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Auto-regulation of Slug mediates its activity during epithelial to mesenchymal transition

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ABSTRACT

Slug, a five C2H2 zinc finger (ZF) motif transcription factor mediates cell migration in development, adult tissue repair and regeneration, as well as during tumor metastases through epithelial to mesenchymal transition. At the molecular level, this involves interactions with E-box (CA**CC**/**GG**TG) consensus elements within target gene promoters to achieve transcriptional repression. However, precise elucidation of events involved in this DNA recognition and binding of specific promoters to regulate target genes have not been achieved. In the present study, we show that besides transcriptional repression, Slug can also directly activate its own expression by preferential binding to specific E-box elements in the distal binding region of its promoter. Our findings suggest that while the first ZF does not contribute to the transcription-associated functions of Slug, all the remaining four ZFs are involved in regulating the expression of target genes with ZF3 and ZF4 being more crucial than ZF2 or ZF5. We also report that recognition and binding preferences of ZFs are defined through intrinsic differences in the E-box core base pairs and/or flanking sequences, with the S2 E-box element being most critical during autoregulation. However, specific target E-box recognition and binding are also defined by the cellular context, which implies that *in silico* and/or biochemical DNA binding preferences may not necessarily be able to accurately predict in situ events. Our studies thus constitute a novel understanding of transcriptional regulation.

1. Introduction

Epithelial–mesenchymal transition (EMT) is a tightly controlled phenomenon occurring during early embryonic development, adult wound healing and cancer metastases [1]. The process involves dramatic changes in epithelial cells including decreased expression of cell junction components and epithelial cell-specific characteristics, altered polarity and acquisition of a motile, invasive mesenchymal phenotype [2]. Slug, a member of the Snail family of zinc-finger transcriptional repressors is known to play an important role in EMT [3,4], and is overexpressed in numerous cancers including lung, esophageal, colorectal, gastric, pancreatic, breast, prostate, ovarian, leukemia, malignant mesothelioma, hepatocellular carcinoma, and glioma [5]. Elevated Slug expression is associated with advanced tumor grade, lymph node metastases, postoperative relapse and shorter patient survival in several cancers [6–8]. A hallmark of EMT and cancer metastases is loss of E-cadherin expression, which was one of the first targets described for Slug [9,10]. Additionally, Slug is also known to represses other epithelial genes, including claudins, occludin, ZO-1, cytokeratins 8 & 18, mucin1, and desmosomal junction components [11,12]. Complementarily, it is reported to indirectly activate mesenchymal genes including N-cadherin, vimentin, and fibronectin [13,14]. It is also probable that exogenous Slug can induce endogenous expression by activating its own promoter [15,16].

Transcriptional repression mediated by Slug is reported to involve the five C2H2 zinc fingers (ZFs) in its C-terminal domain that interact with specific sequences in promoter regions of target genes viz. the canonical E-box sequence: 5′-CA**CC**/**GG**TG-3′ [10,17]. ZFs are short tandem repeats of 23–24 amino acids organized as a classical ββα motif that provides specific interactions and binding affinities for target sequences. Also involved during repression is an N-terminal 20 amino acid (a.a) — a long SNAG domain that interacts with different co-repressor molecules to mediate transcriptional repression; and the SLUG domain which is a unique conserved sequence motif positioned before the ZFs [18]. Despite such understanding of its protein structure, molecular sequence of events in the discrete recognition and specific binding of promoters by Slug to regulate target genes remain largely unknown. We had earlier identified a possible recognition of Slug binding to its own promoter

Abbreviations: EMT, epithelial–mesenchymal transition; TF, transcription factor; ZF, zinc finger; TSS, transcription start site; MD, molecular dynamics; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation assay; PDB, Protein Data Bank; SS, steady state; TGF-β, transforming growth factor beta; MMPSBA, molecular mechanics–Poisson Boltzmann surface area.

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in ovarian cancer [19]. In the present study, we further investigated this suggested auto-regulation through detailed analysis of binding kinetics and interactions of Slug with the seven consensus E-box elements in its promoter in cell-free systems as well as *in situ*. This identified the preferred order of binding to specific E-boxes in the promoter, and indicated that *in vitro* binding does not necessarily predict similar *in situ* affinities, which assign a cellular context for auto-regulation. The interactions of Slug ZF domains with *CDH1* and Slug promoter E-box elements predicted through molecular dynamics (MD) simulation-based computational were further validated in an ovarian cancer model, and suggest the involvement of four Slug ZFs in regulating E-cadherin and Slug expression. Together, our study reveals crucial facets of Slug-mediated transcriptional regulation during EMT.

2. Materials and methods

2.1. Promoter analysis

Slug promoter sequences were extracted from NCBI gene database (http://www.ncbi.nlm.nih.gov/gene; chromosome 8q11; NC_000008.11; 48,917,690–48,921,740, 4051 bp) from which a secondary database consisting of sequences 2.5 kb upstream to 0.5 kb downstream from the transcription start site (TSS) and a tertiary E-box sequence database were derived (Fig. 1A; Supplementary Fig. 1). Similarly, E-cadherin and Slug promoter and associated E-box elements were identified in 9 other vertebrate species (chimpanzee, gorilla, rhesus monkey, horse, mouse, rat, pig, chicken and zebrafish; Supplementary Fig. 1). E-box conservation was determined as a percentage of sequence availability within the species through genomewide alignment using ClustalW tool.

2.2. Cells, culture conditions and transfections

The A4 cell line was established from malignant ascites of a patient diagnosed with serous ovarian adenocarcinoma [20], cultured in MEM(E) containing 5% fetal bovine serum (Sigma-Aldrich) and 1% non-essential amino acids (Invitrogen Life Technologies, USA). Cells were treated with 10 ng/ml TGF-β (Sigma-Aldrich) for 48 h in a serum-free medium. All transfection experiments were performed with Lipofectamine 3000 (Invitrogen Life Technologies) in exponentially growing cells according to the manufacturer's instructions.

2.3. Semi-quantitative reverse transcription (RT)-PCR and quantitative PCR (q-PCR)

Total RNA was extracted from cells using TRIzol and cDNA synthesized by reverse transcription cDNA Synthesis Kit as per the manufacturer's instructions (Invitrogen Life Technologies). Semi-quantitative RT-PCR was performed under standard conditions, and PCR products were visualized and quantified as described earlier [21]. qPCR analyses with specific promoter primers were carried out with Step one plus Real-Time PCR System in a 96-well plate format using SYBR Green Mix (Life Technologies). Primer sequences used can be provided on request. PCR consisted of denaturation at 94 °C for 45 s followed by 34 cycles (denaturation at 94 °C for 30 s — annealing at an optimized temperature of specific primers for 45 s — extension at 72 °C for 45 s) followed by final extension at 72 °C for 60 s. Data are presented as mean ± SEM from triplicate experiments. Changes in threshold cycle (C_T) and fold difference values were calculated as: $\Delta C_T = C_T(\text{test}) - C_T(\text{control})$ and fold difference $= 2^{-\Delta(\Delta C)}_T$. Actin to normalize gene expression levels and non-template controls to diminish the possibility of contaminating DNA amplification in reaction mixture were used.

