in epithelial tumors (16). This EMT allows cells to dissociate from epithelial tissue and become more motile. Furthermore, EMT also participates in mesoderm and neural crest formation during normal development. The putative role of SIP1 in EMT processes was suggested by the phenotype of the *Zfx1b*-knock-out mouse, which displays delamination arrest of cranial neural crest cells resulting in the loss of migratory behavior of these cells (17). This delamination is normally mediated by the triggering of EMT (16), indicating that SIP1 is a key player in EMT processes during development. The adherens junctions are not the only cell–cell junctions nullified during EMT processes. For tight junctions, which are adjacent to the adherens junctions, it was recently shown that the transcriptional repressor Snail directly represses claudin and occludin expression, and induces EMT concomitantly with the disappearance of tight junctions (18,19). Disappearance of the desmosome, another cell junction complex, was also reported in several EMT events (19,20).

It is unlikely that E-cadherin is the only SIP1 target gene that is involved in the triggering of SIP1-induced conversion of epithelial cells to a more fibroblast-like morphology. Using human epithelial cell lines with conditional SIP1 expression, we examined the molecular mechanism involved in the SIP1-induced EMT-like process. This study revealed that SIP1 expression downregulates several cell junctional genes. Furthermore, we illustrate that this downregulation is caused by SIP1-induced repression of promoter activity. Our results demonstrate that by binding promoter regions containing SIP1 recognition sites, SIP1 performs its role in EMT-like processes by altering in a coordinated fashion the functionality of adherens junctions, tight junctions, desmosomes and gap junctions.

MATERIALS AND METHODS

Cell culture and transfection

The colon cancer cell line DLD1 was provided with the T-REX system (Invitrogen) by Van de Wetering *et al.* (21) to yield DLD1Tr21 Tet-on cells. This Tet-on system activates transcription of the gene of interest in the presence of

doxycycline (Dox). The cells were cultured in RPMI with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin. The cDNAs for wild-type SIP1 and for mutant SIP1 (mutated in both zinc finger clusters) (1) were cloned in the pcDNA4/TO vector (Invitrogen). To this end, the wild-type and mutant SIP1 fragments (1), both of which contain an N-terminal myc-tag, were cut from the pCS3 vectors, using XbaI and ClaI, and blunted. Fragments were cloned into the EcoRV digested pcDNA4/TO vector. Both constructs were stably transfected in DLD1Tr21 cells by electroporation using 30 µg of the SIP1 expression plasmid. Transfectants were selected in 500 µg/ml Zeocin (Invitrogen) and 10 µg/ml blasticidin (Invitrogen) for 2 weeks. Clones were isolated using cloning cylinders and designated DLD1Tr21/WTSIP1 and DLD1Tr21/mutSIP1. SIP1 expression was induced using Dox (2 µg/ml, Sigma). As the transfected vectors encode myc-tagged SIP1 fusion proteins, resistant colonies were tested for induction of SIP1 expression by immunofluorescent staining with the anti-myc antibody 9E10 (22). A squamous epidermoid carcinoma cell line A431 with Tet-on Dox-regulated SIP1 expression was constructed (J. Mejlvang *et al.*, manuscript submitted). These cells, designated A431/WTSIP1 were cultured in DMEM with 10% FCS, 100 µg/ml penicillin and 100 µg/ml streptomycin.

SDS-PAGE and immunoblotting

Cells were rinsed with phosphate-buffered saline (PBS) and proteins were extracted with 1× Laemmli lysis buffer. Total protein lysates of cultured cells were loaded on an 8% one-dimensional SDS-PAGE gel and the separated proteins were transferred on to Immobilon-P membranes (Millipore Corp.). After blocking with 5% non-fat dry milk in Tris buffered saline (TBS, pH 7.4) containing 0.01% Tween-20, the membranes were incubated with primary antibody. After several washing steps in TBS, the membranes were incubated with secondary horseradish peroxidase (HRP)-conjugated antibodies (dilution 1:3000; Amersham Pharmacia Biotech). Proteins were detected using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech).

Antibodies and immunofluorescence

Fixation and immunofluorescence were performed following standard procedures (23). Primary antibodies used for both immunofluorescence and immunoblotting were mouse monoclonal antibody HECD1, raised against human E-cadherin (1:75; Takara), polyclonal anti- α E-catenin antibody (1:500; Sigma), polyclonal anti- β -catenin antibody (1:1000; Sigma), mouse anti-myc antibody 9E10 [1:500; (22)], rabbit anti-myc antibody (1:100; Upstate), mouse anti-p120ctn antibody pp120 (1:200; Transduction) and mouse anti-N-cadherin antibody (1:1000; Transduction). Mouse anti-plakophilin 2 antibody (1:10; Progen), mouse anti-desmoplakin I + II antibody (1:10; Boehringer Mannheim) and mouse anti-claudin 4 antibody (1:300; Zymed) were used for immunofluorescence only.

A specific monoclonal antibody for SIP1 was generated by immunizing C57/BL6 mice with a fusion protein composed of glutathione S-transferase (GST) coupled to mouse SIP1 amino acids 26–129, according to a described protocol (24). After characterization of the antibody, supernatant from hybridoma 7F7 was purified on a protein-G Sepharose column

(Amersham Pharmacia Biotech, Rainham, UK). Secondary antibodies were Alexa-488 and Alexa-594 coupled anti-mouse, anti-rat or anti-rabbit Ig (1:500; Molecular Probes).

Real-time quantitative RT-PCR (Q-RT-PCR)

Primers and probes used for amplification were designed using Primer Express 1.0 software (Perkin-Elmer Applied Biosystems). cDNA synthesis and PCR amplification were described previously (25). The average threshold cycle of triplicate reactions was used for all subsequent calculations using the delta Ct method. Sequences of primers for mouse SIP1 cDNA amplification were: 5'-AGGCATATGGTGACG-CACAA-3' and 5'-CTTGAAGTTGCGGTTACCTGC-3'. The Taqman probe sequence was: 5'-FAM-CAGATCAGCAC-CAAATGCTAACCCAAGG-TAMRA-3' (Eurogentec). For human E-cadherin, the primers were: 5'-GTCAGTACAC-CAACGATAATCCT-3' and 5'-TTTCAGTGTGGTGATTA-CGACGTTA-3'. The E-cadherin Taqman probe sequence was: 5'-FAM-TTCAATCCCACCACGTACAAGGGTCAG-TAMRA-3'. For human N-cadherin, the primers were: 5'-AGCCTGACACTGTGGAGCCT-3' and 5'-TCAGCGTG-GATGGGTCTTTC-3' and the Taqman probe sequence was: 5'-FAM-ATGCCATCAAGCCTGTGGGAATCCG-TAMRA-3'. For human claudin 4, the primers were: 5'-GGCCGG-CCTTATGGTGATA-3' and 5'-GCCACCAGCGGATTGTA-GA-3'; for human tight junction protein 3 (*ZO-3*), the primers were: 5'-CGTCGCCTCTACGCACAAG-3' and 5'-TGAAG-AGGTGGCTGCTGTGTT-3'; for human P-cadherin (*CDH3*), the primers were: 5'-ATGACGTGGACCAACCAT-3' and 5'-GTTAGCCGCCTTCAGTTCTC-3'; for human plakophilin 2 (*PKP2*), the primers were: 5'-CGGAAATCTTACC-GAACCA-3' and 5'-AACGGCCTCCAACAAAATCAT-3'; for human desmoplakin (*DSP*), the primers were: 5'-CAG-TGGTGTCAGCGATGATGT-3' and 5'-TGACGCTGGATA-TGGTGAA-3'; for human connexin 26 (*GJB2*), the primers were: 5'-CTGGCTCACCGTCTCTTCA-3' and 5'-GCAGC-CACAACGAGGATCA-3' and for human connexin 31 (*GJB3*), the primers were: 5'-TCTGGCATGGCTTCAATA-TGC-3' and 5'-GGCAATGTAGCAGTCCACGAT-3'. For human *TBP* (TATA-box binding protein), the primers were 5'-CGGCTGTTAACTTCGCTTC-3' and 5'-CACACGCC-AAGAAACAGTGA-3' and the Taqman probe sequence was: 5'-FAM-CATAGTGATCTTTGCAGTGACCCAG-CAGC-TAMRA-3'; for human UBC (Ubiquitin C), the primers were: 5'-ATTTGGGTCGCGGTTCTTG-3' and 5'-TGCCCTTGACATTCTCGATGGT-3' and for human GAPD (Glyceraldehyde-3-phosphate dehydrogenase), the primers were: 5'-TGCACCACCAACTGCTTAGC-3' and 5'-GGCAT-GGACTGTGGTCATGAG-3'. Primers for PCR analysis for chromatin immunoprecipitation (ChIP) of a proximal fragment of the human E-cadherin promoter (-86 to +60) were: 5'-GGCCGGCAGGTGAAC-3' and 5'-GGGCTGGAG-TCTGAACTGAC-3'; primer sequences for the human plakophilin 2 proximal promoter (-529 to -391) were: 5'-GCGA-CAAAGCCTGACTAACCA-3' and 5'-GGATGGATTTCC-GCTCGAT-3'; primer sequences for the human tight junction protein 3 proximal promoter (-784 to -563) were: 5'-CTG-CAACTCAGGCGCTGTTTC-3' and 5'-CCTGAGTAGCTGG-GCTCCTGAG-3' and primer sequences for the human connexin 26 proximal promoter (-1088 to -1017)

