SUPPLEMENTARY INFORMATION

for

ZEB1 promotes invasiveness of colorectal carcinoma cells through the opposing regulation of uPA and PAI-1

Ester Sánchez-Tilló \(^1,2\) *, Oriol de Barrios \(^1\) *, Laura Siles \(^1\), Pier G. Amendola \(^1\), Douglas S. Darling \(^3\), Miriam Cuatrecasas \(^4\), Antoni Castells \(^2,5\), Antonio Postigo \(^1,2,6,7\) †

\(^1\) Group of Transcriptional Regulation of Gene Expression, Dept. of Oncology and Hematology, IDIBAPS, 08036 Barcelona, Spain
\(^2\) CIBERehd (Pancreatic and Gastrointestinal Team), IDIBAPS, 08036 Barcelona, Spain
\(^3\) Dept. of Oral Health and Center for Genetics and Molecular Medicine, University of Louisville, KY 40202
\(^4\) Dept. of Pathology (CDB) and Tumor Bank. IDIBAPS-Hospital Clinic, 08036 Barcelona, Spain.
\(^5\) Institute of Digestive and Metabolic Diseases, Hospital Clinic, 08036 Barcelona, Spain
\(^6\) James Graham Brown Cancer Center. Louisville, KY 40202
\(^7\) ICREA. 08010 Barcelona, Spain

* These authors contributed equally to the work

† Corresponding author: idib412@clinic.ub.es
SUPPLEMENTARY MATERIALS AND METHODS

Cell culture

Cells were cultured in Dulbecco’s modified Eagle medium (DMEM) (Lonza) or RPMI (Lonza) supplemented with 10% FCS (Sigma). L-cells stably carrying an expression vector for mouse Wnt3a (L-Wnt3a cells) or its corresponding empty vector (L-Ctl cells) were cultured in the presence 0.4 mg/ml of geneticin (G418 disulfate, Fisher Scientific). Conditioned media from L-Ctl and L-Wnt3a cells was collected in the absence of geneticin and, where so denoted, added to cells during 48 h. Where indicated, cells were treated with Leptomycin-B (LMB) (Sigma, 10 ng/ml, 30 min), recombinant human Wnt3a (R&D Systems, 100 ng/ml for 24 h in the case of HCT116 cells or 48 h in SW480 cells) or amiloride (Sigma, 100 µM, 24 h).

Cell transfections

Cells were transfected with plasmids and siRNA oligonucleotides using Lipofectamine 2000 (Life Technologies) or, when exclusively transfected with siRNA oligonucleotides, using Lipofectamine RNAiMAX (Life Technologies) as described in (1). After 48 h (by default, or at the time indicated in specific experiments), cells were processed for protein, mRNA or transcriptional analyses. SW480 cells were also stably infected with lentiviral particles encoding shRNAs (see below) followed by selection in puromycin (10 µg/ml)-containing media as described below. In some experiments, the resulting pooled stables were subsequently stably transfected with pCI-neo (CMV empty expression vector) (Promega Corp.) or pCI-neo-uPA (with the cDNA encoding human uPA) followed by selection in geneticin (0.7 mg/ml)-containing media.

Antibodies

Antibodies used in this article originated as follows: ZEB1 [H-102, E-20, and E-20-X from Santa Cruz Biotechnology (SCBT) and Zhep as in (2)], uPA (3689/HD-UK1 from American Diagnostica and H-140 from SCBT), PAI-1 (H-135 from SCBT), ß-cat (Ab6302 from Abcam, C2206 from Sigma), and α-tubulin (B5-1-2 from Sigma). Secondary antibodies were obtained from Jackson ImmunoResearch (JIR), namely, horseradish peroxidase (HRP)-conjugated donkey antimouse IgG, HRP-conjugated goat-anti-rabbit IgG, Donkey Dylight™ 488-anti-rabbit IgG, Donkey Cy3™ anti-goat IgG, Donkey Dy-light™ 649 anti-mouse IgG, and Donkey Rho RedX™ anti-mouse IgG. For blocking in immunostaining and as control for ChIP assays, normal donkey Ig G (JIR) or normal goat Ig G (JIR, 5–8 mg/mL of IgG in normal serum), respectively, were used.