2.4. Plasmid constructs, expression and purification of GST fusion proteins

Slug deletion mutants ΔZF1 and ΔZF5 were generated by PCR and cloned into pEGFP-C1; ΔZF2, ΔZF3, and ZF4 were generated using QuikChange Mutagenesis kit (Stratagene, USA); mutants were verified by sequencing. Human Slug cDNA was amplified from A4 cells, cloned into pGEX-6P-1 GST vector followed by subcloning into pEGFP-C1 GFP vector and transformed into *Escherichia coli* BL21 codonPlus (Agilent

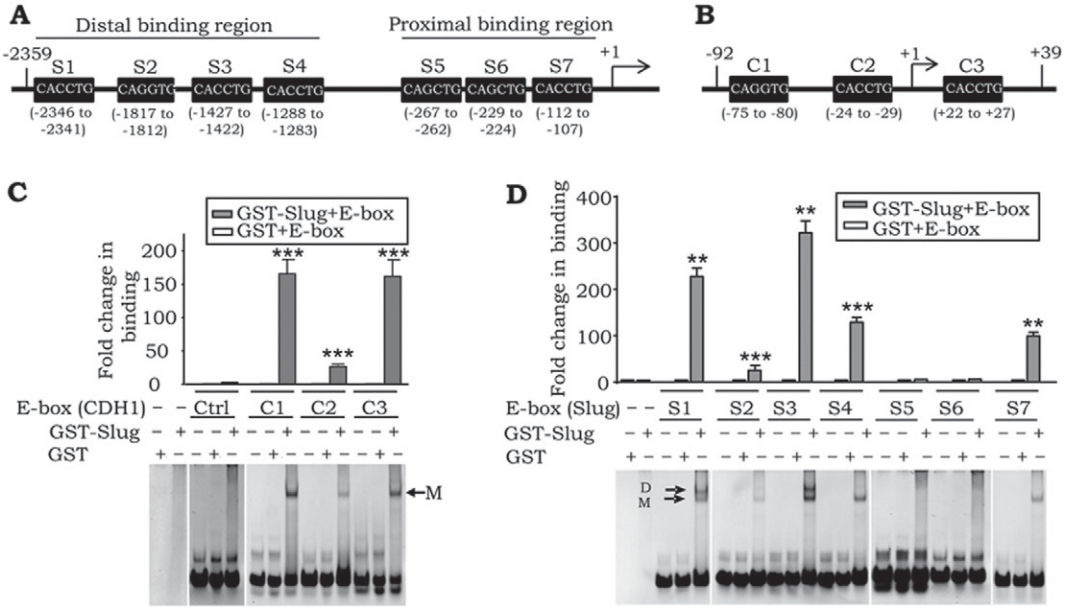

Fig. 1. Slug recognizes and preferentially binds to specific E-box elements within *CDH1* and Slug promoters. A. Schematic representation of Slug (−2.5 kb to +1 from TSS) promoter indicating localization of specific E-box sequences in distal and proximal binding regions; B. schematic representation of known E-box sequences in the *CDH1* promoter; C. top panel — Densitometry-based quantitation of GST–Slug fusion protein to E-boxes in the *CDH1* promoter, bottom panel — Representative EMSA for this binding in which a single mobility band shift is seen (arrow), GST proteins and probes without E-box sequences were included as controls; D. top panel — Densitometry-based quantitation of GST–Slug fusion protein to E-boxes in the Slug promoter, bottom panel — Representative EMSA for this binding in which mobility shift is seen as two bands (arrows) that suggest its binding either as a monomer alone (E-boxes S2, S4, S7) or as a dimer (E-boxes S1, S3), GST proteins and probes without E-box sequences were included as controls; all data are representative of experiments carried out in triplicate and are depicted as mean ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Technologies, USA). Cells grown overnight in LB medium (100 μg/ml ampicillin and 34 μg/ml chloramphenicol) at 37 °C were subcultured in Terrific Broth medium (100 μg/ml ampicillin + 34 μg/ml Chloramphenicol) at 37 °C to an optical density of 0.6–0.8 at 600 nm. Protein expression was induced by 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 16 h at 22 °C. Cells were sonicated in lysis buffer [50 mM Tris–HCl, 300 mM NaCl, 5 mM DTT, 1 mM PMSF and Protease inhibitor cocktail, pH 7.5] and equilibrated with Glutathione sepharose (Amersham Biosciences). GST-tagged Slug fusion proteins were purified by affinity chromatography under native conditions and concentration estimated by DC protein assay kit (Bio-Rad), using bovine serum albumin as protein standard. Protein aliquots were boiled with buffer [0.125 M Tris (pH 6.8), 4% SDS, 20% glycerol, 2% 2-ME, 0.03 mM bromphenol blue], separated on SDS-PAGE and visualized with FastGel Blue R-350 (GE Healthcare, UK) staining. Human Slug and E-cadherin promoters (WT, containing 7 — S1 to S7 and 3 C1, C2, C3 E-box elements respectively) were isolated by PCR from genomic DNA of A4 cells and cloned into pGL3 luciferase plasmid (Promega, USA). Deletion Slug promoter mutants ΔS1, ΔS1–S2, ΔS1–S3, ΔS1–S4, ΔS1–S5 and ΔS1–S6 were generated through PCR amplification of the WT promoter and cloned into pGL3 luciferase plasmid. Slug-S2 mutants with GG to CC, GC and CG (S2.MUT1, S2.MUT2, S2.MUT3 respectively) were generated by QuikChange Mutagenesis kit (Stratagene, USA) from pGL3-Slug-WT. After cloning and mutagenesis, all clones were verified by sequencing.

2.5. Immunoprecipitation, immunoblotting and mass spectrometry (MALDI TOF/TOF)

A4 cells lysed in buffer (50 mM Tris–HCl-pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.25% deoxycholate, 0.1 mM PMSF and protease inhibitor cocktail), 2 mg lysates were precleared with protein-A beads and incubated overnight with 5 μg monoclonal Slug antibody at 4 °C. On the following day 25 μl protein-A agarose (Amersham, GE Healthcare) beads were added and incubated for 3 h, washed with lysis buffer and the immunoprecipitated protein complex resolved on 12.5% SDS-PAGE. Immunoblotting was performed as described earlier [12], primary antibodies used included mouse monoclonal α-Slug, α-GST, α-E-cadherin, α-fibronectin, α-CBP, α-Smad4, α-TFIIB, goat polyclonal α-claudin-3, rabbit polyclonal α-p300, RNA pol II (Santa Cruz Biotechnology), rabbit monoclonal α-N-cadherin (Cell Signaling), mouse monoclonal α & β-actin and rabbit polyclonal α-vimentin (Sigma-Aldrich), α-mouse, α-rabbit (Amersham Pharmacia Biotech) and α-goat (Millipore) horseradish peroxidase (HRP) linked secondary antibodies. Proteins were excised for in-gel digestion by trypsin and subjected to mass spectrometry analysis as described earlier [22].

2.6. Immunofluorescence

Immunofluorescence was performed as described earlier [12]. Briefly, cells grown on coverslips were fixed with 4% paraformaldehyde for 10 min at 4 °C, permeabilized with chilled methanol (−20 °C) for 10 min, blocked with 5% BSA for 1 h. Fixed cells were incubated with mouse α-Slug (1:50; Santa Cruz Biotechnology), followed by incubation with Alexa fluor 568 conjugated α-mouse antibody (1:300; Invitrogen, USA), and nuclei were stained with Hoechst. Images were acquired on an inverted laser scanning confocal fluorescence microscope (Carl Zeiss, Jena, Germany; 63× oil immersion objective) analyzed using Carl Zeiss software.