were: 5'-CCCCCAGCAGGTGTG-3' and 5'-AAGGGG-GAAACTGATAGGAT-3'. Primers for a distal region of the E-cadherin promoter (-4834 to -4779) were: 5'-TGCCAGGTGACAGGGTCTCT-3' and 5'-AGAGGCCTT-GCCCTTCAGAT-3'; primers for a distal region of the plakophilin 2 promoter (-6039 to -5974) were: 5'-GGCAGCTG-TGGTCATCCAT-3' and 5'-GGGCATGCAGAAGCACAGTAC-3' primers for a distal region of the tight junction protein 3 promoter (-4416 to -4337) were: 5'-CCGTGAAACATGTCC-CAGATT-3' and 5'-ACCTCACAGCCCACCTCATC-3' and primers for a distal region of the connexin 26 promoter (-4294 to -4232) were: 5'-AAAAGCTACTGCCGTCCATCA-3' and 5'-ACAAGGGCAATAGAGCGATGA-3'.

Collagen invasion and fast aggregation assay

For the collagen invasion assay, cells were seeded on gelified Collagen S (type I, 0.22%) solution (Seromed, Biochrom KG, Berlin, Germany) and the invasion assay was performed as described (26). For a fast cell aggregation assay, single cell suspensions were prepared according to an E-cadherin protein saving procedure (27). Cells were incubated in an isotonic buffer containing 1.25 mM Ca²⁺ under continuous shaking. E-cadherin was functionally blocked using DECMA-1 (Sigma; 1:500). Particle diameters were measured in a Coulter particle size counter LS200 (Coulter, Electronics Ltd) at the start (N0) and after 30 min of incubation (N30); these results were plotted against the percentage of volume distribution (expressed as percent of the total cell volume).

Isolation of promoter fragments and reporter assays

The human P-cadherin, claudin 4 and connexin 26 promoter sequences were identified by screening the public human genomic DNA database (<http://genome.ucsc.edu>) with the respective cDNA fragments. These promoter sequences were amplified by PCR from genomic DNA isolated from MDA-MB-435S1 cells. Primers used for P-cadherin were: 5'-ACGGGAGGTGGAGAAAGAG-3' and 5'-AGAGAGAG-GGGTGAAGCAG-3'; for claudin 4: 5'-GGGGTACCTTCT-GGGGGACCTGTTCA-3' and 5'-CCCAAGCTTCTTAACG-TTCGAGAGTG-3' and for connexin 26: 5'-GGGGTACC-GGGCGCCAATTTTTCAAG-3' and 5'-CCCAAGCTTGGC-CGCAACACCTGTCTC-3'. Amplified fragments were cloned in the pGL3basic vector (Promega, Madison, WI), in which the multicloning site was exchanged for the promoter fragments. The 1554 bp connexin 26 promoter, containing 5 SIP1-binding sites, was shortened to a 1294 bp fragment by removing the most distal SIP1-binding element using KpnI and NruI. Transient transfection with the luciferase reporter constructs and cotransfection with the pCS3SIP1FS expression vector in MCF7/AZ cells was performed using FuGENE 6 reagent (Roche). Approximately 200 000 cells were seeded per 10 cm² well. After 24 h, 500 ng of each plasmid DNA was transfected. Luciferase activity was measured with a Galacto-Star kit (Tropix) 48 h after transfection. Transfection was normalized by measuring β-galactosidase (Galacto-Star kit; Tropix), encoded by the co-transfected pUT651 plasmid (Eurogentec). Mutagenesis of the SIP1-binding sites in the human connexin 26 promoter segment was performed with the QuickChange Multi Site-Directed Mutagenesis Kit

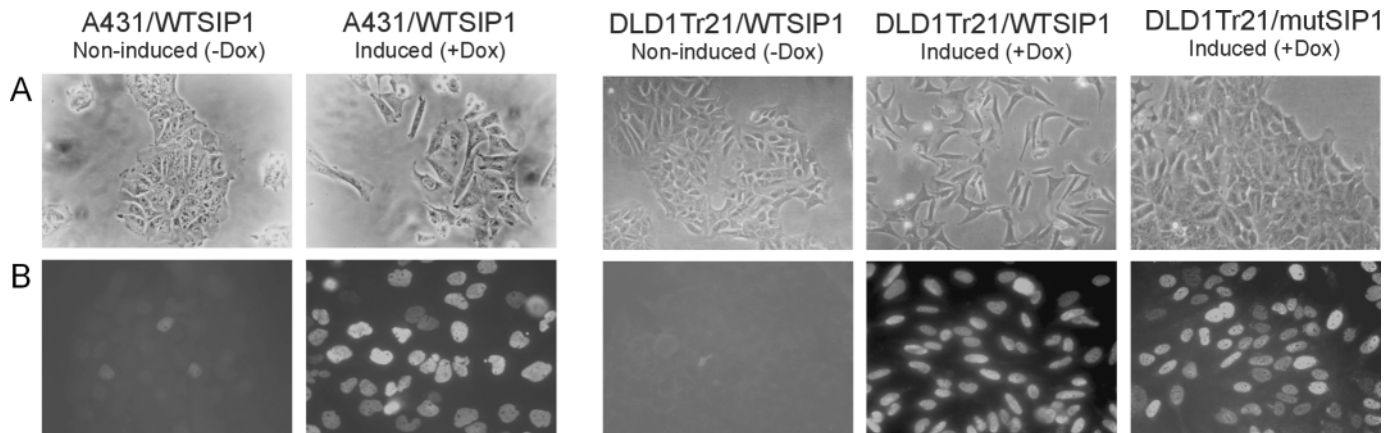


Figure 1. The stable transfectants DLD1Tr21/WTSIP1 and A431/WTSIP1 expressing SIP1. (A) Phase contrast images demonstrate the morphological changes that follow induction of SIP1 *in vitro*. The non-induced DLD1Tr21/WTSIP1 and A431/WTSIP1 cell lines display a general epithelial phenotype. WTSIP1 induces a clear morphological change in these cells whereas mutant SIP1 does not. (B) Immunofluorescence analysis with a monoclonal antibody directed against the myc-tag confirmed the absence of SIP1 protein from the non-induced cell lines and its presence in the induced cell lines.

(Stratagene) using 4 primers, each mutated in the SIP1-binding sequence: Mut 1: 5'-AAGTGGGTGCCCGAGATGGGGCGGGGGTTG-3'; Mut 2: 5'-CCAGAAAGCCCCAGCAGATGTGCAGTGCAGAGC-3'; Mut 3: 5'-CCTCACCCGAAAGGAGTCATCTCCTTGCAGTTCC-3'; Mut 4: 5'-CCACGGCGGGAGACAGATGTTGCGGCCAAGC-3'.