Plasmids

ZEB1 and ZEB1_NTH expression vectors have been previously described (1). Other expression vectors were obtained from the following researchers: ß-cat from J. Woodgett (Mount Sinai Hospital, Canada), uPA from GR Nemerow and S Huang (The Scripps Research Institute, La Jolla, CA) (3), CMV-p300-VP16AD from D Livingston (Dana Farber Cancer Institute, Boston, MA). Firefly luciferase reporters for the promoters tested here originated as follows: -1.94 kb of the human uPA from A Varro (University of Liverpool, Liverpool, UK) (4), -0.8 and -3.4 kb of human PAI-1 promoter from DE Vaughan (North Western University, Chicago) (5). pCI-neo was purchased from Promega, CMV-ß-galactosidase from Clontech, and pBluescript SK from Stratagene-Agilent.
siRNAs and shRNAs

siRNAs were ordered from Invitrogen or Integrated DNA Technologies (IDT) using sequences reported elsewhere: two different siRNAs against human ZEB1 [siZEB1 as described in (6) (5'-UGAUCAGCCUCAAUC UGCA-3') and si2ZEB1 as in (7) (5'-AACUG AACCUGUGGAUUA-3')] and β-catenin (8). As negative control, we used a scramble control siRNA (5'-GGUUACGAACUAAGC UAUA-3') as well as (in Western blot/qRT-PCR studies) a siRNA against firefly luciferase as described in (9). Lentiviral particles encoding shRNAs against human ZEB1, and whose target sequences are different from those in si1ZEB1 and si2ZEB1, consisted of a pool of three shRNAs (referred as shZEB1) (5'-GAAGCAG GAUGUACAGUAA-3', 5'-GGCGAUAGAUG GUAAUGUA-3', 5'-CCAGAACAGUGUUUA UUCU-3') were purchased from SCBT (sc-38643-V). Lentiviral particles for a control shRNA (also different from the siRNA control) were also obtained from SCBT (sc-108080-V).

Western Blot assays

Western blot assays were performed as previously described (1). Briefly, cells were lysed in RIPA buffer (150 mM NaCl, 1% NP40, 0.5% SDS, 50 mM Tris pH 8, 2 mM EDTA plus protease inhibitors) and loaded onto 8% or 12% polyacrylamide gels. Gels were then transferred to a PVDF membrane (Immobilon-P, Millipore). Following blocking for nonspecific antibody binding with 5% nonfat milk, membranes were incubated with the corresponding primary and HRP-conjugated secondary antibodies before the reaction was developed using Pierce’s ECL Western Blotting Substrate or SuperSignal West Pico Chemiluminescent Substrate (Pierce-Thermo Fisher Scientific). Western blots shown in the article are representative of at least three independent experiments.

RNA Extraction and Quantitative Real-Time PCR

Total RNA extracted with Trizol reagent (MRC Inc.) followed by RNase free DNase I treatment (Promega) or SV Total RNA Isolation System (Promega) was used to synthesize cDNA with random hexamers/oligodT with a reverse transcription kit (GoScript, Promega or iScript™ Reverse Transcription, BioRad) according to manufacturer’s instructions. mRNA levels were determined by quantitative real-time PCR (qRT-PCR) at 60°C using either SYBR Green/ROX (GoTaq, Promega) or SYBR®Green (Biorad) and oligonucleotide primer sequences previously described in the literature: human ZEB1 (10), human E-cadherin (11), human uPA (12), human PAI-1 (13), human GAPDH (14), human β-actin (15), mouse uPA (16), mouse Id2 (17), mouse β-actin (18) or mouse GAPDH (19). Relative mRNA levels were determined by Opticon Monitor 3.1.32 software (BioRad) by ΔCt method normalizing values of genes under study to housekeeping GAPDH as reference gene. qRT-PCR data shown in the study are the average of at least three independent experiments performed in triplicate.