2.7. Electrophoretic mobility shift assay (EMSA) and native PAGE

30-bp double-stranded oligonucleotide probes were designed and synthesized around each E-box element in the putative human *CDH1* and Slug promoter regions. Annealing was carried out with complementary oligonucleotides in NEB2 buffer at a final concentration of 50 pm/λ by heating at 95 °C for 2 min followed by Ramp cooling to room temperature (25 °C) over 70 min for use in EMSA, using 30-bp probes lacking E-box sequences as controls. Binding was assayed with 50 ng purified protein, 5 μg oligonucleotide probe and buffer (100 mM HEPES (pH 7.5), 2.5 mM DTT, 50% glycerol, 25 mM $MgCl_2$, 0.25 mM $ZnSO_4$) at room temperature for 20 min. EMSAs were run at 4 °C on 6% DNA polyacrylamide gel in 0.5× tris–boric acid–EDTA (TBE) buffer, electrophoresed at 100 V for 60 min and visualized by ethidium bromide staining. 30-bp oligonucleotide probes were used for binding assay with GST and GST–Slug fusion protein as performed in EMSA. DNA–protein complexes were run on native 6% PAGE gels at 4° in 0.5× TBE buffer, electrophoresed at 100 V for 60 min; and proteins were visualized by Coomassie brilliant blue staining.

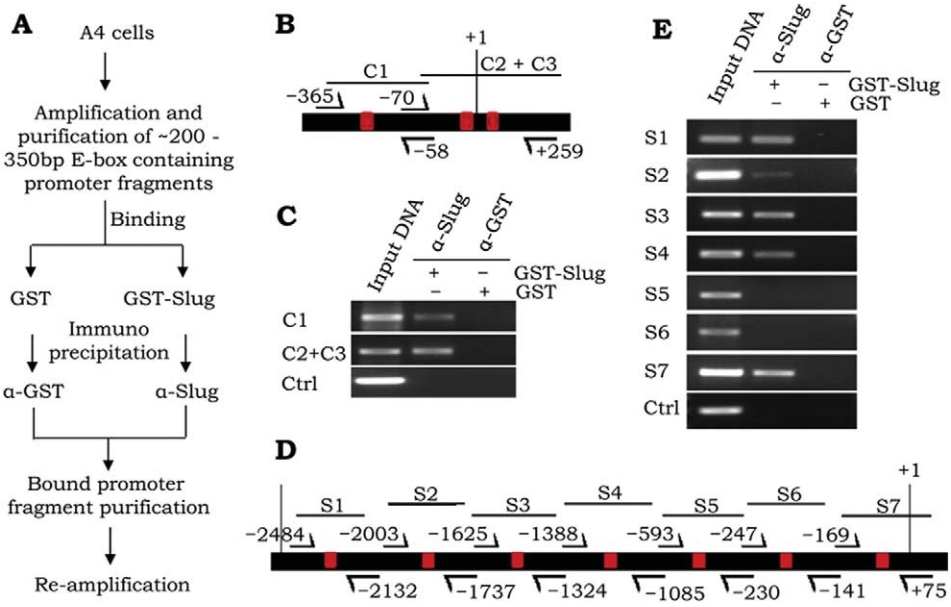

Fig. 2. Affinity and specificity of Slug recognition and binding to E-box elements within the *CDH1* and Slug promoters. A. Flow-chart representing synthetic ChIP-PCR assay; B, D. schematic of the *CDH1* and Slug promoters respectively indicating locations of E-box elements (red boxes) and primer sets for synthetic ChIP-PCR; C, E. Synthetic ChIP-PCR analyses for GST–Slug binding to the *CDH1* and Slug promoter respectively, dsDNA fragments without E-box elements and GST were used as negative controls; β-actin used as control; all data are representative of experiments carried out in triplicate and are depicted as mean ± SEM;*$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.001$.

2.8. In vitro migration assay

Cell migration assay was performed as described earlier [12]. Briefly, cells were grown to confluence and a wound created with a pipette tip. After washing with 1× PBS and media to remove floating cells, the wound was monitored for healing at fixed time intervals. Images were captured on an Olympus IX71 microscope at 10× magnification, analyzed by TScratch software and migratory cells were counted by ImageJ Software [23].

2.9. Promoter fragments and synthetic ChIP-PCR assay

A synthetic ChIP-PCR assay was designed to evaluate the specificity of Slug binding to E-box elements. Primers were designed around individual E-box sequences in the Slug promoter and used for amplification. Binding of amplicons with purified proteins was assayed overnight in buffer (100 mM HEPES (pH 7.5), 2.5 mM DTT, 50% glycerol, 25 mM $MgCl_2$, 0.25 mM $ZnSO_4$). Bound protein–DNA complexes were immunoprecipitated using specific antibodies (α-Slug/α-GST), washed with PBS and eluted with buffer (1% SDS and 100 mM $NaHCO_3$). Cross-links were reversed by overnight incubation at 65 °C in 8 M NaCl, and DNA recovered using Qiaquick DNA purification kit (Qiagen, USA).

2.10. Chromatin immunoprecipitation assay and ChIP-PCR

ChIP was performed as described earlier [19]; ChIPped DNA amplified using the GenomePlex Complete Whole Genome amplification kit as per manufacturer's instructions (Sigma-Aldrich). Specific primers were used to amplify E-boxes within promoter regions of genes, amplified products run on 1.5% agarose gels, and band intensities measured by densitometric analysis using Genetool3.6 (Syngene).

2.11. Luciferase assays

A4 cells were transfected with 0.8 μg Luciferase reporter plasmids and either pEGFP-C1 or pEGFP-C1-Slug in 24-well plates using Lipofectamine 3000 (Invitrogen, USA). Renilla luciferase was added (10 ng) to each transfection as control. For TGF-β treatment, the medium was replaced 12 h after transfection. Luciferase activity was measured using a Dual Luciferase assay kit (Promega, USA) either 24 h post-transfection or TGF-β treatment.

2.12. Statistical analysis

Unless mentioned otherwise, all experiments were carried out in triplicate; data are expressed as mean ± SEM of at least three

