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Direct Repression of Cyclin D1 by SIP1 Attenuates Cell Cycle Progression in Cells Undergoing an Epithelial Mesenchymal Transition[D]

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Zinc finger transcription factors of the Snail/Slug and ZEB-1/SIP1 families control epithelial-mesenchymal transitions in development in cancer. Here, we studied SIP1-regulated mesenchymal conversion of epidermoid A431 cells. We found that concomitant with inducing invasive phenotype, SIP1 inhibited expression of cyclin D1 and induced hypophosphorylation of the Rb tumor suppressor protein. Repression of cyclin D1 was caused by direct binding of SIP1 to three sequence elements in the cyclin D1 gene promoter. By expressing exogenous cyclin D1 in A431/SIP1 cells and using RNA interference, we demonstrated that the repression of cyclin D1 gene by SIP1 was necessary and sufficient for Rb hypophosphorylation and accumulation of cells in G1 phase. A431 cells expressing SIP1 along with exogenous cyclin D1 were highly invasive, indicating that SIP1-regulated invasion is independent of attenuation of G1/S progression. However, in another epithelial-mesenchymal transition model, gradual mesenchymal conversion of A431 cells induced by a dominant negative mutant of E-cadherin produced no effect on the cell cycle. We suggest that impaired G1/S phase progression is a general feature of cells that have undergone EMT induced by transcription factors of the Snail/Slug and ZEB-1/SIP1 families.

INTRODUCTION

An important event in the development of malignant epithelial tumors is epithelial-mesenchymal transition (EMT), a process of generation of motile and invasive mesenchymal cells from polarized epithelia. Because EMT plays a fundamental role at certain stages of normal development (gastrulation, neural crest migration, somitogenesis), it has been suggested that some elements of embryonic transdifferentiation programs are exploited by cells of growing carcinoma (Thiery, 2003). Cells undergoing EMT are characterized by massive alterations in gene expression patterns. They acquire expression of mesenchymal but loose epithelial markers. A central event in EMT is loss of epithelial cadherin (E-cadherin), a surface receptor that plays an essential role in the formation of adherens junctions and that is often mutated or lost in cancer cells (Thiery and Chopin, 1999, Thiery, 2003).

In recent years, several direct transcriptional repressors of E-cadherin (Snail, Slug, ZEB-1, SIP1, and E47) have been identified (Batlle *et al.*, 2000; Cano *et al.*, 2000; Comijn *et al.*, 2001; Perez-Moreno *et al.*, 2001; Bolos *et al.*, 2003; Eger *et al.*, 2005). These proteins act downstream in EMT-inducing signal transduction pathways activated by TGFβ, FGF, and EGF growth factors, integrin engagement, and hypoxia (Imai *et al.*, 2003; De Craene *et al.*, 2005a; Krishnamachary *et al.*, 2006; Imamichi *et al.*, 2007). ZEB-1/SIP1 and Snail/Slug family members directly interact with the response elements in the proximal *e-cadherin* gene promoter and actively repress transcription recruiting transcriptional corepressors such as CtBP or mSinA (Furusawa *et al.*, 1999; Shy *et al.*, 2003; Peinado *et al.*, 2004). More recently, direct repression of other epithelial genes by Snail and SIP1 has been reported (De Craene *et al.*, 2005b; Vandewalle *et al.*, 2005; Moreno-Bueno *et al.*, 2006). In addition, Snail/Slug and ZEB-1/SIP1 proteins mediate up-regulation of genes implicated in cell invasion and motility (e.g., vimentin, members of the matrix metalloproteinase (MMP) family of proteases, fibronectin). The mechanisms of transcriptional activation is less clear; in some cases, indirect activation of genes implicated in EMT by Snail and SIP1 takes place (Jorda *et al.*, 2005; Taki *et al.*, 2006). In contrast to Snail and Slug, ZEB-1, and SIP1 proteins interact with transcriptional coactivators pCAF and p300 (Postigo *et al.*, 2003; van Grunsven *et al.*, 2006). This biochemical difference may indicate that Snail and SIP1 family members activate expression of mesenchymal markers via fundamentally different mechanisms. In vivo studies demonstrated that Snail/Slug and ZEB-1/SIP1 proteins have different functions in embryonic development and are involved in the control of distinct EMT programs. Snail regulates gastrulation, and *snai1*$^{-/-}$ mutant embryos exhibit severe defects in EMT required for generation of the mesoderm cell layer

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(Carver *et al.*, 2001). On the other hand, experiments with *snai2*-deficient mice (Jiang *et al.*, 1998) and generation of conditional *snai1*$^{-/-}$ knockout embryos demonstrated that neither Snail nor Slug is required for the delamination and migration of neural crest cells (Murray and Gridley, 2006). In contrast, homozygous mutant embryos lacking *zfhx1b*, the gene encoding SIP1, display early arrest in cranial neural crest migration (van de Putte *et al.*, 2003).

In a number of clinical studies, transcription of genes encoding Snail/Slug and ZEB-1/SIP1 proteins has been detected in breast (Blanco *et al.*, 2002; Elloul *et al.*, 2005), ovarian (Elloul *et al.*, 2005), gastric (Rosivatz *et al.*, 2002), and hepatocellular (Sugimachi *et al.*, 2003) carcinoma cells, and Snail immunoreactivity significantly correlated with breast cancer metastasis (Zhou *et al.*, 2004). Activation of Snail, Slug, E47, ZEB1, and SIP1 is an important, but not the only instrument that is utilized by cancer cells to acquire motile characteristics. Inactivation of *e-cadherin* by gene mutations (Berx *et al.*, 1998; Guilford *et al.*, 1998) or consistent cleavage of the E-cadherin extracellular domain chronically exposed to matrix metalloproteinases secreted by stromal cells may be sufficient to trigger a process ultimately leading to EMT in tumor cells (Lochter *et al.*, 1997). Recently, we explored a model of functional inhibition of E-cadherin in squamous carcinoma cells A431 by a dominant negative E-cadherin mutant (Andersen *et al.*, 2005). Expression of this mutant triggered a program of gradual EMT, which eventually resulted in activation of vimentin and increased cell motility.

In nonpathological conditions, EMT represents the profound de-differentiation program that must be incompatible with cell proliferation (Burstyn-Cohen and Kalcheim, 2002). Indeed, in 8.5 dpc mouse embryos, cells expressing Snail are characterized by decreased incorporation of bromodeoxyuridine (BrdU; Vega *et al.*, 2004). In Madin-Darby canine kidney (MDCK) cells and in primary keratinocytes, Snail family members induce cell cycle arrest in G1 phase and hypophosphorylation of the retinoblastoma (Rb) protein (Vega *et al.*, 2004; Turner *et al.*, 2006). Complex cell cycle–regulating networks are dependent on cell–cell adhesion, integrin signaling, cell spreading, and actomyosin contractility (Walker *et al.*, 2005). Therefore, there are many potential molecular schemes by which EMT may affect cell proliferation in embryonic development and cancer. However, in cancer cells, the interrelationship between cell growth and EMT can be circumvented by the defects in the molecular pathways controlling the cell cycle. In this study, we analyze cell cycle progression in two EMT models based on conditional expression of either SIP1 or a dominant negative E-cadherin mutant Ec1WVM in the same cell line. We show that SIP1, but not Ec1WVM, induces accumulation of cells in the G1 phase of cell cycle. This effect is largely mediated by the direct transcriptional repression of the cyclin D1 gene by SIP1.