Site directed mutagenesis

Identification of ZEB1 consensus binding sequences was conducted using MacVector software. ZEB1 consensus binding sites at positions −1624 bp and −1480 bp in the human uPA promoter were mutated to sequences known to not bind ZEB1 using the QuickChange Lightning site-directed mutagenesis kit (Agilent) as per manufacturer’s instructions. Complementary mutant primers to opposite strands of the target DNA were purchased from IDT. For the ZEB1 binding site at −1624 bp of human uPA promoter, the upper strand oligonucleotide sequence used is 5’-TCTCCA GAAGACAGTGGGCTATTGCCTCCAAA AGCTGAAAGGC-3’. For the ZEB1 binding site at −1480 bp of the human uPA promoter, the
upper strand oligonucleotide sequence used is 5’-GCCTTCCCTTCTCTCTCTAATGGACCCAGACCCAAGGTCCAG-3’. For the ZEB1 binding site at 230 bp of human uPA promoter, the upper strand oligonucleotide sequence used is 5’-CACTGGGGCAGGCCCCCGCCTATTGCATGGGAGGAAGCACGGAG-3’. All three mutations were confirmed by sequencing with BigDye® Direct Cycle Sequencing Kit (Life Technologies-Applied Biosystems) with the following primers: 5’-TGGGAGTTTCGGGGTAAGTCCTC-3’ and 5’-TAACTTGTACTTTCGCCAGCAGG-3’, and 5’-GGTCTGAGGCAGTCTTAGGCAAGTTGG-3’, respectively.

Transcriptional Assays

In transcriptional experiments, cells were transfected with firefly luciferase reporter vectors and equal molar amounts of either cDNA-containing or reference control empty expression vectors. As internal control for transfection efficiency, all points included cotransfection with 0.5 µg pCMV-β-gal. Total DNA was brought to the same amount by adding promoterless pBluescript SK vector. Firefly luciferase activity was assessed with a Luciferase Assay System kit (Promega Corp.), whereas β-galactosidase was determined with Luminiscent β-galactosidase Detection Kit II (Clontech). Relative luciferase activity values (RLU) throughout the article are expressed as the mean of duplicate and are representative of at least four independent experiments.

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation (ChIP) assays were performed using EpiQuick ChIP kit (Epigentek) as per manufacturer’s instructions. Briefly, SW480 cells were incubated during 10 min with 1% formaldehyde solution (Electron Microscopy) at room temperature followed by incubation with 125 mM glycine. Lysates were sonicated as described (20). Antibodies used for ChIP were as follows: goat anti-ZEB1 (E-20X) and normal goat IgG (JIR). DNA fragments were quantified by qRT-PCR as detailed above. Identification of DNA binding sequences for ZEB1 and design of primers for qRT-PCR was conducted using MacVector software. For the ZEB1 binding site at position -1624 bp of the human uPA promoter, the primers used were as follows: forward 5’-CAGCAAGCAGAAGCTCTCCAG-3’ and reverse 5’-GAGTGACAGAAGGAAGGCAGGGAAGGCAGG-3’. For the ZEB1 binding site at position -1480 bp of the human uPA promoter, the primers used were as follows: forward 5’-TTTCTCTCTCTCTACCTTTGAGG-3’ and reverse 5’-TTTCTGTACGCCGACCATCTCTTTTC-3’. For the ZEB1 binding site at position -230 bp of the human uPA promoter the primers used were as follows: forward 5’-CAGGACCCAGGCTCATCTTCG-3’ and reverse 5’-GAGTGACAGAAGGAAGGCAGG-3’. Identification of DNA binding sequences for ZEB1 and design of primers for qRT-PCR was conducted using MacVector software. For the ZEB1 binding site at position -1624 bp of the human uPA promoter, the primers used were as follows: forward 5’-CAGCAAGCAGAAGCTCTCCAG-3’ and reverse 5’-GAGTGACAGAAGGAAGGCAGGGAAGGCAGG-3’. For the ZEB1 binding site at position -1480 bp of the human uPA promoter, the primers used were as follows: forward 5’-TTTCTCTCTCTCTACCTTTGAGG-3’ and reverse 5’-TTTCTGTACGCCGACCATCTCTTTTC-3’. For the ZEB1 binding site at position -230 bp of the human uPA promoter the primers used were as follows: forward 5’-CAGGACCCAGGCTCATCTTCG-3’ and reverse 5’-GAGTGACAGAAGGAAGGCAGG-3’.