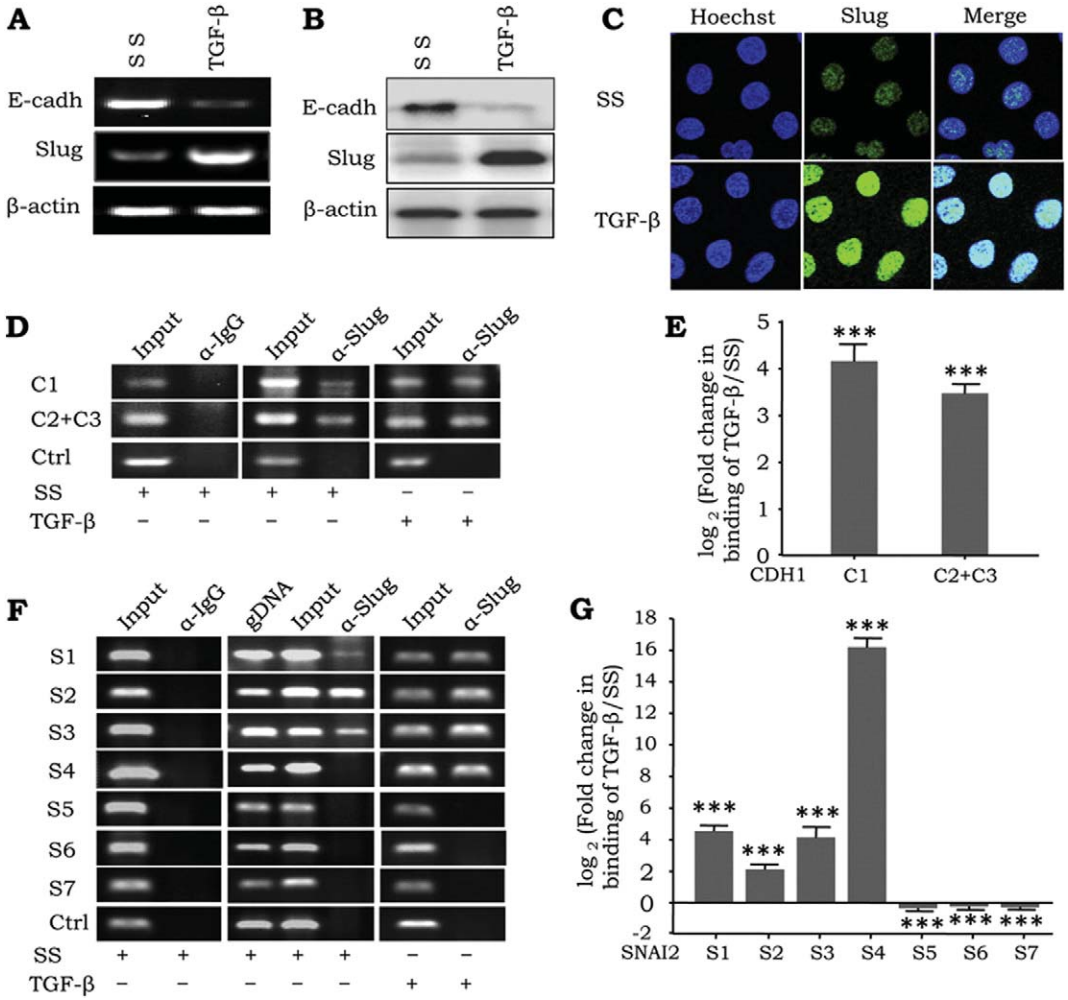

Fig. 3. *In situ* binding of Slug to E-box elements within its own promoter in steady-state (SS) and TGF-β treated A4 cells. A, B. mRNA and protein expression analysis of E-cadherin (E-cadh) and Slug in A4 cells treated with TGF-β; C. Expression and localization of Slug in A4 cells treated with TGF-β; D. *in situ* binding analysis of Slug to E-box elements within *CDH1* promoter in SS and TGF-β treated A4 cells using ChIP-PCR assay; E. quantitative ChIP-PCR for analyzing the enrichment of Slug on the *CDH1* promoter in SS and TGF-β treated A4 cells; F. *in situ* binding analysis of Slug to E-box elements within its own promoter in SS and TGF-β treated A4 cells using ChIP-PCR assay; G. quantitative ChIP-PCR for analyzing the enrichment of Slug on the Slug promoter in SS and TGF-β treated A4 cells; IgG was used as isotype control; β-actin was used as control (Ctrl). gDNA — genomic DNA; input — fragmented DNA for ChIP. All data are representative of experiments carried out in triplicate and are depicted as mean ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

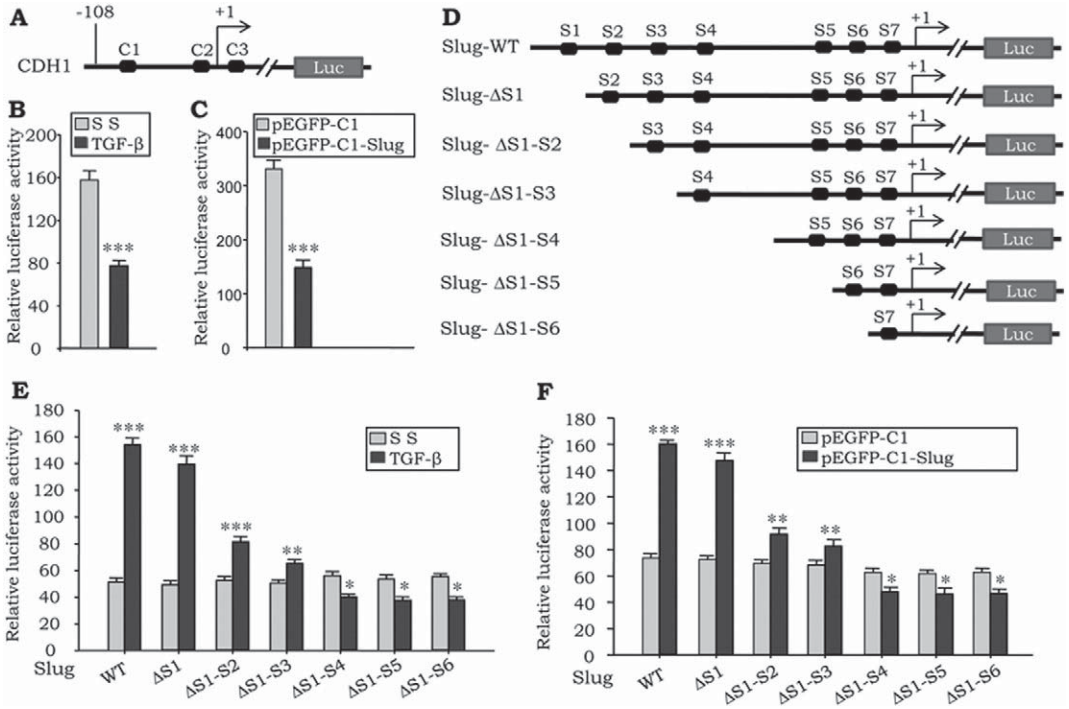

Fig. 4. Binding of Slug to its own promoter is able to induce promoter activation. A. Schematic representation of luciferase reporter construct of proximal *CDH1* promoter containing three E-box elements. CDH1-Luc contains wild type promoter sequences from −108 to +125 of the endogenous *CDH1* promoter upstream of a *Luciferase* reporter gene. +1 indicates the transcription start site; B, C. Luciferase reporter assays with TGF-β exposure and with wild type Slug on *CDH1* promoter in A4 cells; D. Schematic representation of luciferase reporter constructs of wild type Slug promoter (Slug-WT) containing seven E-box elements and its deletion mutants Slug-ΔS1, Slug-ΔS1–S2, Slug-ΔS1–S3, Slug-ΔS1–S4, Slug-ΔS1–S5 and Slug-ΔS1–S6. +1 indicates the transcription start site; E, F. luciferase reporter assays with TGF-β exposure and with wild type Slug on wild type Slug promoter and its individual deletion mutants in A4 cells; all data are representative of experiments carried out in triplicate and are depicted as mean ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