MATERIALS AND METHODS

Plasmids

Vectors expressing myc-tagged wild-type SIP1 (pUHDmyc-SIP1) and myc-tagged SIP1 with the mutated C-terminal Zn finger (pTREmyc-SIP1ZFmut) have been described (Comijn *et al.*, 2001; van Grunsven *et al.*, 2003). To generate a doxycycline (DOX)-regulated cyclin D1 expression vector (pBIcyclD1), the cyclin D1 coding sequence was amplified and cloned into pBI vector (BD Bioscience, Clontech, Palo Alto, CA). To analyze cyclin D1 promoter activity, we generated two luciferase reporter vectors, p*wt*CCND1LUC and p*mut*CCND1LUC. To generate p*wt*CCND1LUC, a fragment of the cyclin D1 5′-flanking sequence (−1025 to +18) was cloned into pGL3 basic vector (Promega Biotech, Madison, WI). To create p*mut*CCND1LUC, three Z-boxes with coordinates −1014 to −1010; −857 to −853, and −300 to −290 were mutated by introducing a single nucleotide substitution (5′-AGGTG replaced by 5′-AGATG) using conventional PCR-based methods.

Cell Lines and Transfections

To generate A431 clones with the inducible expression of wild-type or mutant SIP1 (Tet-On system), we used a clone of A431 cells expressing Tet-responsive transcriptional activator rtTA (Andersen *et al.*, 2005). Cells were transfected either with the pUHDmyc-SIP1 or pTREmyc-SIP1ZFmut along with the pTK-Hyg vector (BD Bioscience Clontech). Selection of stable clones was carried out in the presence of 60 μg/ml hygromycin B. Clones with concurrent DOX-regulated expression of SIP1 and cyclin D1 were obtained by cotransfecting A431/SIP1 cells with pBIcyclD1 and pPuro (BD Bioscience Clontech; conveys resistance to puromycin), followed by the selection of puromycin-resistant cells in the presence of 0.5 μg/ml puromycin. Transfections of plasmid DNA were performed by electroporation with a single pulse of 250 V and 250 μFd by using the Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Hercules, CA). Established cell lines were cultured in DMEM supplemented with 10% fetal bovine serum with or without DOX (2 μg/ml).

Immunofluorescence

For immunofluorescent staining, cells were grown for 2 d in 10-well glass microscope slides (VWR International, Fontenay-sous-Bois, France). Cells were washed and fixed in acetone/methanol (1:1) solution for 3 min on ice. After rinsing, the slides were incubated with primary antibodies for 1 h at room temperature, rinsed, and incubated with Alexa 488–conjugated rabbit anti-mouse IgG (Pierce, Rockford, IL) for 1 h. The anti-vinculin antibody was from BD Biosciences, Transduction Laboratories. Cells were examined and photographed using a confocal inverted microscope (Axiovert 200M; Zeiss, Oberkochen, Germany). To monitor BrdU incorporation, cells were pulse-labeled with BrdU for 40 min and stained with DAPI and an anti-BrdU antibody (Roche, Mannheim, Germany) according to the protocols supplied with the Detection kit I (Roche). Proportion of BrdU-positive cells was quantified in several microscopic fields and are presented as mean ± SD.

Western Blotting

Proteins (10 or 20 μg) were denatured, separated on 6% or precast 4–20% gradient SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA), and then transferred to Immobilon-P membranes (Millipore, Bedford, MA) by the standard procedure. After protein transfer, blots were incubated in blocking solution with primary antibody at a dilution of 1:1000 (for anti-myc tag antibody, clone 9E10; Santa Cruz Biotechnology, Santa Cruz, CA) and 1:500 (for anti-cyclin D1, anti-p21, anti-p16, anti-p27, and anti-Rb antibodies; Santa Cruz Biotechnology). Immunoreactive proteins were detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ).

cDNA Microarray Analysis

Construction of 20K microarrays, probe labeling, hybridization, and scanning were carried out at the MicroArray Facility of the Flanders Interuniversity Institute for Biotechnology. Changes in spot intensities >1.8 or <0.55 were regarded as significant in this system.

RNA Interference

Purified and annealed synthetic oligonucleotides specific for cyclin D1 or control small interfering RNA (siRNA) were purchased from Ambion (Austin, TX). Target sequence for cyclin D1 was validated previously by the company. Cells ($n = 2 \times 10^6$) were transfected with 0.2 nmol of siRNA by nucleofection technique in buffer V (nucleofection protocol T-20). The nucleofector device and a nucleofection kit were obtained from Amaxa (Köln, Germany) and used in accordance with the manufacturer's recommendations. At 48 h after transfection, cells were harvested, counted, and processed for fluorescence-activated cell sorting (FACS) analysis or Western blotting.

Determination of Cyclin D1 mRNA Stability

Cells were maintained in the presence or absence of DOX for 48 h. Then, actinomycin D (ActD) was added at the concentration of 5 μg/ml for various time periods. Total RNA was isolated, and transcription of *cyclin D1*, 28S gene, and *fosl1* was analyzed by RT-PCR or quantitative real time PCR.

FACS Analysis

A431/SIP1 and A431/SIP1/cyclD1 cells or cells nucleofected with siRNA were grown in the presence or absence of DOX for 48 h, harvested, fixed in 70% ethanol, treated with RNase (1 mg/ml), and stained with propidium iodide (PI; 50 μg/ml). The cellular DNA content was evaluated using FACS flow cytometer.

Three-dimensional Matrigel Invasion Assay

Invasion was analyzed in inverse invasion assay as previously described (McGarry *et al.*, 2004) with minor modifications. A431/SIP1 and A431/SIP1/cyclD1 cells were maintained with or without DOX for 48 h. Cells ($n = 6 \times 10^4$) were seeded on the underside of the polycarbonate filter of a Transwell chamber containing 100 μl of matrigel basement membrane matrix (Becton

Dickinson, Oxford, United Kingdom) diluted 1:1. Cells were allowed to adhere for 3 h and washed by DMEM. Transwell chambers were placed in wells filled with 1 ml of DMEM with or without DOX. In 3 d, cells were fixed in methanol and stained for 1 h in PI solution (10 μg/ml). Optical sections were scanned at 10-μm intervals using the confocal microscope Zeiss Axiovert 200M. To perform statistical analysis of the invasive potential of A431/SIP1 and A431/SIP1/cyclD1 cells, the amount of cells entering matrigel and remaining at the filter was calculated in 12 optical fields. The values were expressed as a percentage of cells that penetrated matrigel.

Cell Adhesion and Transwell Migration Assays

Cell adhesion assay was carried out essentially as previously described (Mejlvang *et al.*, 2007). Ninety-six–well tissue culture plates were coated with 50 μg/ml human fibronectin or 50 μg/ml rat collagen type I (all from BD Biosciences). Cells were allowed to adhere for 15 min. In some experiments, a blocking antibody AIIB2 known to prevent adhesion to both substrates (Brockbank *et al.*, 2005) has been mixed with the cells for 10 min before the assay.