ChIP assays

DLD1Tr21/WTSIP1 and A431/WTSIP1 cells were grown for 24 h up to 80% confluency in the absence or presence of Dox. Cells were then crosslinked with 1% formaldehyde and processed using the ChIP-IT kit from Active Motif as described (28). Purified immunoprecipitated DNA was used for real-time quantitative PCR.

RESULTS

Exogenous SIP1 expression induces morphological changes in human epithelial cells

We reported previously that SIP1 induces downregulation of endogenous E-cadherin in the MDCK cell line (7). One way to further elucidate the functional role of SIP1 in dedifferentiation and invasion of epithelial cells is to analyze SIP1-mediated differential gene expression. The human DLD1Tr21 cell line is an E-cadherin positive colon cancer cell line expressing high levels of the tetracycline repressor (TetR) protein. Using stable transfection with expression vectors under control of the Tet responsive promoter for myc-tagged wild-type and mutant SIP1, we created the inducible model cell systems DLD1Tr21/WTSIP1 and DLD1Tr21/mutSIP1, respectively. Furthermore, a similar Tet-on inducible human epidermoid cancer cell line, A431/WTSIP1 was constructed (J. Mejlvang *et al.*, manuscript submitted). Addition of Dox to the cell cultures resulted in a dose-dependent expression of SIP1. Nuclear expression of SIP1 was detectable by immunofluorescence after 4 h of Dox treatment. The cells expressing SIP1 underwent a dramatic morphological conversion, from an epithelial cell state to a fibroblast-like phenotype, which was most apparent after 4 days of SIP1 expression (Figure 1). This conversion was not due to changes in the expression status of the other

E-cadherin repressing transcription factors Snail or Slug, as they showed no detectable mRNA expression in the DLD1Tr21 cell line conditionally expressing SIP1 (data not shown). The status of the E-cadherin–catenin complex was studied in detail by immunofluorescence and western blot analysis (Figure 2). Expression of SIP1 resulted in the loss of membranous E-cadherin and α E-catenin expression and in their internalization. Western blot analysis revealed that protein levels of E-cadherin and α E-catenin were significantly decreased only after 4 days of SIP1 expression. However, Q-RT-PCR experiments showed that extensive repression of endogenous E-cadherin mRNA was already evident after 12 h of SIP1 protein expression (Figure 3). On the other hand, the α E-catenin mRNA levels remained unchanged after SIP1 expression (data not shown). β -catenin protein was relocalized, but unlike α E-catenin, it was not strongly downregulated. It should be noted that APC is mutated in the DLD1Tr21 cell line, and so β -catenin cannot be degraded by the ubiquitin-proteasome machinery in the DLD1Tr21 derivatives. β -catenin was translocated to the cytoplasm and putatively also to the nucleus, even though nuclear β -catenin staining was barely detected (Figure 2A). The SIP1-induced changes in p120ctn protein levels are also remarkable. Immunofluorescence analysis indicated that expression of p120ctn decreased at the cell contacts (Figure 2A). Due to alternative splicing of internal exons and multiple translation initiation sites, several p120ctn isoforms can be expressed from a single gene (29,30). For the induced SIP1-expressing DLD1Tr21/WTSIP1 cell line, western blot analysis revealed an upregulation of a protein of ~120 kDa, representing p120ctn isoform 1 (Figure 2B). On the other hand, isoform 3 was clearly downregulated upon SIP1 expression. This inverse regulation of p120ctn isoforms by SIP1 was also detected in A431/WTSIP1 (data not shown).

Wild-type SIP1 induces loss of aggregation and invasion but mutant SIP1 does not

The adhesion function of the E-cadherin–catenin complex is lost in the induced SIP1-expressing cells. In a fast aggregation assay, induced DLD1Tr21/WTSIP1 cells showed loss of cell–cell aggregation, whereas expression of the mutant

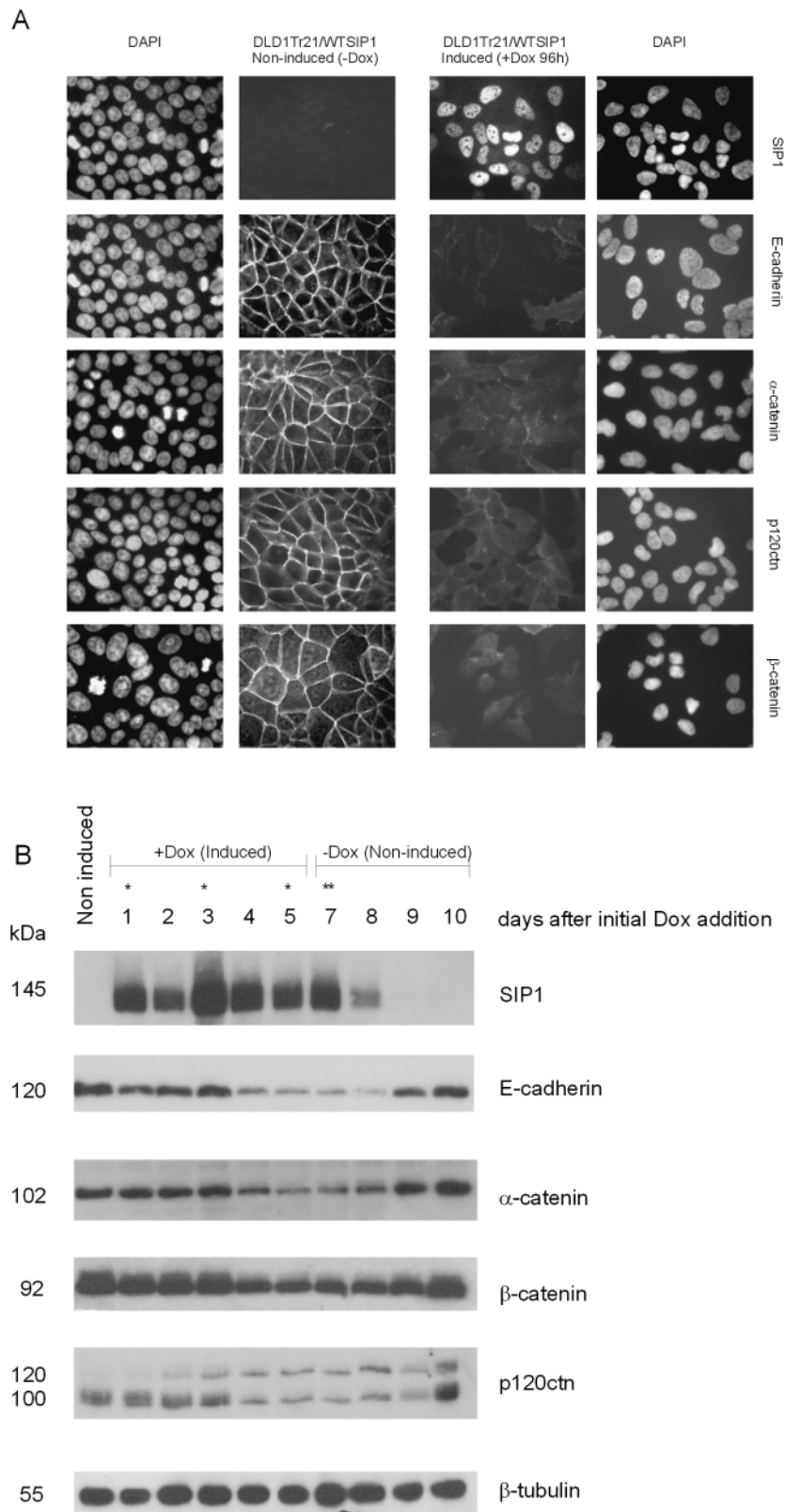


Figure 2. Behavior of the different proteins of the cadherin–catenin complex upon SIP1 induction. (A) Immunofluorescence microscopy of non-induced and induced DLD1Tr21/WTSIP1 cells using antibodies specific for adherens junction components. E-cadherin as well as α E-catenin, p120ctn and β -catenin became nearly undetectable at cell–cell contacts in the SIP1-induced cells. (B) Western blot analysis of the non-induced and induced DLD1Tr21/WTSIP1 cell line. E-cadherin and α E-catenin were downregulated at the protein level in the SIP1-expressing cells. β -catenin protein levels were unaltered in the SIP1-induced compared to non-induced cells. Protein expression of p120ctn isoform 1 was upregulated and that of isoform 3 downregulated after SIP1 induction (*: addition of Dox every 2 days, **: washing away Dox from the cell culture medium).