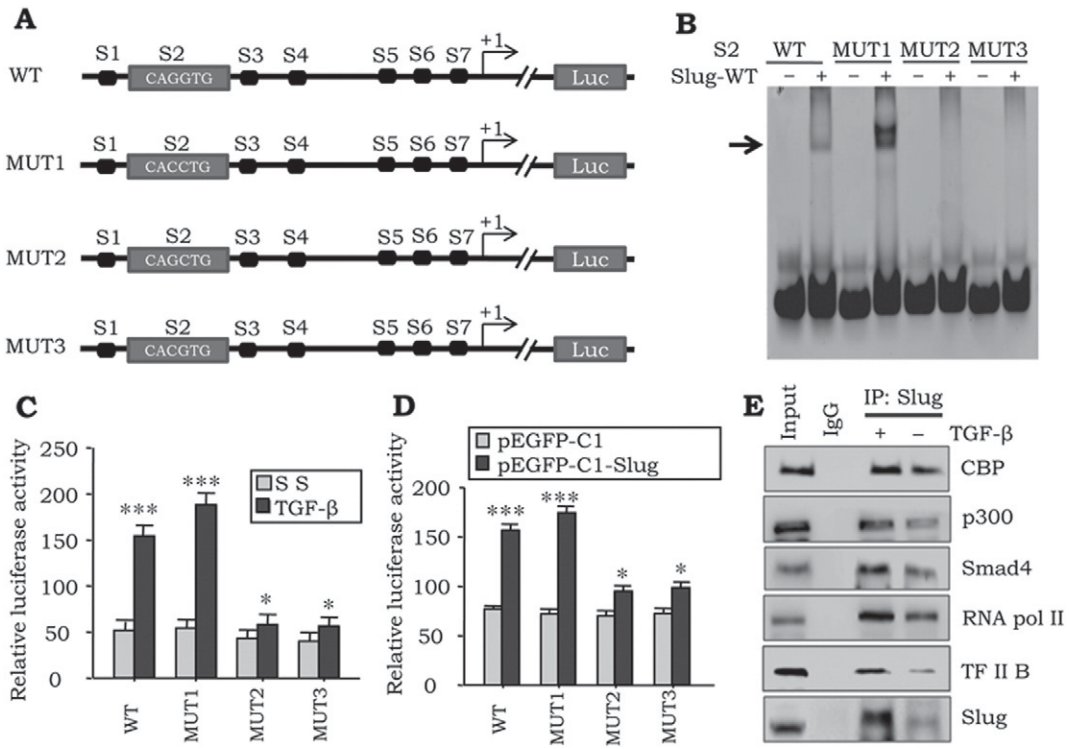

Fig. 5. Identification of the role of S2 E-box elements. A. Schematic representation of luciferase reporter constructs of Slug-WT.Luc and S2-site specific mutants (MUT1, MUT2, MUT3 in which central GG of 5′-CAGGTG-3" sequence is replaced by either G and/or C; B. Representative EMSA for Slug fusion protein binding to WT, MUT1, MUT2, and MUT3 oligonucleotides. Arrow represents the mobility shift of Slug; C, D. schematic representation of luciferase reporter assays of WT Slug binding following TGF-β exposure and exogenous expression of WT Slug respectively on WT, MUT1, MUT2, and MUT3 S2 E-boxes in A4 cells. E. Slug was immunoprecipitated from cell lysates of steady state and TGF-β treated A4 cells and association of CBP, p300, Smad4, RNA polymerase and TFIIB probed by immunoblotting. All data are representative of experiments carried out in triplicate and are depicted as mean ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

independent experiments. The significance of difference in the mean values was determined using two-tailed Student's t-test. One way ANOVA using Sigma Stat (SPSS Inc. USA) was applied to data generated in EMSA, Synthetic ChIP-PCR, ChIP-PCR and luciferase assays to determine the significance of means within the seven E-box elements of Slug promoter; $p < 0.05$ was considered significant.

3. Results

3.1. Slug recognizes and preferentially binds to specific E-box elements within CDH1 and Slug promoter regions

Slug promoter harbors seven E-boxes corresponding to three different consensus sequences viz. four 5′-CACCTG-3′ (S1, S3, S4, S7), one 5′-CAGGTG-3′ (S2) and two 5′-CAGCTG-3′ (S5, S6; Fig. 1A). S1 to S4 are present in the distal promoter region (1283 to 2346 bases upstream of TSS), while S5, S6, and S7 are present in the proximal promoter region (500 bases upstream of TSS; Fig. 1A). Probing the evolutionary conservation of these E-box elements in ten vertebrate species (human, chimpanzee, gorilla, rhesus monkey, horse, mouse, rat, pig, chicken and zebrafish) revealed S2, S3 and S7 to be maximally conserved; S1, S4 and S5 to be moderately conserved, while S6 was poorly conserved (Supplementary Fig. 1).

To ascertain functional interactions, we generated and purified N-terminal GST–Slug fusion protein using Sepharose GST resin affinity columns and further validated its integrity and purity through immunoblotting and mass spectrometry (Supplementary Figs. 2, 3). Functionality of the fusion protein in recognition and binding to the E-cadherin (*CDH1*) promoter as extensively reported earlier [9,24] was confirmed through EMSA using 30-bp double-stranded oligonucleotide probes for each of the three E-box promoter elements and validated by native PAGE (Fig. 1B, C; Supplementary Fig. 4A, B). This revealed strong affinity of C1 and C3 and weaker affinity of C2 E-box elements in recognition and binding to Slug as a monomer (as indicated from a single band in mobility shift; Fig. 1C, bottom panel). The presence of E-box elements in its own promoter further presented the possibility of Slug autoregulation through recognition and binding to some of these regulatory elements. We thus probed for affinity of the GST–Slug fusion protein to each E-box element (S1 to S7) in the Slug promoter using specific 30-mer oligonucleotide probes. This identified E-box elements S1 and S3 to have the highest recognition and binding (5–7 fold as compared to S2); S4 and S7 to have moderate interactions (3.5–2.5 fold as compared to S2), and S2 to have weak affinity, while S5 and S6 failed to demonstrate any binding with Slug (Fig. 1D). Strikingly, EMSA revealed that the higher affinity for S1 and S3 also involves binding of Slug both as a fast migrating monomer and a slower migrating dimer [25–27] as indicated from the presence of two band shifts in EMSA (Fig. 1D, bottom panel). These preferential interactions of Slug with E-box elements (S3 > S1 > S4 > S7 > S2) were affirmed by native PAGE (Supplementary Fig. 4C, D).

A synthetic ChIP-PCR assay was designed to further dissect out and validate Slug binding specificity (Fig. 2A). Briefly, ~200–350 bp amplicons around each target E-box from the A4 cell genome were generated and allowed to bind purified Slug fusion protein; such bound protein–DNA complexes were immunoprecipitated using α-Slug and α-GST (control) antibodies. DNA recovered by reverse cross-linking was probed through PCR to identify specific binding sites in the *CDH1* and Slug promoters (Fig. 2B, D). Higher specificity for the C2 + C3 amplicon over C1 in *CDH1* promoter (Fig. 2C), strong affinity for S1 and S3, moderate affinity for S4 and S7, low affinity for S2 and none for S5 and S6 in Slug promoter were detected (Fig. 2E). Associations of binding specificity of Slug with these E-box elements of *CDH1* and Slug promoters were also affirmed using qPCR (Supplementary Fig. 5A, B). Thus, cell-free EMSA as well as synthetic ChIP-PCR assays affirmed similar, specific interactions of Slug with E-boxes in the *CDH1*