A directed transwell migration assay was performed using 24-well transwell plates containing 8-μm pore-size polycarbonate filters (Corning Costar, Cambridge, MA). Cells ($n = 10^5$) were cultured with or without DOX for 48 h, seeded in culture inserts, and maintained overnight. Adhered cells were allowed to migrate toward gradient of serum used as a chemoattractant in the lower chamber for 2 h. Cells that migrated to the underside of transwell filters were fixed, stained with a Gurr rapid staining kit (BDH, Dagenham, United Kingdom), and counted by bright-field microscopy at a magnification of $\times$200 in four random fields using the ImageJ program.

Nuclear Run-On Assay

Nuclear run-on assay was based on the incorporation of biotin-16-uridine-5′-triphosphate (biotin-16-UTP) in nascent transcripts according to Patrone *et al.* (2000). Briefly, cells were maintained with or without DOX for 48 h. Cells were harvested and consequently resuspended in buffer I (10 mM Tris-Cl, pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl, 150 mM sucrose, 0.5% NP40) or buffer II (10 mM Tris-Cl, pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl, 150 mM sucrose) and centrifuged at 500 $\times$ *g* for 10 min. Nuclei were then resuspended in buffer III (40% glycerol, 50 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$, 0.1 mM EDTA) and quickly frozen.

To perform nuclear run-on reactions, 2×10^6 nuclei were incubated in a reaction buffer (4 mM of each NTP, 200 mM KCl, 20 mM Tris-Cl, pH 8.0, 5 mM $MgCl_2$, 4 mM dithiothreitol, 200 mM sucrose) for 30 min at 29°C and stopped by adding RNase-free DNase I. In some reactions (negative controls), 0.5 mM UTP instead of biotin-16-UTP was used. Total RNA was isolated by TRIzol (Invitrogen) extraction, and biotinylated RNA was purified using agarose-conjugated streptavidin beads. Beads were washed two times with 15% formamide and five times with 2$\times$ SSC. Isolated biotinylated RNA was used for RT-PCR.

Real-time Quantitative PCR

RNA was isolated using TRIazol reagent. cDNA synthesis was carried out using random hexamers and Superscript II (Invitrogen). PCR was performed using SYBR Green PCR Master Mix in the PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Primers were designed to cross exon–exon boundaries and used at the concentration 900 nM. Each sample was run in triplicate. The C_T (threshold cycle when fluorescence intensity exceeds 10 times the SD of the baseline fluorescence) values for the target amplicon and endogenous control (28S) were determined for each sample. Quantification was performed using the comparative C_T method ($\Delta\Delta C_T$).

Luciferase Reporter Assay

To determine transcriptional activity of cyclin D1 reporters, A431/SIP1 cells were transfected with 1 μg reporter constructs. The efficiency of each transfection was monitored using 400 ng cotransfected β-galactosidase expression vector, pCMVβ-gal (BD Biosciences). Cells were maintained with DOX for 48 h and lysed, and the luciferase activity was measured with a Lumat LB9501 tube luminometer (Berthold Detection Systems, Pforzheim, Germany). The luciferase activity was normalized to the activity of β-galactosidase determined using *o*-nitrophenyl-β-D-galactopyranoside (Sigma, Poole, Dorset, United Kingdom) as a chromogenic substrate.

Chromatin Immunoprecipitation Assay

A431/SIP1 cells were cultured for 24 h in the presence or absence of DOX. Cross-linking, immunoprecipitation, and DNA purification were performed using chromatin immunoprecipitation (ChIP)-IT kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. Immunoprecipitated DNA was analyzed by real-time quantitative PCR.

Statistics

Results are expressed as the mean $\pm$ SD. Student's *t* test was used to evaluate the differences between groups.

RESULTS

SIP1-mediated EMT in A431 Cells: Switch from a Proliferative to an Invasive State

To study the physiological effects of SIP1 in carcinoma cells, we generated clones of the squamous carcinoma cell line A431 with the DOX-regulated expression of 6xMyc-tagged SIP1 (clone A431/SIP1). Treatment with DOX resulted in rapid accumulation of SIP1 in 95–98% of nuclei of A431/SIP1 cells, leading to cell scattering and dramatic morphological conversion from an epithelial cell state to a fibroblast-like phenotype. On the other hand, A431 cells expressing 6xMyc-tagged SIP1 with the mutated C-terminal Zn-finger retained entirely polarized epithelial morphology (Figure 1A). Expression of wild-type SIP1 promoted cytoplasmic redistribution of the adherens junctions and tight junction proteins (data not shown). The staining of DOX-treated cells

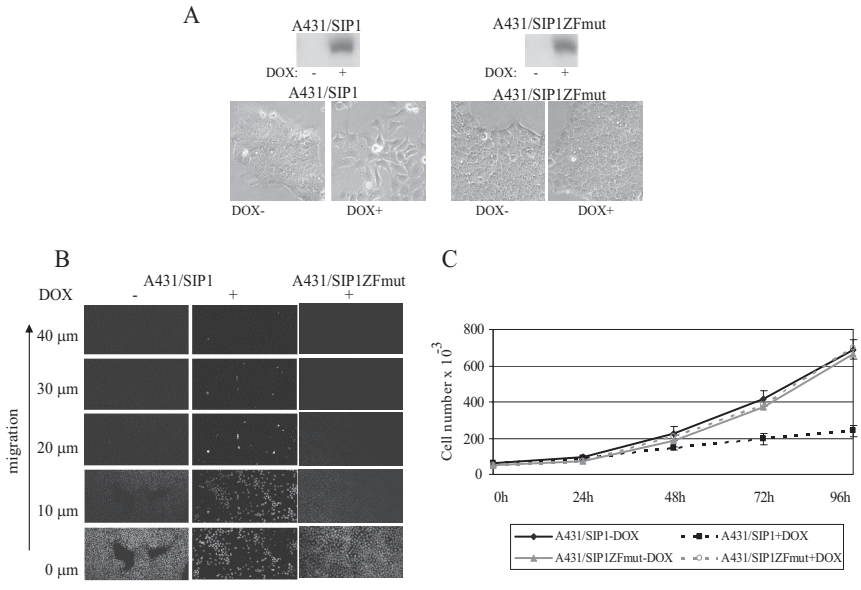

Figure 1. SIP1 induces cell invasion and inhibits cell growth. (A) Characterization of DOX-regulated A431 clones expressing SIP1 or SIP1ZFmut. Phase-contrast images of DOX-inducible A431 cell clones expressing myc-tagged SIP1 or SIP1ZFmut. Western blots show the expression of wild-type or mutant SIP1 in cells maintained with or without DOX for 48 h. (B) Inverse invasion assay of DOX-treated or untreated A431/SIP1 and A431/SIPZFmut cells. Confocal microscope sections of PI-stained cells were used to analyze the invasion assay. The row corresponding to 0 μm shows cells on the underside of the filter. Other sections show cells invaded into matrigel at different distances as indicated. The experiment was repeated four times, and results of a typical experiment are shown. (C) SIP1 inhibits cell growth. A431/SIP1 or A431/SIP1ZFmut cells were cultured with and without DOX, and cell number was counted at different time points as indicated. Experiments were repeated three times with similar results. Data shown are mean $\pm$ SDs of triplicate experiments.