CRC cell migration and invasion assays

6.5 mm diameter/8 µm pore polycarbonate membrane Transwell™ inserts (Costar, Corning Inc.) were used directly for migration assays. For invasion assays, these Transwell inserts were first coated overnight with a 1/8 dilution of Matrigel™ matrix (BD Biosciences) in DMEM. Next, 1 x 10^5 cells in 100 µl of DMEM were added to Matrigel-Transwell inserts that were then placed on 24-well plates containing 650 µl of DMEM supplemented with 10% FCS. After incubation at 37°C during 6 h for migration or 24 h for invasion assays, cells on the upper
surface of inserts were removed by washing with PBS and a cotton swab. Migration or invasive cells on the lower side of inserts were detached by incubation (30 min, 37°C) with Trypsin-EDTA, collected and counted by staining with Trypan blue. Results shown are the average of four independent experiments with triplicates of each condition, and cells counted thrice.

**Immunohistochemistry and immunofluorescence analysis**

Tissue samples were first immunostained by the horseradish peroxidase and 3, 3’-diaminobenzidine (DAB) method and, where indicated, processed for multiplex immunofluorescence analysis. Antigen retrieval was performed with 10 mM sodium citrate (pH 6.0). Slides for immunohistochemistry were then treated with 0.3% H2O2 in methanol to block endogenous peroxidase, whereas slides for immunofluorescence staining were first incubated for 30 min with 0.1% sodium borohydride. In either case, slides were next incubated with a non-specific binding blocking solution at 37°C (5% donkey normal serum, 4% BSA and 0.5% Tween 20 in PBS) followed by the corresponding primary (overnight at 4°C) and HRP-conjugated or fluorochrome-conjugated secondary (1 h at 37 °C) antibodies. The immunohistochemistry reaction was developed with a DAB substrate Kit (Vector Labs) before slides were counterstained with hematoxylin and mounted in Di-N-butylPhthalate in Xylene solution (DPX, Sigma). The number of positively stained tumor cells in the CRC tissue array was scored by microscopic analysis at 400X magnification according to the following scale: 0 (0-10 positively stained cells), 1 (11-20), 2 (21-30), 3 (31-40), 4 (41-50), 5 (over 50). Slides for immunofluorescence were mounted in Vectashield-DAPI (Vector Labs). Immunofluorescence was examined in a TCS SP5 Spectral confocal microscope (Leica) at the Microscopy Unit of the University of Barcelona, whose staff is here acknowledged for their technical advice. Images were analyzed using ImageJ software applying a median filter of 0.5–1 pixels. We are also grateful to E Jimenez for help in assembly of immunohistochemistry figures.