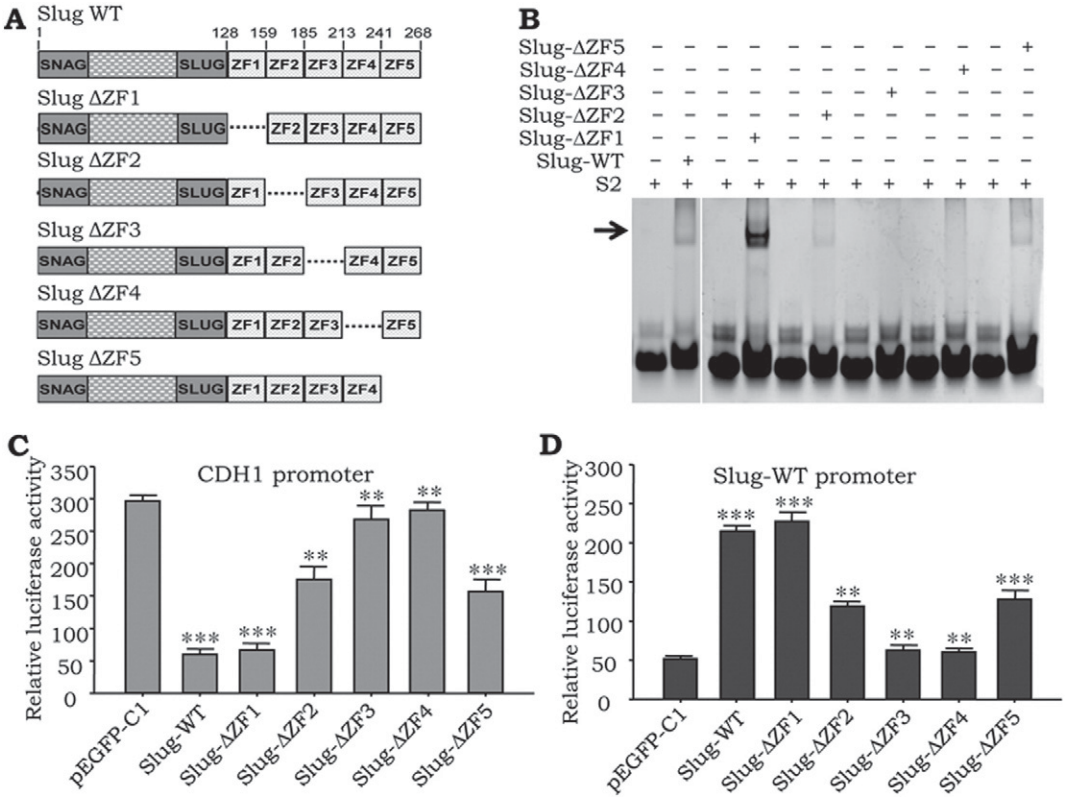

Fig. 6. DNA binding and regulatory activity of full-length Slug or ZF mutants. A. Schematic representation of human Slug and its ZF mutants; B. representative EMSA for the binding of Slug and individual ZF mutant protein to E-box S2 element. Arrow represents the mobility shift of Slug and ZF mutants; C. Luciferase reporter assays of Slug and individual ZF mutants on proximal *CDH1* promoter in A4 cells; D. Luciferase reporter assays of Slug and individual ZF mutants on wild type Slug promoter in A4 cells. All data are representative of experiments carried out in triplicate and are depicted as mean ± SEM. *$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.001$.

and Slug promoters like other members of the Snail family [28–33], with Slug displaying a moderate to strong affinity for 5′-CACCTG-3′, weak affinity for 5′-CAGGTG-3′ and none for 5′-CAGCTG-3′ sequences [34,35]. Taken together, these results suggest possible auto-regulation of Slug.

3.2. In situ dynamic binding of Slug to E-box elements in ovarian cancer cells

Since TGF-β exposure triggers Slug expression and mediates EMT [36–39], we tested if these interactions would be relevant to EMT *in situ*. Confluent, steady state (SS) cells express a basal level of Slug in the nucleus which is enhanced on TGF-β treatment, with concurrent decrease in E-cadherin expression (Fig. 3A, B, C). Using a Slug antibody validated for specificity (Supplementary Fig. 6), we further probed for the specific binding of Slug to E-box elements under these conditions. In SS cells Slug recognizes and binds to C1 as well as C2 + C3 elements in the *CDH1* promoter to repress its expression; these binding affinities increase following TGF-β treatment (Fig. 3D, E). Exploring Slug autoregulation along similar lines revealed dynamic E-box binding affinities in an order of S2 > S3 > S1 in SS cells. On stimulation with TGF-β, not only was the binding enhanced, but also an additional affinity for the S4 E-box was evident (2–16 fold enhanced binding; $p \leq 0.001$; Fig. 3F, G). S5, S6, and S7 E-boxes were not recognized by Slug. The binding order (S2 > S3 > S1 > S4) also suggests a preferential affinity for 5′-CAGGTG-3′ over 5′-CACCTG-3′, and none for 5′-CAGCTG-3′ sequences. Surprisingly, these results varied from those identified in the cell-free evaluation, although both *in situ* conditions indicated Slug autoregulation to involve the distal promoter region. Together, the data establish that the Slug promoter contains a higher number of E-box binding sites than can be actually occupied, and that preferential binding within diversity of available binding sites may provide for differential regulation under varying environmental conditions.

3.3. Binding of Slug to its own promoter is able to induce promoter activation

To further affirm these effects of enhanced cellular levels of Slug, we transfected A4 cells with luciferase reporter-tagged *CDH1* promoter construct (Fig. 4A) either with or without WT Slug and compared the response to that on TGF-β exposure. As in the case of the response to TGF-β, exogenous expression led to a ~2 fold decrease in luciferase activity of *CDH1* promoter over control (Fig. 4B, C). To probe for autoactivation along similar lines, we generated and transfected a series of luciferase reporter-tagged Slug promoter deletion constructs (Fig. 4D), in A4 cells with WT Slug and compared the response to that on TGF-β exposure. TGF-β treatment significantly increased luciferase activity in A4 cells (~3-fold; Fig. 4E); similarly Slug overexpression led to an enhanced increase in luciferase activity indicating effective Slug autoactivation (~2.5-fold; Fig. 4F). Deletion of S2 showed marked effects on luciferase activity and marginal influences of S1, S3 and S4 binding. This suggests the involvement of distal promoter region in autoactivation. Importantly, these results emphasize on the key involvement of S2 E-box in autoactivation with S1, S3 and S4 possibly being supportive for the process. We thus explored a contribution of specific sequences to regulation by mutating the internal residues of the S2 E-box "CA**GG**TG" to either CC, GC or CG (MUT1, MUT2, MUT3 respectively; Fig. 5A). Assaying Slug binding efficiencies in terms of EMSA based binding as well as luciferase