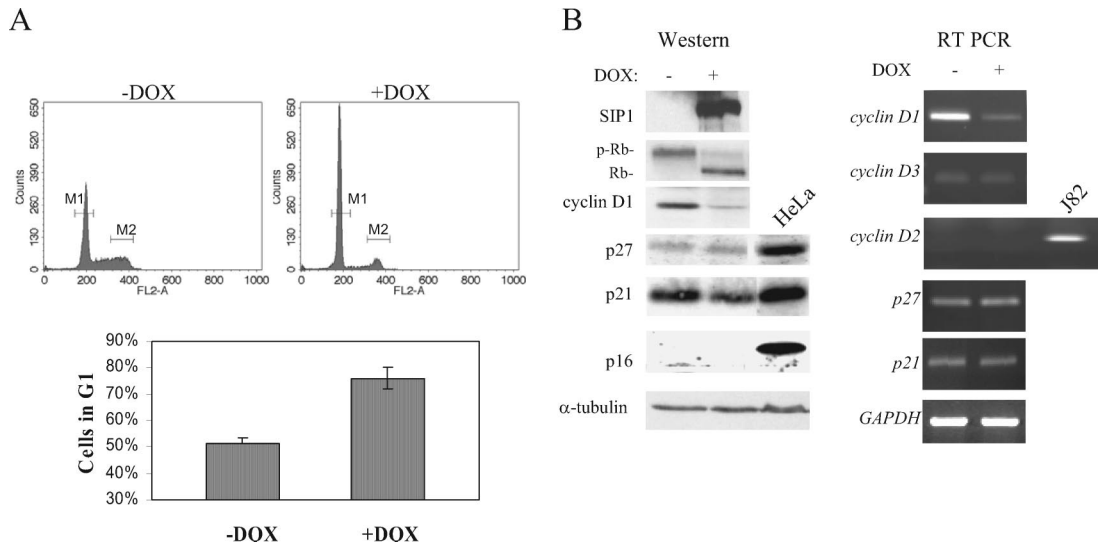

Figure 2. SIP1 negatively regulates G1/S transition. (A) FACS analysis of A431/SIP1 cells maintained in the absence or presence of DOX for 48 h. The diagram shows mean ± SDs of triplicate experiments. $p < 0.0001$ (*t* test). (B) Expression of molecules regulating G1/S transition in A431/SIP1 cells. Cells were cultured with or without DOX for 48 h, and the expression of indicated genes was analyzed by Western blotting or RT-PCR. Note the appearance of the hypophosphorylated form of Rb in DOX-treated cells.

with phalloidin or an anti-vinculin antibody indicated that SIP1-inhibited cell–cell adhesion, but promoted formation of focal adhesions and disappearance of F-actin from intercellular borders (Supplementary Figure S1A). In addition, SIP1-activated cell adhesion to collagen and fibronectin (Supplementary Figure S1B). We used a cDNA microarray technique to obtain a global view on the number of genes regulated by SIP1 in A431 cells. Forty-eight hours of SIP1 induction led to prominent changes in gene expression profiles. We found that out of 20,000 genes analyzed, SIP1 repressed 281 genes by a factor of 0.55 or less and activated 204 genes by a factor of 1.8 or more (corresponds to the ~2.4% of human genome; Supplementary Tables SIA and SIB).

RT-PCR analysis of the selected genes demonstrated the reliability of the microarray hybridization (Supplementary Figure S2). Transcription of all genes tested was not affected in cells expressing SIP1 with the inactivated C-terminal Zn-finger domain. The largest cluster in a group of genes down-regulated by SIP1 contained markers of epithelial differentiation (components of epithelial microfilaments and junctional proteins). Given that essential mesenchymal genes (vimentin, fibronectin, and N-cadherin) were significantly up-regulated by SIP1, we concluded that SIP1 activated a program of EMT in A431 cells. We aimed to examine how the mesenchymal conversion of tumorigenic cells influenced their invasive and proliferative properties. We examined effects of SIP1 on tumor cell invasion in an inverse three-dimensional (3D) in vitro invasion assay. As we expected, in the experimental conditions used in this study, cells maintained without DOX were only minimally invasive. SIP1 induction strongly activated invasion, and in the presence of DOX A431/SIP1 cells penetrated matrigel at the distance of more than 40 μm (Figure 1B).

To analyze the effects of SIP1 on cell growth, we seeded equal amounts of cells on six-well culture plates, maintained them with and without DOX, and counted them in 24, 48, 72, and 96 h. Already after 24 h of DOX-treatment, SIP1 significantly decreased the doubling time of A431 cells ($p < 0.05$; Figure 1C). Consistent with this observation, A431/SIP1 cells incubated with DOX for 48 h incorporated 3.2-fold less BrdU than cells maintained in the absence of DOX (see Figure 3C). As expected, expression of SIP1 with the mutated C-terminal Zinc-finger domain produced no effect on cell proliferation or matrigel invasion (Figure 1, B and C). Taken together, these data demonstrated that SIP1-induced EMT program encompasses a global genetic reprogramming and switch from a proliferative to an invasive type of cell behavior.

Transition into S Phase of the Cell Cycle Is Inhibited by SIP1

Having demonstrated inhibition of cell growth by SIP1, we analyzed the effect of SIP1 on cell cycle distribution. FACS analysis of A431/SIP1 cell cultures maintained with or without DOX for 48 h showed that SIP1 increased proportion of cells in G1 phase (Figure 2A). Percent of cells passing through S phase, G2, and mitosis was two times lower in cells undergoing EMT (24 ± 4 vs. 49 ± 3%). Because G1/S transition in mammalian cell cycle is regulated by Rb pathway and phosphorylation of the Rb protein is critical for G1/S progression, we examined the effect of SIP1 on the Rb phosphorylation. We found that in our system, accumulation of cells in G1 phase of the cell cycle was concomitant with the hypophosphorylation of Rb (Figure 2B). Microarray analysis revealed strong (6.7-fold) down-regulation of the *CCND1* gene, which encodes cyclin D1, a critical regulator of Rb phosphorylation (Supplementary Table SIB). We confirmed SIP1-mediated repression of cyclin D1 on both mRNA and protein levels. Next, we analyzed expression of other key proteins regulating Rb phosphorylation and cell cycle progression through G1 phase (Figure 2B). Whereas the mRNA levels of cyclin D3, p21(Cip1), and p27(Kip1) remained not altered upon SIP1 induction, transcription of cyclin D2 was not detectable independently on the presence of DOX. Western blot analysis demonstrated similar levels of p27(Kip1) and lack of the expression of p16 protein in SIP1-expressing and nonexpressing cells. Unexpectedly, in the presence of SIP1, the expression of p21(Cip1) was