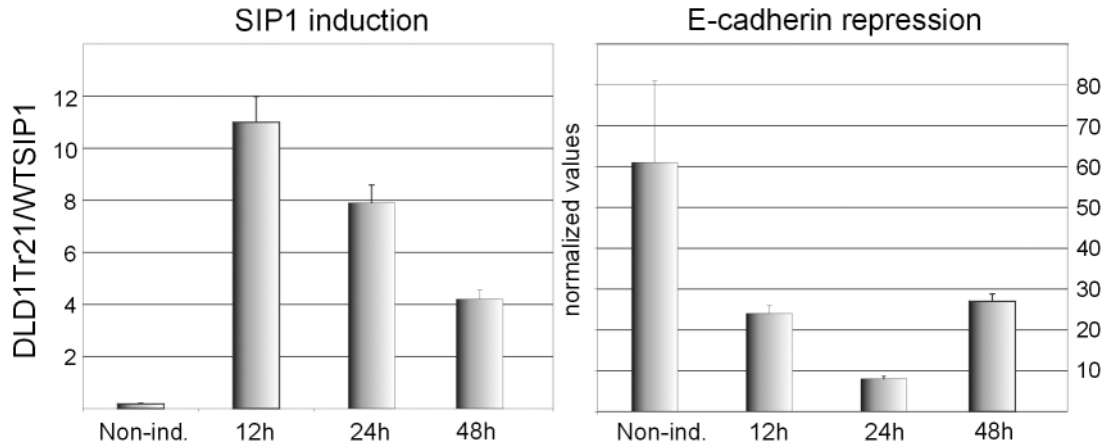


Figure 3. Downregulation of E-cadherin mRNA due to induction of SIP1 expression in DLD1Tr21/WTSIP1 cells. Q-RT-PCR for mRNA expression of SIP1 and E-cadherin. E-cadherin downregulation is already clear after 12 h of SIP1 induction. Maximum inhibition was observed after 24 h of induction. *TBP* amplification was used to normalize the values.

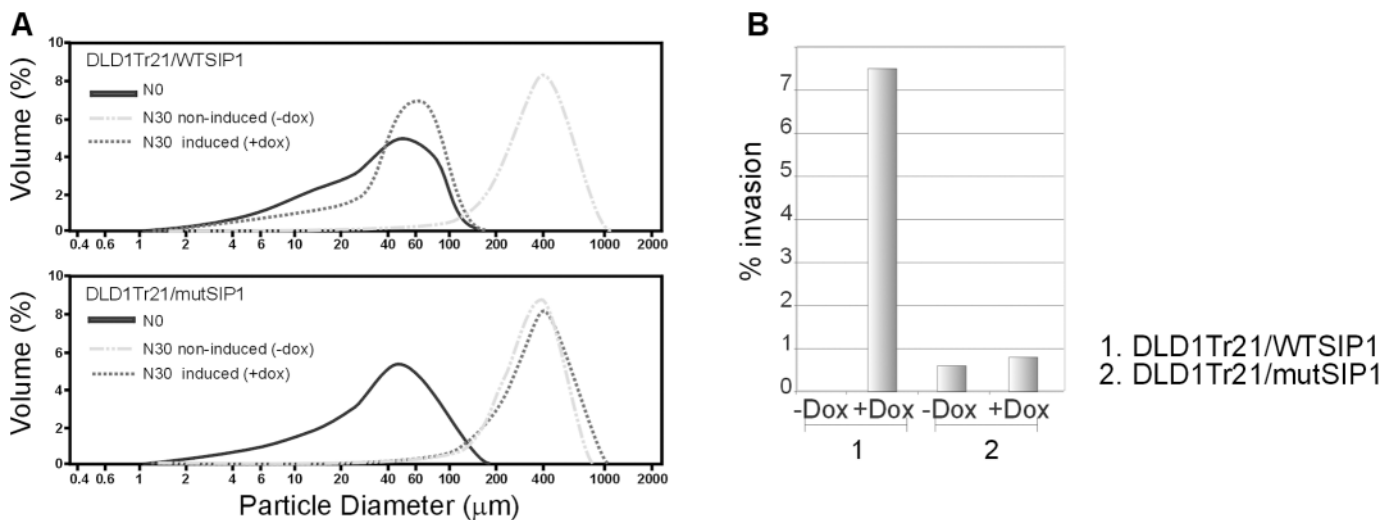


Figure 4. Wild-type SIP1 induces loss of cell aggregation and invasion whereas mutant SIP1 does not. (A) Fast aggregation assay. No cell aggregates were detected in liquid cell suspensions at time 0 (N0). After 30 min, cell–cell aggregation was detected for the DLD1Tr21 cell line expressing mutant SIP1, but no aggregates were detected when the cell line expressed wild-type SIP1. (B) Invasion into type I collagen was induced by induction (+Dox) of expression of wild-type SIP1 but it was not induced by mutant SIP1.

SIP1 had no effect on the aggregation capacity compared to the non-induced cells (Figure 4A). SIP1 expression in DLD1Tr21/WTSIP1 results in the induction of an invasive phenotype (Figure 4B). Invasion into collagen type I was induced as efficiently by SIP1 as by the E-cadherin-blocking antibody DECMA-1 (data not shown). This is in line with the demonstrated invasive behavior of MDCK cells upon SIP1 expression (7). In contrast, the induction of SIP1 protein, mutated in the zinc finger domains, in DLD1Tr21/mutSIP1 cells had no influence on the *in vitro* invasive behavior of these cells (Figure 4B). These data indicate that functional DNA-binding of the SIP1 protein is needed to convert the cells to a more mesenchymal phenotype.

Wild-type SIP1 downregulates expression of tight junction, adherens junction, desmosome and gap junction proteins at the mRNA level

In order to gain better insight into the functional impact of SIP1 expression in epithelial cells, a comparative differential

gene expression analysis using cDNA microarrays was performed (will be reported elsewhere) 12, 24 and 48 h after SIP1 induction. Besides E-cadherin, a distinct but large set of genes encoding proteins localized in the different epithelial cell junctions showed modified expression. The differential expression of transcripts encoding tight junction, adherens junction, desmosome and gap junction proteins was confirmed by Q-RT-PCR in the DLD1Tr21/WTSIP1 versus DLD1Tr21/mutSIP1 cell line (Figure 5). SIP1-mediated downregulation was shown for transcripts encoding E-cadherin, P-cadherin, claudin 4, tight junction protein 3 (*ZO-3*), plakophilin 2, desmoplakin, connexin 26 (*GJB2*) and connexin 31 (*GJB3*). None of the genes that were downregulated by wild-type SIP1 were repressed by mutant SIP1. To further verify the authenticity of the observations made on the transcript level, we performed immunofluorescence analysis for proteins encoded by SIP1-repressed genes from both the non-induced and SIP1-induced DLD1Tr21/WTSIP1 and A431/WTSIP1 cell lines. The desmosomal proteins desmoplakin and plakophilin 2