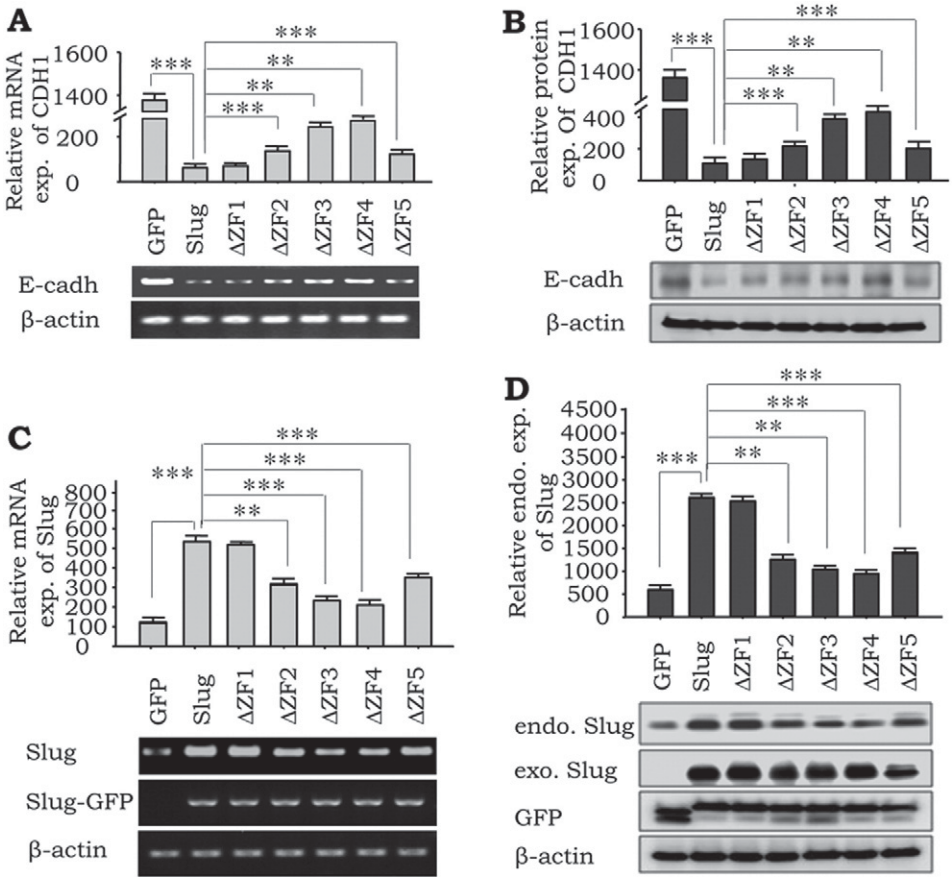

Fig. 7. Identification of ZF(s) required for transcriptional regulation. A, B. Comparative repression of human *CDH1* by full-length Slug or ZF mutants in A4 cells at transcription and protein levels respectively; C, D. comparative auto-regulation of Slug by full-length Slug or ZF mutants in A4 cells, expression was normalized with control GFP expression. β-Actin was used as loading control. All data are representative of experiments carried out in triplicate and are depicted as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

reporter activity indicated increased affinity to 'CC' as compared to the wild type 'GG', while 'GC' and 'CG' failed to demonstrate any binding (Fig. 5B). The same observations were consistent in A4 cells in which Slug expression was enhanced either by TGF-β treatment or transient transfection (WT Slug; Fig. 5C, D). These data imply the S2 E-box element to be a critical component during autoregulation.

The co-activator functions of Slug involve associations with other co-factors such as CBP/p300, both of which are activated during TGF-β mediated EMT by acetylated Smads and Smad4 [40–44]. CBP/p300 itself functions as histone acetyltransferase to remodel chromatin by exposing DNA regions through relaxation of its superstructure that enables direct interaction with RNA polymerase II and general transcription factors to initiate the transcription of genes [45]; while Slug- associated recruitment of RNA-polymerase II to the promoter binding sites is indicative of transcriptional activation [46,47]. Hence towards validation of autoactivation, we immunoprecipitated protein from A4 cells at SS and on TGF-β treatment with Slug antibody, and probed for its association with CBP/p300, Smad4, RNA polymerase II and TFIIB. All these cofactors were detected in the immunoprecipitated protein complex from SS cells and also upon TGF-β treatment (Fig. 5E). This demonstrates that CBP/p300, Smad4 and Slug can interact with the transcriptional initiation complex and induce active transcription that is different from the more frequently demonstrated role of Slug as a transcriptional repressor. Importantly, these data together suggest the possibility of Slug autoactivation by creating a positive regulatory transcription loop.

3.4. Slug auto-activation and identifying the Slug ZFs required for transcriptional regulation

Following the identification of regulation through the S2 E-box, we simulated all possible interactions of this element with individual ZFs (Supplementary Note). MD simulation data classification indicating interactions based on the length of contact time as being either stable (present for >85% of simulation time), moderate (70–84% of simulation time) or transient (40–69% of simulation time) revealed a lack of ZF1 involvement in S2 recognition and/or binding, while the remaining four ZFs surround the DNA double helix and establish hydrogen bonds with DNA bases of the S2 E-box element (Supplementary Note Figs. 3, 4). This suggests that ZF integrity is crucial for proper recognition and binding to consensus E-box sequences. To validate these predictions, we generated individual Slug ZF mutants (ΔZF1, ΔZF2, ΔZF3, ΔZF4, ΔZF5; Fig. 6A) and analyzed their binding efficacies to WT-*CDH1* and WT-Slug promoters. ΔZF1 showed slightly increased affinity, ΔZF2 and ΔZF5 were associated with reduced affinity, while ΔZF3 and ΔZF4 underwent complete loss of binding (Fig. 6B; Supplementary Fig. 7A, B, C, D). Luciferase Reporter assays of Slug ZF mutants with proximal *CDH1* promoter also indicated that ZF1 is not required for repression; either ΔZF3 or ΔZF4 leads to complete loss of Slug repressor activity, while ΔZF2 or ΔZF5 mediates partial loss of function (Fig. 6C). Similar analyses with Slug promoter similarly indicated affirmed an essential role of Slug ZF3 and ZF4, partial contribution of ZF2 and ZF5 and lack of involvement of ZF1 in transcriptional regulation by Slug (Fig. 6D). These regulatory effects were also validated at a functional level by comparing E-cadherin and Slug expression at mRNA and protein level in A4 cells transfected with full length Slug or individual ZF deletion constructs. As expected, full length Slug effectively repressed E-cadherin and mediated autoactivation at the mRNA and protein level; ΔZF1 demonstrates comparable effects that affirm its lack of involvement in regulation (Fig. 7A, B, C, D). ΔZF3 or ΔZF4 led to strong loss of Slug activity implied by *CDH1* de-repression or Slug activation, while ΔZF2 or ΔZF5 exhibited a partial involvement in this regulation.