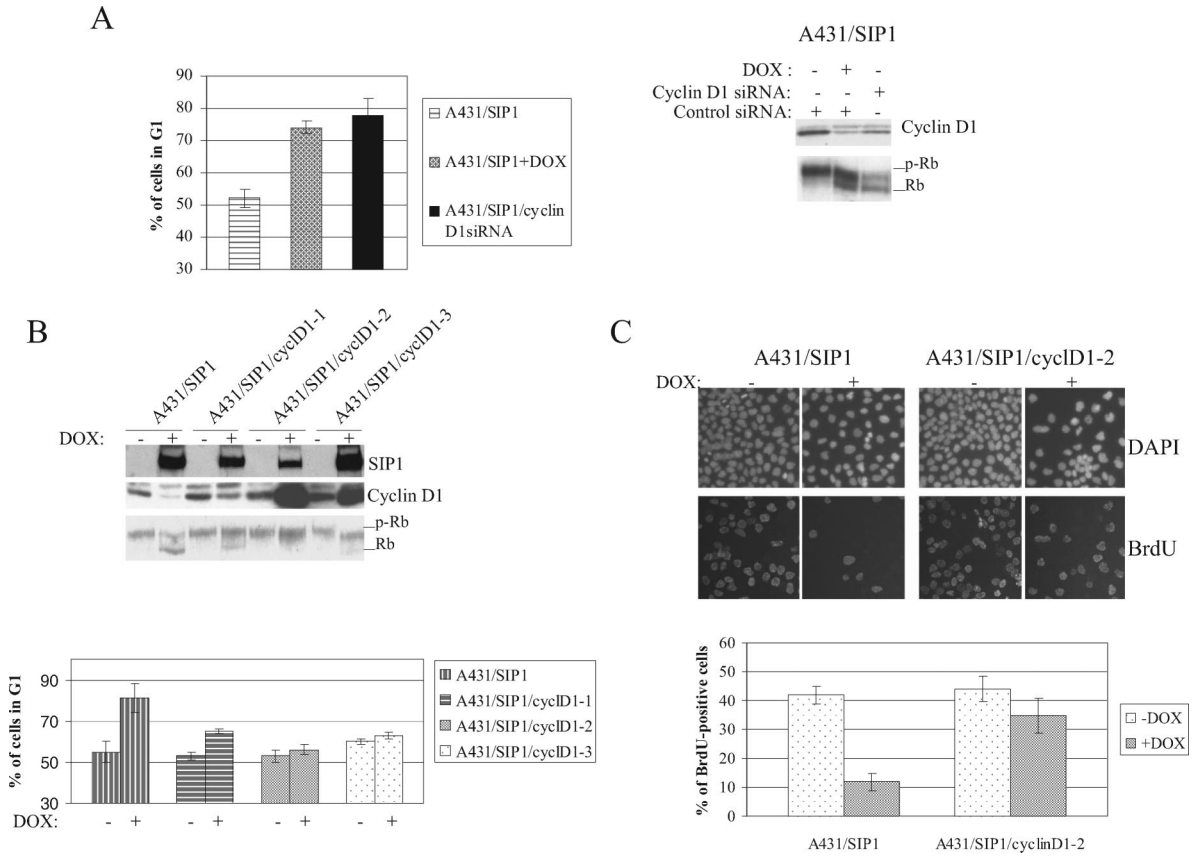

Figure 3. SIP-1 attenuates cell cycle progression via cyclin D1 down-regulation. (A) RNAi-mediated repression of cyclin D1 leads to accumulation of A431/SIP1 cells in G1 phase of the cell cycle. FACS analysis of cell cycle distribution of A431/SIP1 cells, in which cyclin D1 expression was reduced either by SIP1 or by RNAi. Control cells were cultured with or without DOX for 48 h. Results are mean ± SD of three experiments. Right panel, Western blot analysis of cyclin D1 and Rb expression in A431/SIP1 cells transfected with the negative control siRNA or cyclin D1-specific siRNA. 20 μg of total proteins was loaded in each lane. (B) Exogenous cyclin D1 counteracts the effect of SIP1 on Rb phosphorylation and cell cycle distribution. Clones with simultaneous DOX-regulated expression of SIP1 and cyclin D1 were generated and the phosphorylation of Rb was analyzed by Western blotting. Bottom, cells were cultured in the presence or absence of DOX for 48 h, and cell cycle distribution was monitored by FACS. (C) DOX-induced A431/SIP1 and A431/SIP1/cyclinD1-2 cells differently incorporate BrdU after 40-min pulse labeling. After the labeling, BrdU incorporation was detected by fluorescence microscopy with the monoclonal anti-BrdU antibody. Total cell number was identified by blue fluorescence (DAPI DNA staining). Proportion of BrdU-positive cells was quantified in six microscopic fields and presented as mean ± SD. The experiment was repeated twice with similar results.

reduced on protein level, although mRNA level was not affected (Figure 2B).

Cyclin D1 Down-Regulation Is Necessary and Sufficient for SIP1-induced Changes in Cell Cycle Distribution

Cyclin D1 down-regulation correlated with Rb hypophosphorylation and accumulation of the cells in G1 phase of the cell cycle. To analyze whether SIP1 affects cell cycle distribution via cyclin D1, we used two approaches. First, we inhibited endogenous cyclin D1 level in A431/SIP1 cells by RNA interference (RNAi). The reduction in cyclin D1 levels in A431/SIP1 cells caused by siRNA resulted in the accumulation of cells in G1 and Rb hypophosphorylation, resembling the effect of SIP1 (Figure 3A). In parallel experiments, we generated clones of A431/SIP1 cells with simultaneous DOX-regulated expression of SIP1 and cyclin D1 (Figure 3B, clones 1–3). Although in the absence of DOX, all clones retained epithelial phenotype, DOX treatment induced morphological transformation and cell scattering, which was identical to the effect produced by SIP1 in parental A431/SIP1 cells (data not shown). In clone 1, activation of exogenous cyclin D1 resulted in partial suppression of the effect of SIP1 on total cyclin D1 level. In DOX-treated cells of clone 1, Rb hypophosphorylation was partly suppressed, and the proportion of cells retained in G1 dropped from 82 to 62%. In clones 2 and 3, DOX treatment led to the very high cyclin D1 expression significantly exceeding cyclin D1 levels in DOX-untreated A431/SIP1 cells. In these clones, overexpression of cyclin D1 completely blocked Rb hypophosphorylation and abandoned the effect of SIP1 on cell cycle distribution (Figure 3B). Moreover, enforced expression of cyclin D1 bypassed the effect of SIP1 on the level of BrdU incorporation (Figure 3C). Taken together, these data indicate that repression of cyclin D1 is indispensable for the effects of SIP1 on cell cycle distribution.

Ectopic Expression Cyclin D1 Does Not Interfere with the Motile Behavior of SIP1-expressing Cells

Using 3D matrigel invasion assay, we found that cells simultaneously expressing SIP1 and cyclin D1 were at least as invasive as cells expressing SIP1 only (Figure 4A). In addition, we analyzed migratory capabilities of A431/SIP1 cells expressing or nonexpressing exogenous cyclin D1 using a transwell motility assay. SIP1 strongly activated migration