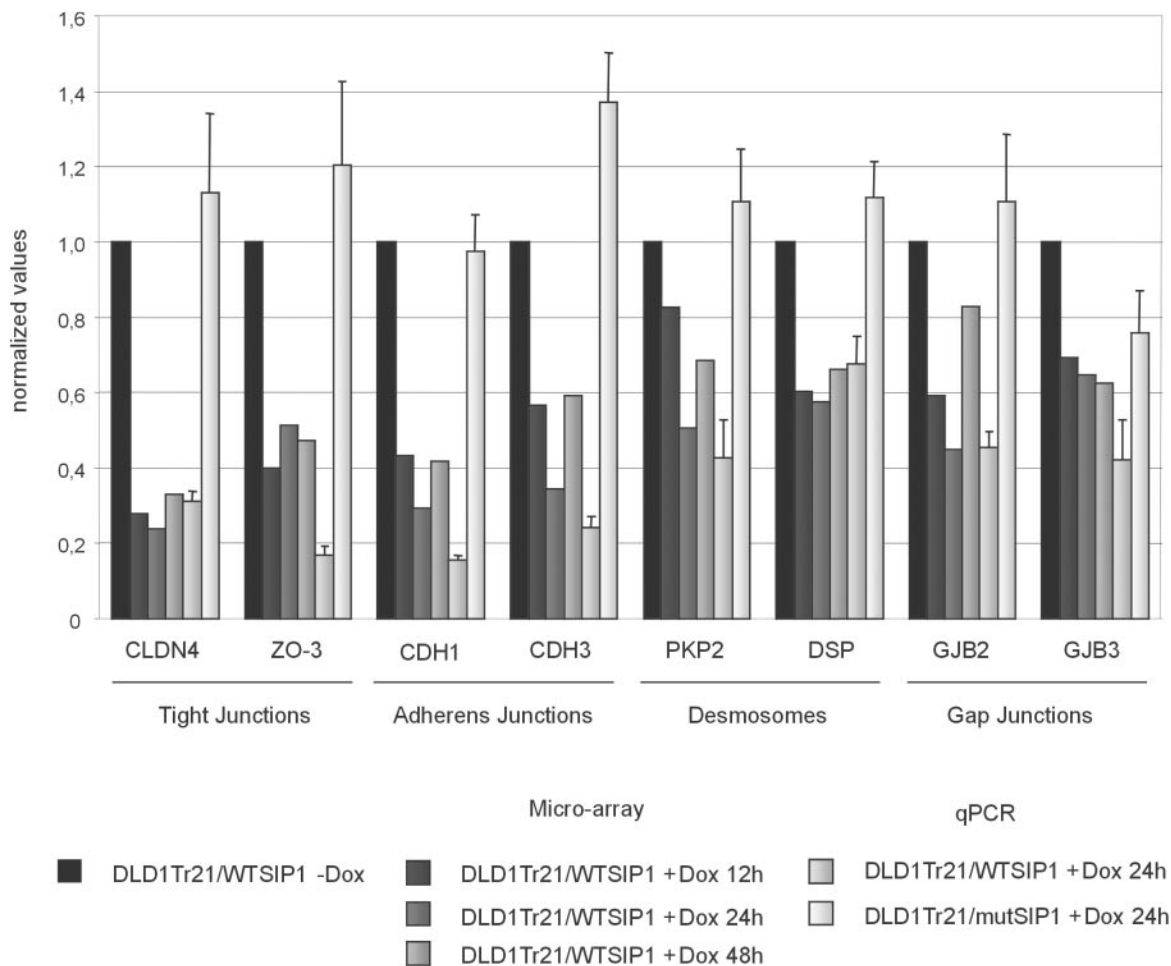


Figure 5. SIP1 downregulates the mRNA expression levels of constituents of tight, adherens, desmosomal and gap junctions in epithelial cells. Total RNA was isolated from DLD1Tr21/WTSIP1 and DLD1Tr21/mutSIP1 cell lines after induction with Dox (at 12 h, 24 h and 48 h) or without Dox. Q-RT-PCR analysis for the genes indicated was compared with the microarray results. It is clear that all these transcripts are downregulated by WTSIP1, but the expression of none of them was affected in the cell line expressing mutant SIP1. Amplification of *TBP*, *UBC* and *GAPD* was used to normalize the values.

were clearly downregulated (Figure 6). The tight junction protein claudin 4 was also repressed at the protein level by induction of SIP1.

SIP1 induces cadherin switching in A431/WTSIP1 cells

In contrast to E-cadherin, N-cadherin is believed to promote cell migration and tumor progression, and has been shown previously to be upregulated in invasive cancer cell lines (31,32). Q-RT-PCR revealed upregulation of N-cadherin mRNA in A431/WTSIP1 cells 48 h after induction of SIP1, while E-cadherin expression was strongly downregulated (Figure 7A). This cadherin switching was confirmed at the protein level (Figure 7B).

Effect of SIP1 expression on promoter activities

After induction of SIP1, morphological changes were observed only in the DLD1Tr21/WTSIP1 cell line, not in the DLD1Tr21/mutSIP1, in which SIP1 is mutated in both zinc finger clusters and is therefore unable to bind promoter sequences (2,7). Indeed Q-RT-PCR analysis revealed that the

SIP1 regulated genes in the SIP1-induced cell line were repressed only by the wild-type SIP1, not by the mutated SIP1 protein. This indicates that the SIP1-induced morphological changes observed are caused by direct functional promoter regulation of target genes. Until now, E-cadherin is the only known target gene of SIP1 which is known to be involved in EMT and invasion processes (7). Therefore, we investigated whether the promoters of several putative SIP1 target genes are directly regulated by SIP1. Initially, the promoters of P-cadherin, claudin 4 and connexin 26 (*GJB2*) were screened for the presence of SIP1-binding sites [CACCT(G)], and suitable fragments were cloned in the pGL3basic vector upstream of the luciferase reporter gene. In the P-cadherin promoter (531 bp) 1 AGGTG and 3 CACCTG sequences are present. One CACCT and two AGGTG sequences were identified in the claudin 4 promoter (635 bp) (Figure 8A). Finally, the isolated connexin 26 promoter fragment that was cloned (1294 bp) contains 1 CACCT, 1 AGGTG and 2 CAGGTG sequences. To elucidate whether SIP1-binding affects the transcriptional activity of these cloned promoters, we transiently co-transfected the reporter plasmids together with a SIP1 expression vector in the E-cadherin positive epithelial cell line