**LEGENDS TO SUPPLEMENTARY FIGURES**

**Supplementary Figure S1**

(A) Expression of ZEB1 associates to uPA in CRC cells. Relative mRNA expression for ZEB1, E-cadherin and uPA were determined in SW480 and HCT116 cells by qRT-PCR respect to GAPDH as reference gene. (B) Stable knockdown of ZEB1 downregulates uPA protein. SW480 cells were stably infected with lentiviral particles encoding shRNAs against ZEB1 (shZEB1) or a control shRNA (shCtl). Cell lysates were immunoblotted for uPA (3689/HD-UK1) or ZEB1 (H-102) along α-tubulin (B5-1-2) as loading control. (C) Stable downregulation of ZEB1 upregulates PAI-1 protein. As in (B), but with cell lysates immunoblotted for PAI-1 (H-135). (D) Transient knockdown of ZEB1 inhibits uPA mRNA expression in CRC Colo320 cells. Colo320 cells were transiently transfected with 100 nM of a siRNA control (siCtl) or two specific siRNAs for ZEB1 (si1ZEB1 and si2ZEB1) and relative mRNA levels for uPA and ZEB1 determined by qRT-PCR respect to GAPDH. (E) Stable downregulation of ZEB1 differentially controls uPA and PAI-1 mRNAs. SW480 cells stably interfered with a shRNA control (shCtl) or against ZEB1 (shZEB1) and assessed for mRNA levels by qRT-PCR as described in Supplementary Materials and Methods. (F) Upregulation of endogenous ZEB1 following nuclear translocation of β-catenin increases uPA and decreases PAI-1. Increase in ZEB1 mRNA levels in HCT116 cells in response to treatment with 10 ng/ml LMB for 30 min resulted in
upregulated levels of uPA mRNA and decrease of PAI-1 mRNA. Cells were also subjected for 30 min to the same volume amount of the solvent in which LMB was resuspended (70% methanol, MeOH). Relative mRNA levels of uPA, PAI-1 and ZEB1 were determined by qRT-PCR respect to GAPDH. (G) Upregulation of endogenous ZEB1 following activation of Wnt signaling increases uPA and decreases PAI-1. HTC116 cells were either untreated (Unt) or exposed during 24 h to 100 ng/ml of recombinant human Wnt3a. Relative mRNA levels of uPA, PAI-1 and ZEB1 were determined by qRT-PCR respect to GAPDH.

Supplementary Figure S2

(A) ZEB1 activates uPA transcription in Colo320 cells. Colo320 cells were transfected with 0.5 µg of a luciferase reporter containing 1.9 kb of the human uPA promoter along with 1 µg of empty expression vector or the corresponding equal molar amount of a ZEB1 expression vector. Transcriptional assays were performed as described in Supplementary Materials and Methods. (B) β-catenin activates uPA transcription. As in (A) but using SW480 cells and either 1 µg of an empty expression vector or equal molar amount of the same vector carrying β-catenin cDNA. (C) As in (A) but replacing expression vectors with 200 nM of siRNA control (siCtl) or a siRNA against β-catenin (siβcat).

Supplementary Figure S3

ZEB1 expression decreases PAI-1 mRNA stability. SW480 cells stably transfected with a shRNA control (shCtl) or against ZEB1 (sh1ZEB1) were incubated for the indicated periods with 10 µg/ml of Actinomycin D (Act D) before relative mRNA levels of PAI-1 were analyzed by qRT-PCR respect to GAPDH as reference gene.

Supplementary Figure S4

The uPA inhibitor amiloride blocks ZEB1-mediated invasiveness. (A) SW480 stable cell lines described in Figure 4A were tested for their ability to invade through a Matrigel matrix during 24 h in the presence of either 100 µM of amiloride or equal volume of its solvent (DMSO). Invasion assays were performed as described in Materials and Methods. A t-test for significance of means difference was used. (B) Amiloride reduces uPA expression in SW480 cells. SW480 cells were treated for 24 h with either 100 µM of amiloride or equal volume of DMSO. Cells lysates were assessed by Western blot for expression of ZEB1 (H-102) and uPA (3689/HD-UK1) proteins using α-tubulin (B5-1-2) as loading control.

Supplementary Figure S5

(A) Expression of ZEB1 in the developing intestine. As in Figure 5A, immunohistochemistry for ZEB1 (Zfhep Ab) in the intestine of ZEB1 wild type (+/+ and null (-/-) E18.5 mouse embryos. (B) Single staining for uPA (blue, 3689/HD-UK1), ZEB1 (red, E-20) and β-catenin (green, Ab6302) corresponding to tumor front pictures in Figure 5D. uPA was originally detected with Dylight 649 (invisible far red) but converted to blue for representation. DAPI staining is also shown. Scale bars represent 25 µm. (C) As in (B) but corresponding to tumor center pictures in Figure 5D. (D) ZEB1 colocalizes with uPA in invading cancer cells at the tumor front of CRCs. As in Figure 5D but for an alternative (more differentiated area) of the tumor front. (E) As in (B), single staining for uPA, ZEB1 and β-catenin corresponding to tumor front pictures in panel (D).
SUPPLEMENTARY REFERENCES