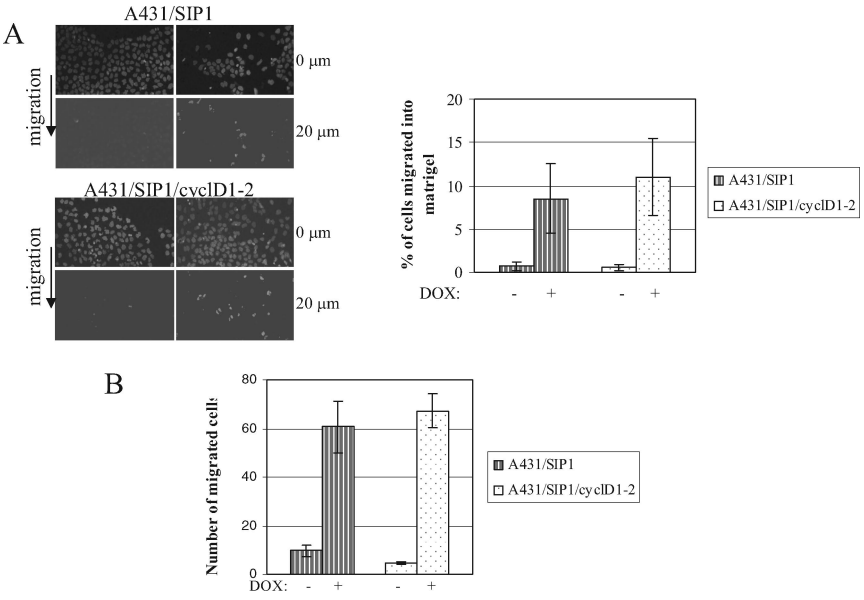

Figure 4. Ectopic expression of cyclin D1 does not influence SIP1-mediated cell invasion and motility. (A) Exogenous cyclin D1 does not compromise SIP1-activated invasion. DOX-treated or untreated A431/SIP1 and A431/SIP1/cyclinD1-2 cells were allowed to invade into matrigel. Cell number on the underside of the filter and at the distance of 20 μm was quantified after staining with PI. The percentage of invaded cells was quantified in twelve microscopic fields. The diagram represents mean ± SD of triplicate experiments. (B) Enforced expression of cyclin D1 does not influence SIP1-activated cell motility. A431/SIP1 and A431/SIP1/cyclinD1-2 cells were maintained with or without DOX for 48 h. Migration was analyzed in transwell motility assay. Bar graphs summarize the results of three separate experiments (mean ± SD).

of cells through the pores of transwell filters in parental A431/SIP1 cells and in cells expressing exogenous cyclin D1 (clone A431/SIP1/cyclD1-2, Figure 4B). Our data show that enforced expression of cyclin D1 uncouples cell cycle effects from key features of SIP1-induced EMT, cell motility, and invasiveness.

SIP1 Directly Regulates Cyclin D1 Promoter Activity

There are two levels controlling cyclin D1 mRNA abundance in mammalian cells. Signaling networks, which coordinate G1/S transition, regulate activity of the cyclin D1 promoter. In addition, regulation of the cyclin D1 mRNA turnover plays an important role in the control of cyclin D1 function (Lin *et al.*, 2000). To explore the possibility that SIP1 activates degradation of cyclin D1 mRNA, A431/SIP cells were treated with DOX for 48 h or left untreated and then incubated with ActD for different time periods. In control experiments, the concurrent treatment of cells with ActD and DOX for 8 h prevented *SIP1* transcription and therefore proved the efficacy of ActD (Figure 5A, right panel). The application of ActD for 4 or 8 h revealed that cyclin D1 mRNA was very stable in DOX-treated and untreated A431/SIP1 cells compared with the stability of *fosl1* or *SIP1* mRNA (Figure 5A). To quantify the effects of SIP1 on cyclin D1 mRNA stability more accurately, we applied real-time PCR. The difference in the effects of 4 h ActD treatment on cyclin D1 mRNA stability in cells maintained with or without DOX was not statistically significant ($p = 0.3695$; $n = 5$; Figure 5B). To examine whether SIP1 regulates the transcription rate of cyclin D1, we carried out nuclear run-on assay with nuclei prepared from DOX-treated or untreated cells. Biotin-labeled UTP was incorporated into nascent transcripts, and after the transcriptional reaction was completed, newly synthesized RNA was affinity-purified and subjected to RT-PCR analysis.

With three primer sets (a scheme in Figure 5C), we demonstrated that SIP1 drastically inhibited the transcription rate of the cyclin D1 gene. In contrast, transcription of a SIP1-up–regulated gene, *prss11* was much more efficient in nuclei isolated from DOX-treated cells. In all control reactions, in which nonlabeled UTP was used, no PCR product was detected (data not shown). From these experiments, we concluded that repression of cyclin D1 promoter rather than mRNA destabilization is responsible for cyclin D1 inhibition in course of SIP1-mediated EMT. To directly address this issue, we analyzed the in vivo binding of SIP1 to potential SIP1-binding sites (Z-boxes) located in the vicinity of the cyclin D1 transcription start site. We carried out ChIP assays in A431/SIP1 cells maintained with or without DOX for 24 h. After formaldehyde cross-linking, chromatin physically associated with SIP1 was pulled-down, and cyclin D1 promoter fragments enriched in SIP1-containing chromatin fraction were identified by quantitative PCR. As negative control a preimmune serum was used. Data indicated that three Z-boxes with coordinates −1014 to −1010 (Z-box 1); −857 to −853 (Z-box 2); and −300 to −290 (Z-box 3) are occupied by SIP1 in DOX-stimulated cells. In contrast, neither sequences upstream of Z-box 1 (Figure 6A), nor sequences containing Z-boxes 4 and 5 located at the first exon/intron boundary (+390 to +409) (data not shown) were detected in association with SIP1. Next, we aimed to test whether the physical binding of SIP1 to Z boxes 1–3 resulted in the repression of cyclin D1 promoter activity. Two luciferase reporters were generated. A wild-type reporter (p*wt*CCND1LUC) contained the −1025 to +18 cyclin D1 promoter sequence cloned upstream of the firefly luciferase gene. The second reporter (p*mut*CCND1LUC) had the same structure but with Z-boxes 1–3 inactivated by a single nucleotide substitution converting 5′-AGGTG to 5′-AGATG. This substitution has been previously shown to block binding of SIP1 to DNA (Remacle *et al.*, 1999). Transient transfection experiments demonstrated that the mutation of Z-boxes 1–3 markedly activated reporter activity in SIP1-expressing cells (Figure 6B). Taken together with the results of ChIP analysis, these data indicate that SIP1 represses cyclin D1 transcriptional activity via direct interaction with Z-boxes 1–3 in the cyclin D1 promoter.

An E-Cadherin Dominant Negative Mutant Induces EMT But Does Not Influence Cell Cycle Progression in A431 Cells

EMT programs encompass deep reorganization of the cytoskeleton and modulation of cell adhesion. Significant body of evidence implicates integrins, cadherins and cytoskeletal