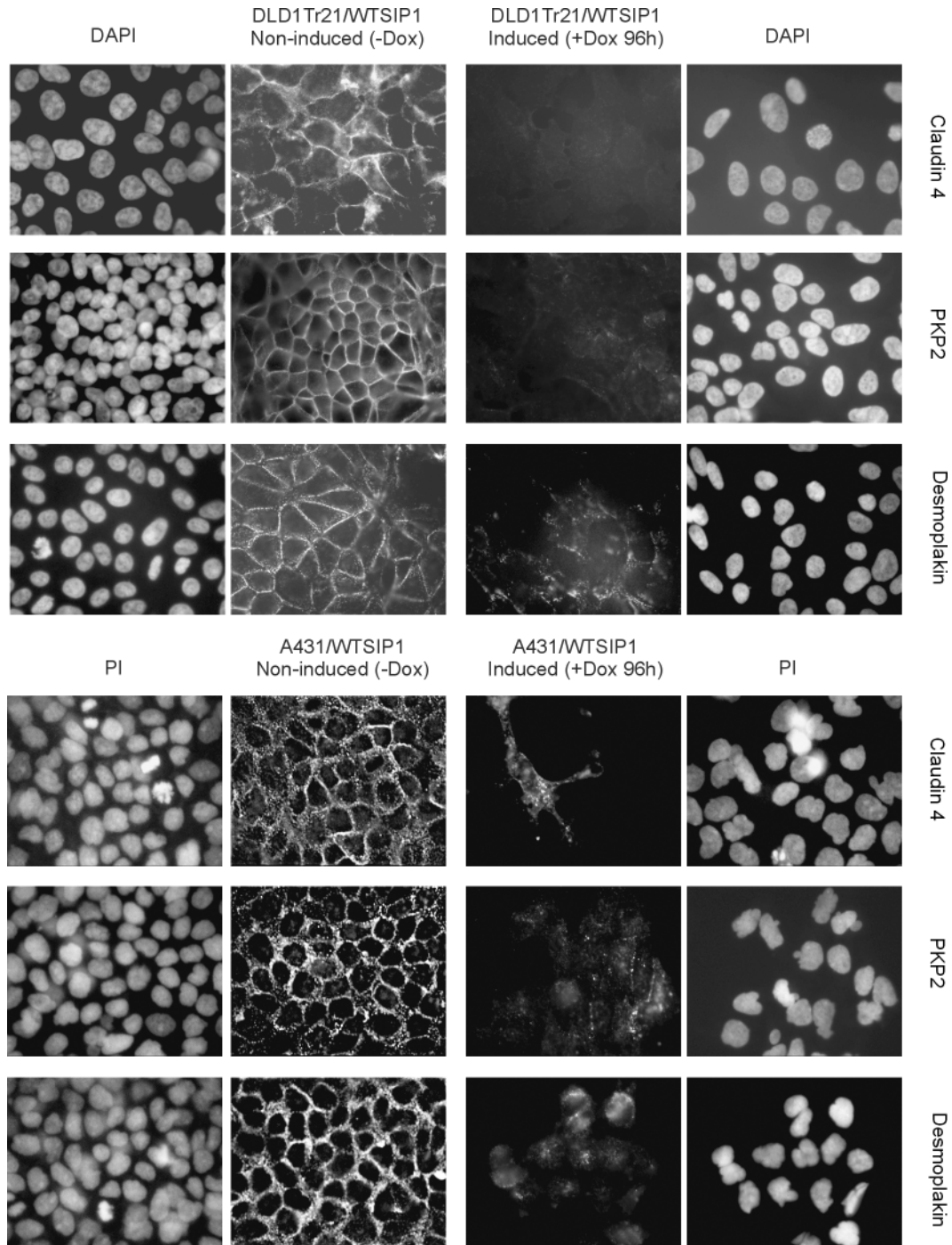


Figure 6. SIP1 downregulates different proteins of the tight junctions and desmosomes. Immunofluorescence microscopy of non-induced and induced DLD1Tr21/WTSIP1 and A431/WTSIP1 cells using specific antibodies for tight junctional and desmosomal components. SIP1 expression decreases expression of desmoplakin and *PKP2* at contact regions. Claudin 4 expression at the tight junctions was affected by SIP1 expression. DAPI staining was done in DLD1Tr21/WTSIP1 and PI (propidium iodide) staining in A431/WTSIP1 to visualize nuclei of the same cell fields.

MCF7/AZ. SIP1 expression caused significant decreases in the promoter activities of P-cadherin, claudin 4 and connexin 26 (Figure 8C). To address the specificity of SIP1 action, the 4 SIP1-binding sites present in the human connexin 26 promoter were mutated, either separately or in different combinations (Figure 8B). When these mutant connexin 26 promoter constructs were co-transfected with SIP1 cDNA, the SIP1

repressive activity was diminished (derepressed) only when all 4 SIP1-binding sites were mutated (Figure 8C). Mutating 1, 2 or 3 SIP1 recognition sequences did not have a large impact on the repressed promoter activity. These data show that the integrity of a single SIP1-binding element in the connexin 26 promoter is sufficient for recruitment of SIP1 to the promoter and significant repressive activity. These findings are in line

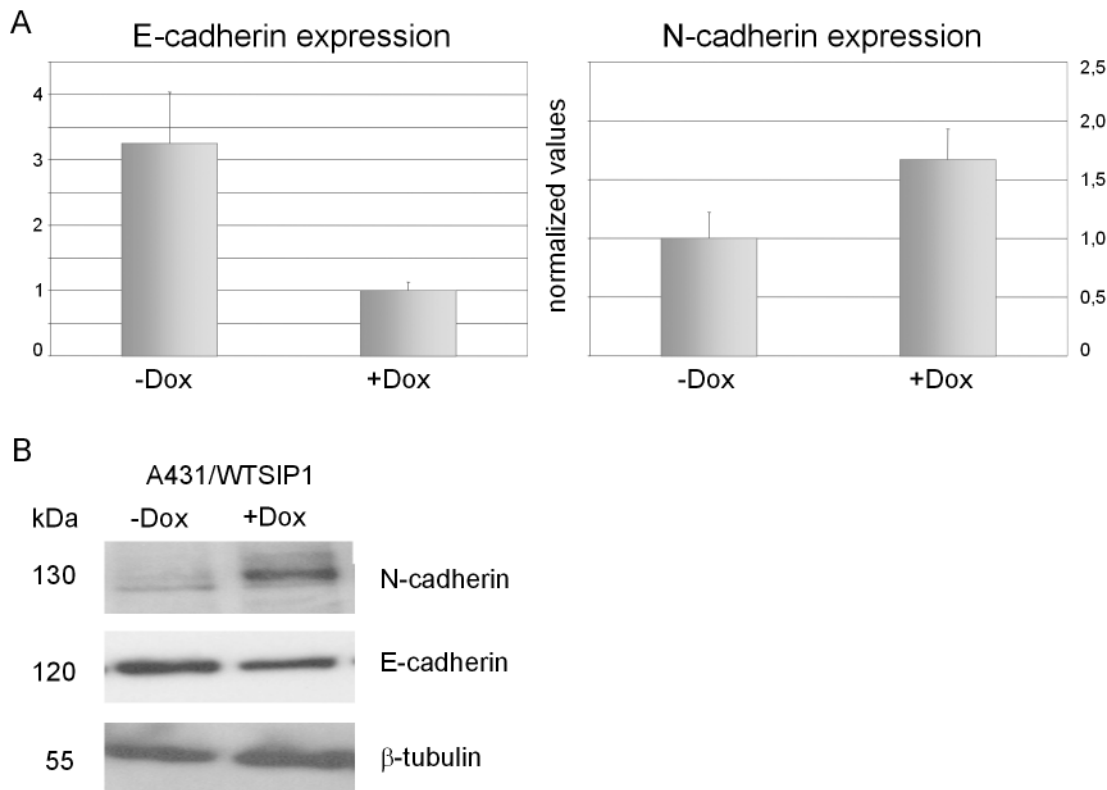


Figure 7. Cadherin switching in A431/WTSIP1 upon SIP1 induction. (A) Q-RT-PCR for E-cadherin and N-cadherin mRNAs in A431/WTSIP1. E-cadherin was clearly downregulated after 48 h of SIP1 induction, while N-cadherin mRNA was upregulated. *TBP* and *GAPD* amplification was used to normalize the values. (B) Western blot analysis showed a similar inverse correlation between E-cadherin and N-cadherin protein levels after induction of SIP1.

with the results obtained previously with the E-cadherin promoter in which both SIP1-binding sites had to be mutated in order for SIP1 to lose its repressive activity (Figure 8C) (7).

SIP1 associates at the chromatin level with promoters containing SIP1-binding sites

We wanted to investigate whether SIP1 associates directly, at the chromatin level, with these new target genes via their SIP1-binding sites. Therefore, we performed chromatin immunoprecipitation (ChIP) assays in both the DLD1Tr21/WTSIP1 and the A431/WTSIP1 inducible cell systems. Cells were grown to 80% confluency in the absence or presence of Dox. After 24 h the cells were crosslinked with 1% formaldehyde and harvested. A SIP1-specific mouse monoclonal antibody was used to pull down any chromatin fragment physically bound by SIP1. Background was determined using an irrelevant IgG antibody. Quantitative PCR performed with primers specific for SIP1-binding site containing promoter fragments of E-cadherin (*CDH1*), plakophilin 2 (*PKP2*), tight junction protein 3 (*ZO-3*) and connexin 26 (*CX26*) revealed an enrichment of these sequences after induction of SIP1 (Figure 9), whereas distal promoter sequences for these genes showed no significant enrichment. This indicates that SIP1 can directly downregulate expression of epithelial cell junctional genes in a direct manner by physically interacting with the promoter regions containing SIP1-binding sites.