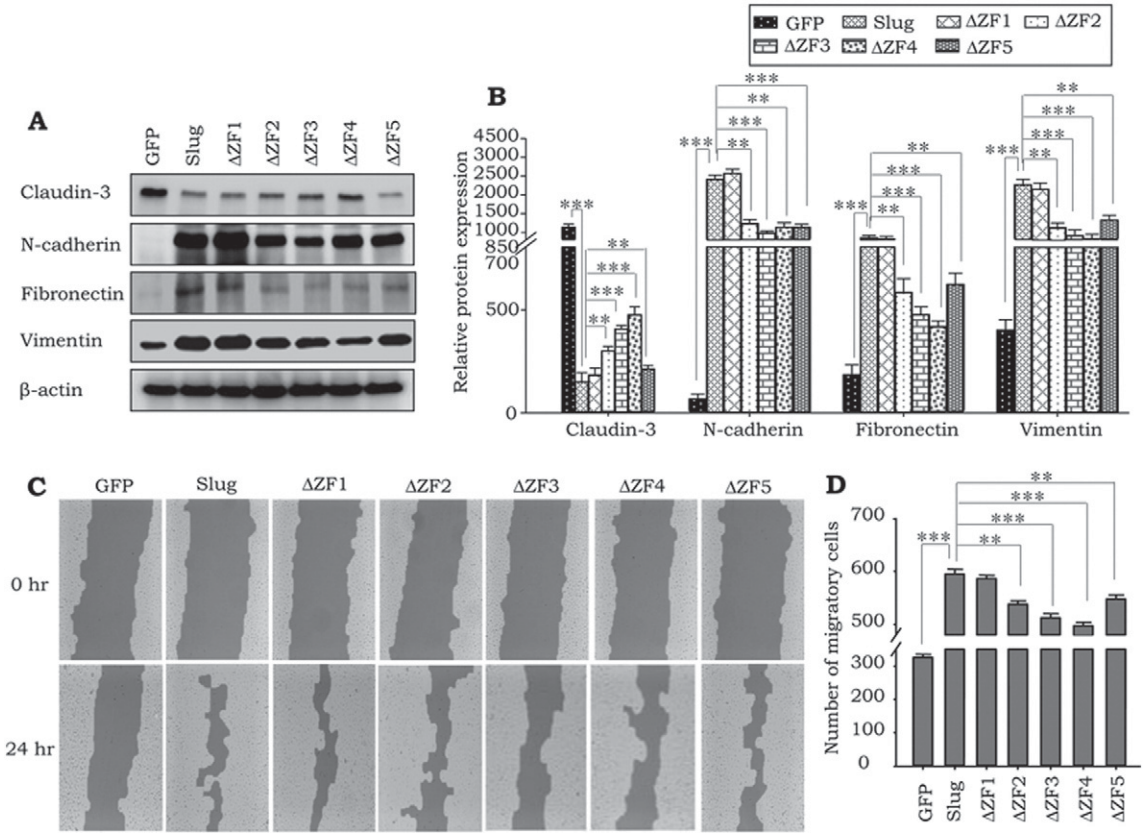

Fig. 8. Identification of ZF(s) regulating the expression of epithelial and mesenchymal markers. A. Comparative profiles of epithelial (claudin3) and mesenchymal (fibronectin, N-cadherin and vimentin) markers in cells expressing either full length Slug vs. ZF-mutants; β-actin was used as loading control; B. quantitative analysis for the expression of epithelial and mesenchymal markers; C. representative images of comparative wound healing by full-length Slug vs. ZF mutants in A4 cells analyzed by TScratch software; D. quantitative wound healing analysis. All data are representative of experiments carried out in triplicate and are depicted as mean ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Table 1
Representative binding of Slug to Slug promoter E-box elements in cell-free (EMSA, synthetic ChIP-PCR) and *in situ* (ChIP-PCR of cells at steady-state or TGF-β−stimulated) systems; + to ++++ indicates weak to maximum binding affinities.

E-boxes	S1	S2	S3	S4	S5	S6	S7
E-box element	CACCTG	CAGGTG	CACCTG	CACCTG	CAGCTG	CAGCTG	CACCTG
EMSA	++++	+	++++	++	–	–	++
Synthetic ChIP-PCR	++++	+	++++	++	–	–	++
ChIP-PCR (Steady state)	+	++++	++	–	–	–	–
ChIP-PCR (TGF-β)	+++	++++	++++	+++	–	–	–

To support these findings, we further examined the expression of epithelial (claudin-3) and mesenchymal (N-cadherin, vimentin, fibronectin) markers in full-length Slug and mutant ZF-expressing cells. Loss of claudin-3 along with increased expression of mesenchymal markers indicated effective EMT in full-length and ΔZF1 expressing cells, but was compromised in the other deletion mutants (Fig. 8A, B). The functional implications of effective EMT on cell migration were also assayed by comparing efficacies of *in vitro* wound healing, wherein full-length and ΔZF1 expressing cells were highly efficient, while other mutants lagged in migratory capabilities (Fig. 8C, D). This functional assay thereby validates the results of biochemical assays and predictions from simulation studies by demonstrating a predominant role for ZF3 and ZF4 and partial contribution of ZF2 and ZF5 in promoter regulation.

4. Discussion

Autoregulation of Slug is earlier suggested in *Xenopus oocytes* during early neural crest development and mouse fibroblast cells [15,16]. However the specific molecular mechanisms in Slug recognition and binding to consensus E-box sequences of target gene promoters are largely unknown. Understanding the same is crucial since Slug contributes to the invasive nature of tumor cells and facilitates metastasis during cancer progression [48]. This study identifies that in cell-free systems (EMSA and synthetic ChIP-PCR), Slug binds to five E-box elements in its own promoter in a preferential order of S3 > S1 > S4 > S7 > S2 (Table 1). However, in steady-state cells, the binding order was altered to S2 > S3 > S1 and on further triggering the cellular context of Slug activation by TGF-β, was reinforced with additional binding to the S4 element. Higher affinity for either 5′-CACCTG-3′ and 5′-CAGGTG-3′ over 5′-CAGCTG-3′ sequences was noted in both systems (cell-free and cell-associated) and is suggested earlier [34,35]. However our study provides the first proof of Slug activating its own expression through specific preferential E-box binding in its distal promoter region, a mechanism reported for some other TFs [40,49–54].

Conservation of sequences during evolution are important in predicting binding sites [55–59]; indeed the strong evolutionary conservation of the S2 Slug promoter element in ten species followed by moderate conservation of S1, S3 and S4 elements correlated with differential binding preferences during autoregulation. However, *in vitro* DNA binding preferences do not completely correlate with *in situ* affinities (Table 1), suggesting that specific E-box recognition and affinity rely on microenvironmental cues. This provides an understanding for the differential modulation of Slug target gene profiles under varying cellular contexts [19]. An additional feature is that S7 was identified as a target E-box element in cell-free systems, but not in either of the in situ cellular situations studied. We hypothesize that recognition and binding of S7 by virtue of its presence in the proximal Slug promoter region could possibly be relevant for other regulatory functions such as autorepression, which is likely to be crucial under discrete microenvironmental contexts necessitating termination of EMT, for instance when migratory tumor cells arrive at secondary sites and re-establish a metastatic tumor.

Resolution of such differential Slug binding further necessitates characterization of Slug ZF interactions with intrinsic and flanking sequences of the E-box elements. Using *CDH1* and Slug as its prototypical gene targets, careful dissection of differential DNA recognition of the five Slug ZFs (ZF1–ZF5) was associated with ionic interactions of specific residues of Slug ZFs with DNA bases that led to differences in conformations of ZFs and DNA helices in the genomic regions flanking the E-box (Supplementary Note). Dissimilar constrains between the E-box structures characterize its bound state, while additional contacts outside the E-box may result in the selection of different flanking sequences. Altering the secondary structures of each ZF identified higher involvement of ZF3, ZF4 and to a lesser extent of ZF2 and ZF5 in DNA binding, repression and/or activation in target promoters. However ZF1, the unique zinc finger in Slug that distinguishes the protein from its highly homologous TF viz. Snail [60] did not exhibit any E-box affinity. This similarity between Slug and Snail accounts for the overlapping functions of these TFs in EMT.

In conclusion, we propose a model in which Slug activates its own expression by creating a positive feedback loop involving its distal promoter region and through association with several co-activators. Further, this loop may regulate not only EMT but also other crucial cellular processes such as senescence, survival and stemness.

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Transparency Document

The Transparency Document associated with this article can be found, in the online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagrm.2015.07.006.

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