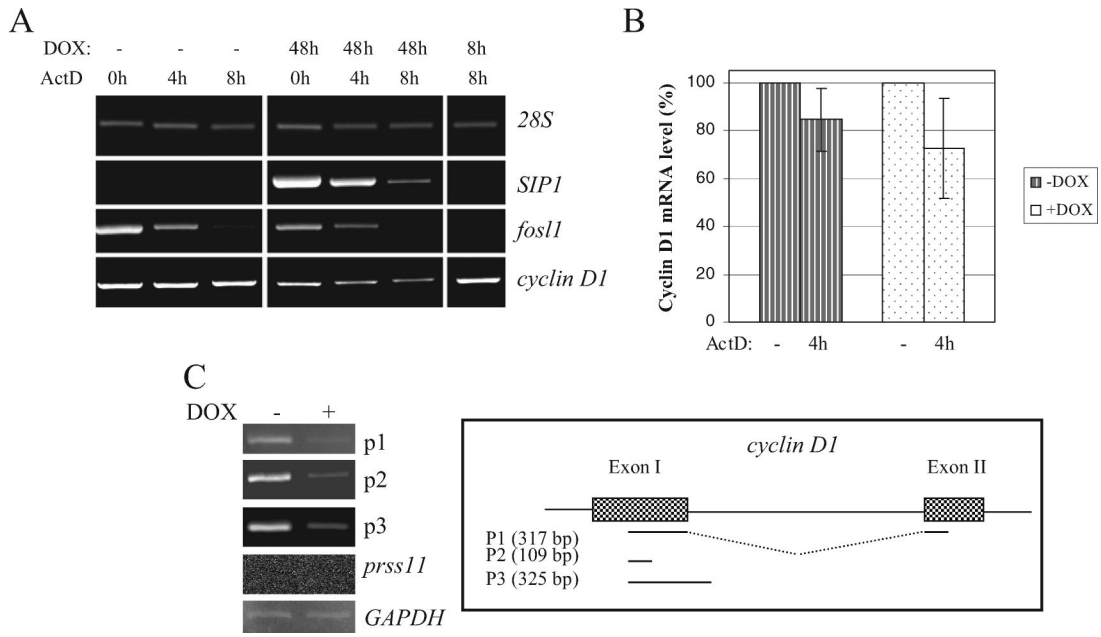

Figure 5. SIP1 regulates cyclin D1 promoter activity rather than the stability of cyclin D1 mRNA. (A) A431/SIP1 cells were maintained with or without DOX for 48 h. ActD was added for 4 or 8 h and expression of cyclin D1, SIP1, and *fosl1* was analyzed by RT-PCR. 28S rRNA was used as an ActD-insensitive control. Right panel, DOX and ActD were added simultaneously, and transcription of cyclin D1, SIP1, *fosl1,* and 28S rRNA was analyzed by RT-PCR 8 h later. (B) Quantification of cyclin D1 mRNA levels in A431/SIP1 cells maintained with or without DOX using real time PCR. Cells were treated with ActD for 4 h, and amplification was performed in triplicate. Data are presented as the mean ± SD. (C) Nuclei were isolated from A431/SIP1 cells maintained with or without DOX for 48 h and subjected to nuclear run-on assay. Isolation of nuclei, conditions of the reaction, and purification of biotinylated uridine-containing transcripts are described in *Materials and Methods*. Isolated transcripts corresponding to GAPDH, *prss11,* and cyclin D1 genes were amplified by RT-PCR and analyzed in an agarose gel. The scheme depicts fragments of the cyclin D1 mRNA detected by RT-PCR. Results are representative of two independent experiments.

tensions in the control of cell cycle (Walker and Assoian, 2005; Walker *et al.,* 2005). However, given that the effect of SIP1 on cyclin D1 expression was direct, cell cycle regulation might be not affected in course of EMT programs, which do not involve SIP1. To test this, we used a recently generated model of EMT based on the expression of a dominant negative E-cadherin mutant (Ec1WVM) in A431 cells (Andersen *et al.,* 2005). This mutant harbors a Trp^2/Ala amino acid substitution in the first cadherin-like repeat, leading to an inability of the mutant protein to form *trans*-dimers. Forty-eight hours of Ec1WVM expression induced cell scattering and activated cell invasiveness (Figure 7A). Prolonged expression of Ec1WVM resulted in activation of vimentin, down-regulation of cytokeratins, and further increase in cell motility (Andersen *et al.,* 2005). However, neither long-term (data not shown), nor short-term Ec1WVM expression (Figure 7B) inhibited G1/S phase transition in A431 cells. In agreement with these data, we observed no effects on Rb phosphorylation or cyclin D1 expression in cells undergoing EMT in response to Ec1WVM (Figure 7B). These data indicate that in different EMT models, the G1/S transition depends on the nature of EMT-inducing signals.

DISCUSSION

Direct repression of *e-cadherin* transcription by Snail/Slug and ZEB-1/SIP1 proteins demonstrated in several epithelial cell lines is highly relevant to EMT and epithelial tumorigenesis. However, the functions of Snail/Slug and ZEB-1/SIP1 are not restricted to the repression of the *e-cadherin* gene. A number of genes encoding components of different epithelial intercellular adhesive complexes are directly or indirectly repressed by Snail, Slug, ZEB-1, or SIP1 (Ohkubo and Ozawa, 2004; De Craene *et al.,* 2005b; Vandewalle *et al.,* 2005; Moreno-Bueno *et al.,* 2006; Aigner *et al.,* 2007; Supplementary Table SIB and this article). Moreover, expression of exogenous E-cadherin in MDCK/Snail or DLD/Snail cells was unable to restore epithelial differentiation or to inhibit Snail-induced invasion (Ohkubo and Ozawa, 2004; De Craene *et al.,* 2005b). Similarly, we found that ectopic expression of E-cadherin in A431/SIP1 cells did not revert EMT initiated by SIP1 induction (data not shown). These data suggest that Snail/Slug and ZEB-1/SIP1 proteins do not act through transcriptional repression of *e-cadherin*, but rather orchestrate EMT programs via independent and coordinated repression of multiple genes controlling epithelial features and by activation of mesenchymal genes.

In addition to the activation of canonical well-described EMT-related processes (cell dissociation, cell motility and invasiveness, global changes in gene expression pattern), SIP1 significantly stimulated adhesion of A431 cells to fibronectin and collagen I (Supplementary Figure S1B). In contrast, Slug inhibited adhesion of human epidermal keratinocytes to fibronectin and laminin-5 as a result of transcriptional repression of genes coding for $\alpha 3$, $\beta 1$, and $\beta 4$ integrin subunits (Turner *et al.,* 2006). In A431 cells, transcription of these genes was not affected by SIP1 (data not shown), and the mechanism by which SIP1 activated cell–matrix adhesion remains unclear. However, results reported by Turner *et al.* and our data represent a rare example of a cell feature oppositely regulated by different Snail/Slug and ZEB-1/SIP1 proteins in two cell lines of common (epidermal) origin.

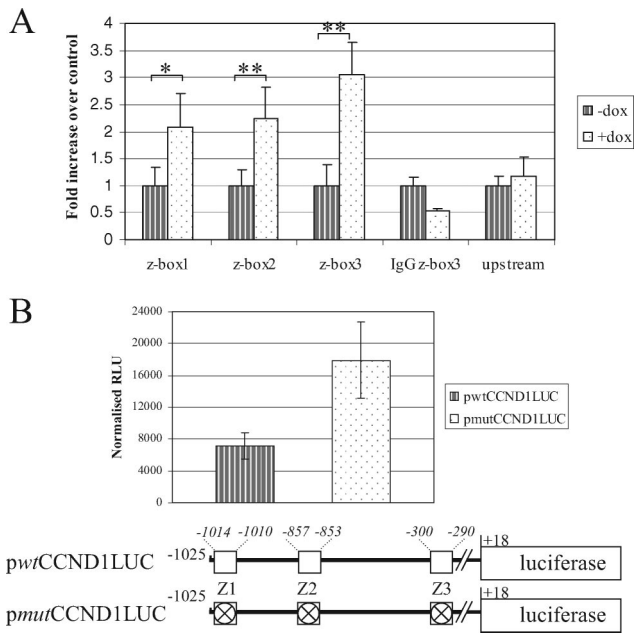

Figure 6. SIP1 directly regulates transcription of the cyclin D1 gene. (A) SIP1 interacts with three Z-box sequences in the cyclin D1 promoter in vivo. ChIP analysis of cyclin D1 promoter sequences in DOX-treated or untreated cells with the 9E10 antibody. As control an irrelevant antibody was used. Enrichment of SIP1-bound sequences was quantified by real-time PCR. Mean ± SD are shown. n = 9; *p < 0.01; **p < 0.001 (*t* test). (B) Luciferase reporter constructs p*wt*CCND1LUC and p*mut*CCND1LUC were transfected into A431/SIP1 cells. Cells were cultured in the presence of DOX for 48 h. Assays were carried out in triplicate, and four independent experiments were performed; a representative result is shown (mean ± SD, n = 3). p < 0.05 (*t* test).