DISCUSSION

EMT occurs frequently during normal development in processes such as mesoderm and neural crest cell formation. During tumor progression, EMT is also crucial for loss of cell polarity of epithelial cells, thus facilitating migratory and invasive behavior. The involvement of the transcription factor SIP1/ZEB2 during EMT in developmental processes was indicated by the phenotype of the SIP1 knock-out mouse (17). Loss of SIP1 expression was correlated with loss of the migratory capacities of neural crest cells. Retroviral insertion mutagenesis suggested that SIP1 could contribute to oncogenic transformation (9). Furthermore, the upregulation of ZEB-family members during EMT was recently demonstrated (33). To study the role of SIP1 in more detail in EMT-like processes, we generated human cell lines with conditional SIP1 expression. In these Tet-on cell systems, adding Dox to the cell culture medium resulted in nuclear expression of SIP1. A drastic morphological change was induced in these epithelial cells as a consequence of exogenous SIP1 expression. As the transmembrane cell adhesion protein E-cadherin is a direct target of SIP1, we analyzed the expression of the different components of the cadherin-catenin complex. Downregulation of protein and mRNA levels was only detected for E-cadherin. The complexing α E-catenin was altered only at the protein expression level, probably as a consequence of loss of E-cadherin expression (34). Different p120ctn isoforms were inversely regulated during the SIP1-induced EMT-like process. The upregulation of p120ctn isoform 1 (~120 kDa)

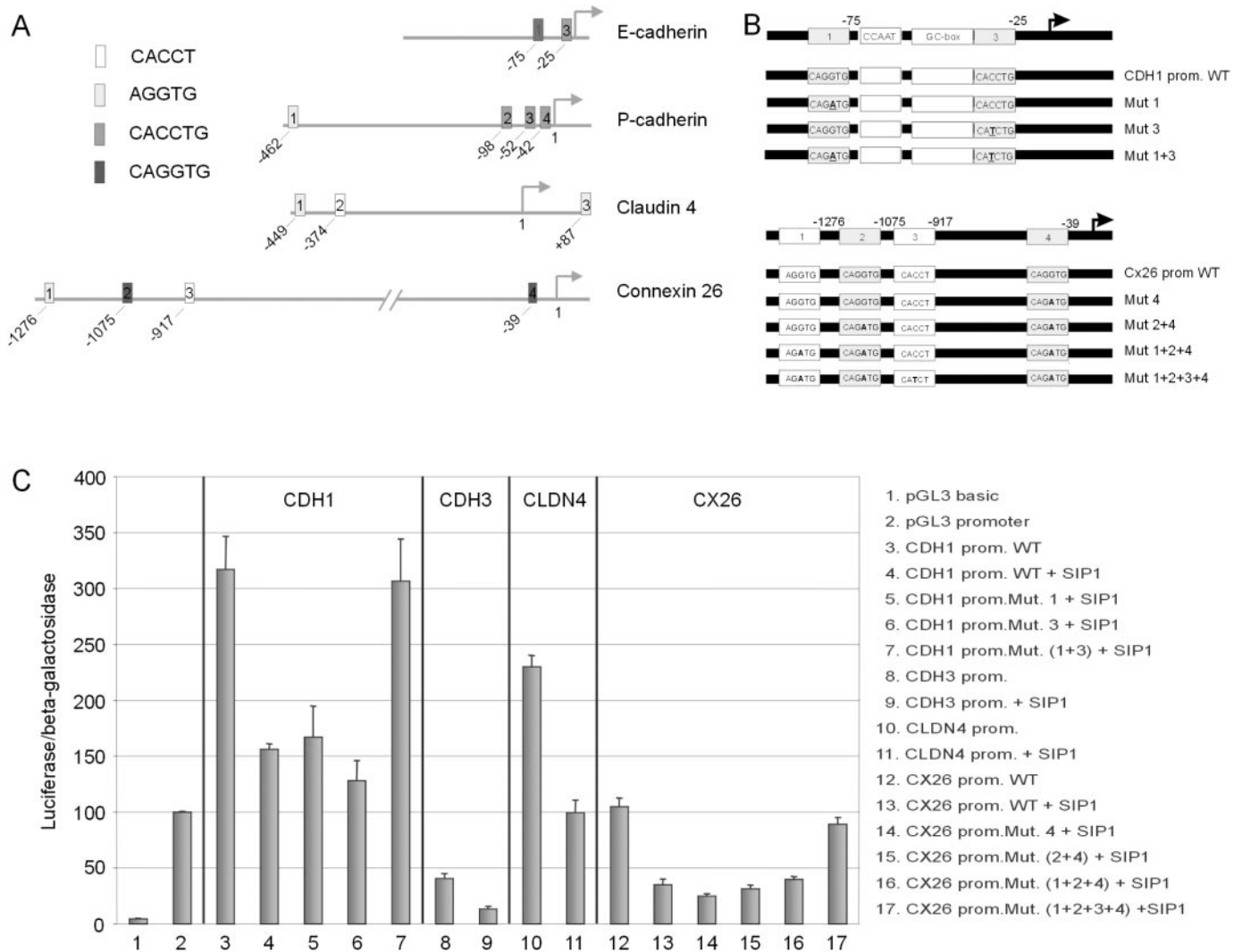


Figure 8. SIP1 transcriptionally downregulates genes of several intercellular junctional complexes. (A) Schematic overview of the cloned promoter regions of E-cadherin, P-cadherin, claudin 4 and connexin 26. The putative SIP1-binding sites are indicated. (B) Mutations generated in the SIP1-binding sites of the human E-cadherin promoter (7) and the human connexin 26 promoter. E2-boxes are shaded gray. Mut 4 carries the mutation CAGGTG → CAGATG; Mut 2 + 4 carries 2 identical CAGGTG → CAGATG mutations; Mut 1 + 2 + 4 carries an additional AGGTG → AGATG mutation; Mut 1 + 2 + 3 + 4 carries the 3 mutations described above and has a CACCT → CATCT mutation in SIP1-binding site 3. (C) Promoter activity assays on extracts of transfected MCF7/AZ cells. Cells were co-transfected with a SIP1 expression vector and luciferase promoter constructs for E-cadherin, P-cadherin, claudin 4 or connexin 26. Co-expression of SIP1 with the promoter constructs resulted in downregulation of promoter activities. Mutation of all 4 SIP1-binding elements in the connexin 26 promoter (see B) relieved the repressive activity of SIP1. Mutation of less than 4 SIP1-binding sequences preserved the repressive effect of SIP1. Luciferase values are normalized with β-galactosidase activities.

and the downregulation of isoform 3 (~100 kDa) indicate putative specific roles for different isoforms in epithelial and mesenchymal states. A similar shift in p120ctn isoform expression was seen in FosER cells, in which EMT is induced as a consequence of FosER activation by estradiol addition (35). This shift is in line with the previously observed predominant expression of 100 kDa and 120 kDa isoforms in epithelial cells and in highly motile fibroblastoid cells, respectively (36). The functional difference between these isoforms remains to be elucidated. The other E-cadherin-binding Armadillo protein β-catenin showed no decrease in mRNA and protein expression levels, but β-catenin was no longer expressed at the cell–cell contacts. SIP1 expression resulted in the redistribution of β-catenin to the cytoplasm and possibly also to the nucleus.