Snail/Slug and ZEB-1/SIP1 family members control distinct EMT programs that are implicated in many aspects of embryonic development, gastrulation, somitogenesis, and neural crest migration. It is therefore plausible to speculate that cancer cells recapitulate some elements of concealed embryonic differentiation programs to acquire metastatic capabilities. Given that normal differentiating cells do not proliferate, the intriguing question arises as to whether the EMT programs affect cell proliferation in cancer as well. However, to our knowledge, this issue has not been scrupulously addressed. In an important study by Vega *et al.* (2004) the expression of Snail has been shown to induce accumulation of MDCK cells in the G1 phase of the cell cycle. In addition, Vega *et al.* demonstrated that Snail inhibited phosphorylation of Rb, lowered expression of cyclins D2 and D1 and increased expression of p21(Cip1). Cyclin D2 has been shown to be a direct Snail target. However, the role of particular cell cycle regulators has not been addressed in this study. Here, we analyzed how an EMT program initiated by the expression of SIP1 affects cell cycle progression. We found that although SIP1 belongs to a protein family only distantly related to Snail/Slug, its effect on cell cycle distribution in human epidermoid A431 cells is similar to the effects of Snail in MDCK cells and Slug in normal keratinocytes (Turner *et al.*, 2006). Moreover, in the present study, we demonstrated the essential role of cyclin D1 whose direct transcriptional repression by SIP1 was necessary and sufficient to affect Rb phosphorylation status and to inhibit progression through G1 into S phase in A431 cells. Taken together, these observations indicate that targeting G1/S checkpoint is a common feature of different EMT-inducing transcription factors in different cell lines, although the actual mechanisms of this targeting might be different.

Immunohistochemical data on the expression of Snail/Slug and especially ZEB-1/SIP1 family members in tumor tissue are limited. A proportion of ZEB1-positive tumors has been identified by immunohistochemical analysis of aggressive endometrial and non-small lung cancer specimens (Dohadwala *et al.*, 2006; Spoelstra *et al.*, 2006). In oral squamous cell carcinoma, SIP1 was detected in 27% of tumor specimens. SIP1 expression correlated with lack of E-cadherin immunoreactivity and low disease-specific survival (Maeda *et al.*, 2005). Similarly, Zhou *et al.* (2004) described extended E-cadherin-negative and Snail-positive areas in breast cancer surgical specimens, and this pattern significantly correlated with cancer metastasis. In another study, only a limited number of single Snail-positive cells has been detected at the periphery of tumor tissue in cervical squamous carcinoma and colon adenocarcinoma (Franci *et al.*, 2006). Studies on EMT of MDCK cells (Vega *et al.*, 2004) and data presented here suggest that cells maintaining control over G1/S transition and undergoing a rapid EMT in response to Snail or SIP1 acquire a growth disadvantage. Therefore, the functional status of the Rb pathway may determine the configuration of EMT programs utilized by cells of growing tumors. In carcinoma cells maintaining partial control over G1/S restriction point, members of the

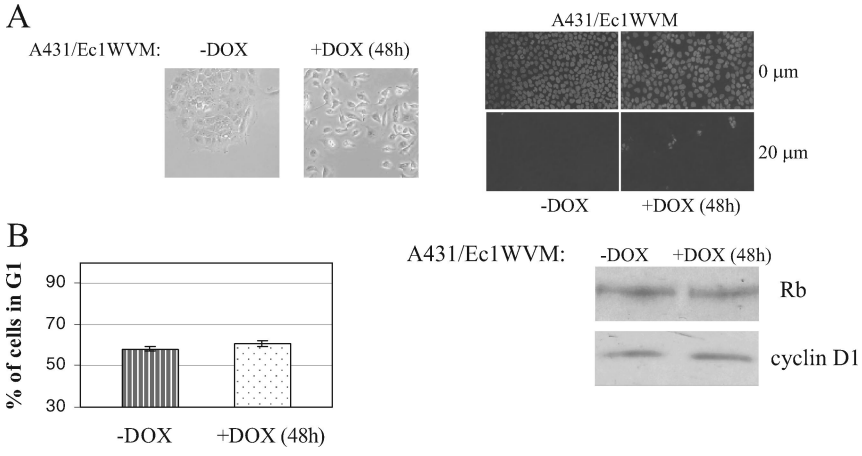

Figure 7. E-cadherin dominant negative mutant (Ec1WVM) induces invasion without affecting cell cycle. (A) DOX-activated expression of myc-tagged Ec1WVM in A431 cells (clone 31D6) induces cell scattering and invasion. Phase-contrast images of 31D6 cells treated with DOX for 48 h or left untreated. Right panel, results of a typical matrigel invasion assay of 31D6 cells. (B) FACS analysis of 31D6 cells cultured in the absence or presence of DOX for 48 h. Results are means ± SD of three experiments. Right panel, Western blot analysis of cyclin D1 and Rb expression levels in DOX-treated (48 h) and untreated A431/Ec1WVM cells.

SIP1 and Snail protein families may induce a transient EMT, which will contribute to metastatic dissemination without stable repression of epithelial markers (e.g., E-cadherin) in primary tumors. This hypothesis may explain why complete EMTs are relatively rarely observed in human cancers (Christofori, 2006). One of the events perturbing the Rb pathway is overexpression of cyclin D1 that is frequently associated with carcinomas in humans (in part, as a result of amplification of the cyclin D1 gene; Malumbres and Barbacid, 2001; Knudsen *et al.*, 2006). Concurrent expression of cyclin D1 and SIP1 in A431 cell line generated cells, which were capable of proliferating and invading into matrigel at the same time (Figures 3 and 4). We suggest that accumulated defects in the Rb pathway in vivo would permit a stable EMT, resulting in the appearance of most aggressive tumor cell variants.

In contrast to the SIP1 model, functional inhibition of E-cadherin by a dominant negative E-cadherin mutant induces a gradual EMT in A431 cells without attenuating the cell cycle (Figure 7). Therefore, prolonged inactivation of epithelial adhesion by matrix metalloproteinases secreted by stroma cells or *e-cadherin* gene mutations may represent a mechanism of a stable EMT in tumor cells retaining partial control over G1/S transition.

In conclusion, we have demonstrated that cyclin D1 is a new direct transcriptional target of SIP1. Taken together with previously published results (Vega *et al.*, 2004; Turner *et al.*, 2006), our data suggest that attenuated G1/S phase cell cycle transition is a common feature of EMT programs induced by Snail/Slug and ZEB-1/SIP1 proteins.

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