The E-cadherin promoter was previously identified as a direct target of SIP1. SIP1 binds to the E2-boxes (CACCTG) present in the E-cadherin promoter, resulting in downregulation of the promoter’s activity (7). Global gene expression analysis using the *in vitro* SIP1-induced cell models revealed that SIP1 expression results in downregulation of major constituents of different cell junctional complexes, such as tight junctions, adherens junctions, desmosomes and gap junctions. Interestingly we found by Q-RT-PCR analysis that SIP1 downregulates several cell junction genes on the transcript level. The fact that expression of a SIP1 mutant, with one missense mutation in each of the zinc finger clusters, has no effect on mRNA expression levels of these regulated genes, suggests that downregulation by SIP1 is mediated mainly via promoter regulation. Both zinc finger clusters are indeed needed for

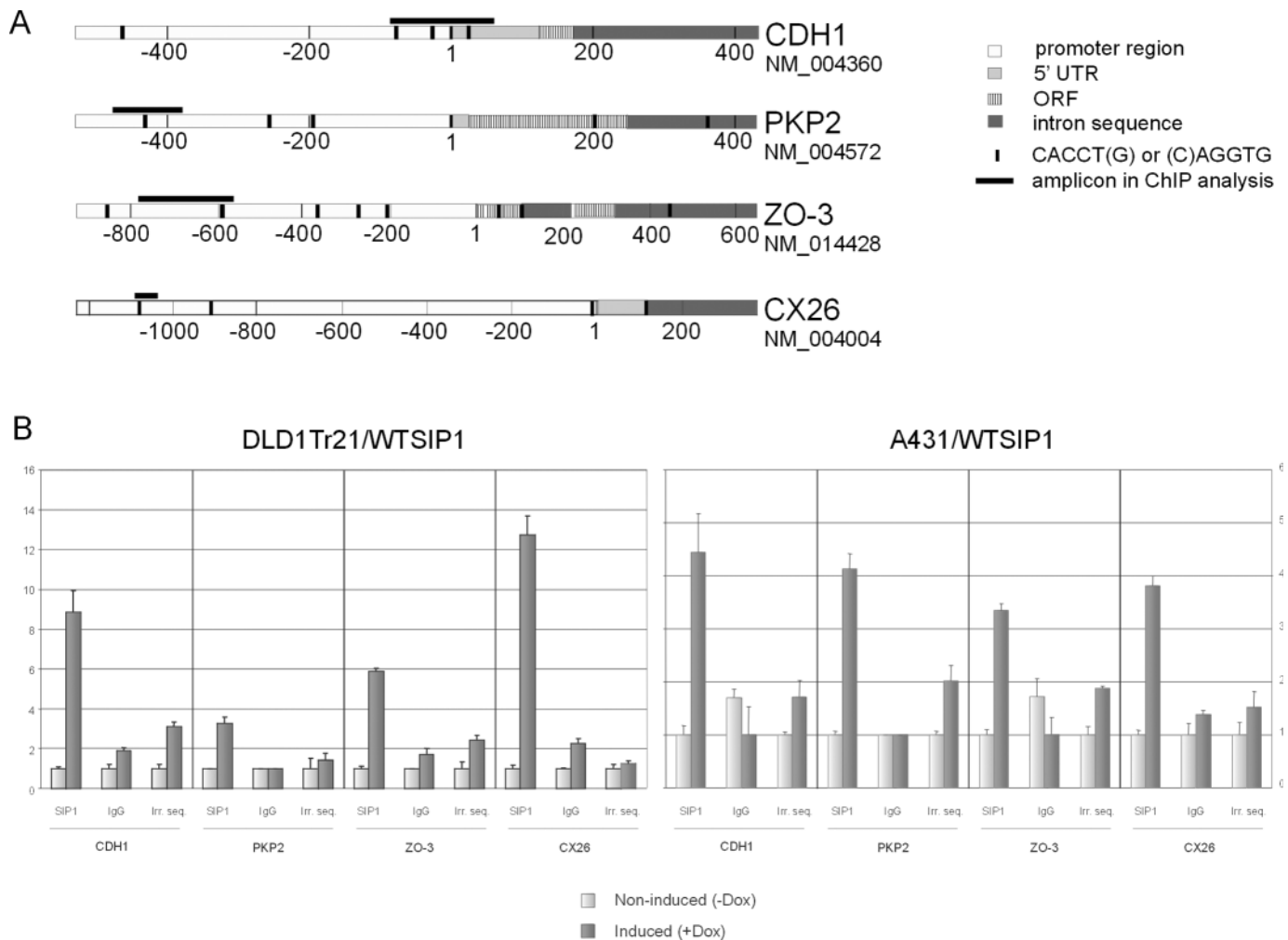


Figure 9. SIP1 associates with the promoter regions of cell junction genes at the chromatin level. (A) Promoter regions of junctional genes repressed by SIP1. The promoter region, 5' untranslated region (5'-UTR), open reading frame (ORF) and intron were defined using the sequence information derived from the Database for Transcriptional Start Sites (<http://dbtss.hgc.jp>) and the public human genomic DNA database (<http://genome.ucsc.edu>) using the Refseq Ids as mentioned under the gene symbol. CACCT(G) and (C)AGGTG boxes were mapped. Amplicons analyzed in the chromatin immunoprecipitation are shown as black bars. (B) DLD1Tr21/WTSIP1 and A431/WTSIP1 cells were either not induced or induced with Dox for 24 h. *In vivo* binding of SIP1 to proximal promoter sequences in DLD1Tr21/WTSIP1 and A431/WTSIP1 cells, as determined by ChIP analysis. Enrichment of bound sequences was quantified by quantitative real-time PCR and is depicted as the fold increase of association of SIP1 detected with a SIP1-specific antibody in the Dox induced cells versus non-induced cells. Background was determined using an irrelevant IgG antibody and is also depicted as the fold increase in the induced versus non-induced cells. Irrelevant sequences (Irr. seq.) are amplified distal promoter sequences, 4 to 7 kb upstream of the transcription start site of E-cadherin, plakophilin 2, tight junction protein 3 and connexin 26.

SIP1-dependent promoter repression via E-box-binding (7). Cloning of the promoter regions of the regulated genes connexin 26 (in the gap junctions), P-cadherin (at the adherens junction) and claudin 4 (in the tight junctions) revealed the presence of several SIP1-binding sequences in each of them. Mutation of these elements in the cloned connexin 26 promoter showed the importance of the integrity of these sequences in the SIP1-dependent suppressive activity. Furthermore, we could demonstrate physical interaction at the chromatin level between SIP1 and the promoter regions of E-cadherin, plakophilin 2, connexin 26 and ZO-3, all of which contain SIP1-binding sites.

The change in expression and distribution of those SIP1 target genes during EMT could be explained as a secondary consequence of repression of E-cadherin. A crucial role for E-cadherin in epithelial cell polarity has been well documented (37–39). However, exogenous E-cadherin expression

in mesenchymal cells expressing EMT inducers such as Snail cannot restore the epithelial phenotype (19,40,41). Moreover, downregulation of the tight junction components, occludins and claudins, by Snail was linked to repression of their promoter activity (18,19). Hence, we have to conclude that E-cadherin and other junctional genes are simultaneously downregulated as part of the SIP1 driven reprogramming during EMT. It remains enigmatic though why these different junctional genes are repressed in a coordinated fashion. We do know that some of these genes can be regarded as NACos, proteins that can localize both to the nucleus and adhesion complexes (42). Such proteins have the intriguing potential to coordinate the regulation of cell adhesion and transcription. The function of several NACos proteins belonging to the Armadillo family such as PKP2, β -catenin and p120ctn seem to be affected in epithelial cells with SIP1 expression. The desmosomal PKP2 has been reported to be present also in

the nucleus at all times. This protein seems to be part of particles containing RNA polymerases (43). Moreover the transcription factor Snail is also able to repress *PKP2* expression very potently (19). This strong downregulation of *PKP2* suggests that inhibiting the potential role of *PKP2* in adhesion and/or transcriptional regulation could be essential in the process of EMT. It is at present not clear if the SIP1-induced isoform switching has functional consequences for a particular nuclear role of p120ctn (23). P120ctn can interact with Kaiso and has as such the potential to influence beta-catenin/TCF signaling (44). On the other hand, downregulation of E-cadherin could result in loss of β -catenin sequestration to sites of cell-cell adhesion, enabling β -catenin/TCF mediated transcription. However SIP1 did not induce clear nuclear β -catenin localization nor enhanced WNT signaling (data not shown).

Enhanced SIP1 expression has so far been reported in a distinct set of cancers comprising gastric, hepatocellular, ovarian and breast carcinomas (8,45,46). Here, the described candidate SIP1 target genes have been documented to show aberrant expression in a variety of human cancer types (47–50). This suggests that repression of these genes could be due to enhanced SIP1 expression, although this has to be examined in detail in the near future.

Taken together, the present results identify the SIP1 protein as an important mediator of epithelial dedifferentiation through direct downregulation of a distinct set of constituents of adherens junctions, tight junctions, desmosomes and gap junctions, which are key determinants of the epithelial phenotype, including epithelial cell polarity.

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Conflict of interest statement. None declared.

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