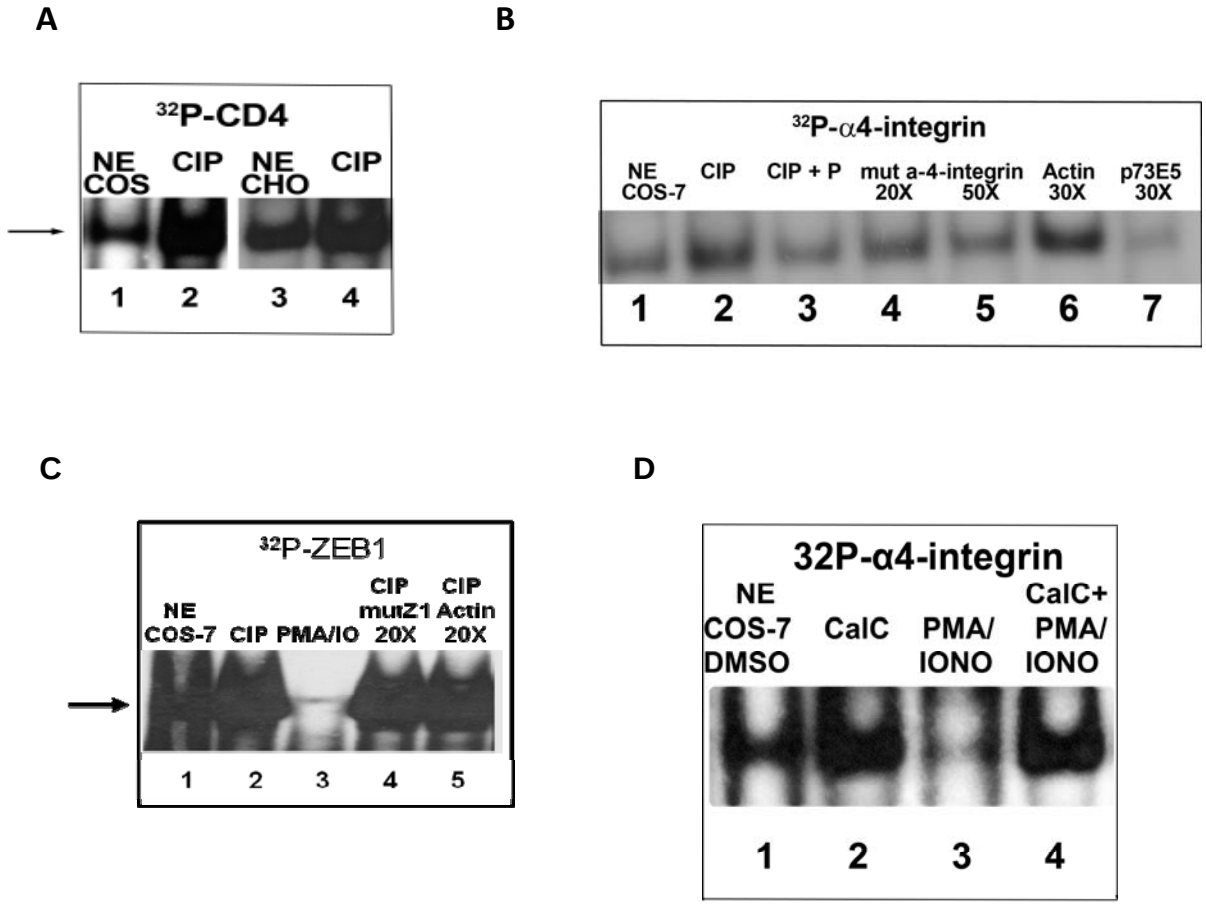
SUPPLEMENTARY MATERIAL AND METHODS

Western blots and immunoprecipitation. Nuclear extracts from COS-7 and CHO-K1 were used for western immunoblots with polyclonal anti-ZEB1 antibody (anti-ZEB1-HD) against the homeodomain of ZEB1. When whole CHO-K1 extracts were assayed, cell lysates were collected using modified RIPA lysis buffer. Lysates included a mixture of protease and/or phosphatase inhibitors (Roche, Indianapolis, IN, USA) as appropriate. For immunoprecipitation, 1.5 x 10^6 CHO-K1 cells were transfected with 60 µg of the ΔZD1 (pcDNA1/ΔZD1) or ZD2E (pcDNA1/ZD2E) DNAs, or the empty vector pcDNA1 by the PEI method. Cell lysates were obtained and 200 µg of protein immunoprecipitated with 4 µg of ZEB1 R17 antibody or an equivalent amount of anti-ZEB1-HD antibody. After immune-complex absorption to Protein A agarose and washing, the samples were boiled in Laemmli buffer, and centrifuged. The eluted protein was loaded onto 10% SDS-PAGE gels. The blots were blocked and incubated with phospho-TP MAPK/CDK substrate mouse monoclonal antibody (1:5000) (Cell Signalling, Danvers, MA, USA), followed by horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Amersham, GE Healthcare, Piscataway, NJ, USA). Subsequently, blots were stripped and incubated with ZEB1 R17 antibody at 4°C overnight, followed by horseradish peroxidase-conjugated anti-goat IgG (Amersham, GE Healthcare, Piscataway, NJ, USA). The HRP conjugates were detected by chemiluminescence (Amersham ECL Western Blotting Reagent, GE Healthcare) detected by X-ray film for evaluation.

Electrophoretic mobility shift assay (EMSA). The source of ZEB1 protein was nuclear extracts (NE) from cell lines that express different amounts of native ZEB1, as described (Costantino et al., 2002). ZEB1-b, ZEB1-ZD2E and ZEB1-ZD2 proteins were synthesized by in vitro transcription/translation in rabbit reticulocyte lysates (RRL) according to the manufacturer's instructions (T7-TnT Quick Coupled Transcription/Translation System Promega, Madison, WI, USA). The oligonucleotides used for EMSA were obtained from Sigma Genosys (Sigma-Aldrich, Saint Louis, MO, USA) and are depicted in Supplementary Table 2. Double-stranded oligonucleotides were labelled with [^{32}P]-dATP by fill-in with Klenow DNA polymerase I.

SUPPLEMENTARY FIGURES AND TABLES

S1



SUPPLEMENTAL FIGURE 1. The phosphorylation state of ZEB1 changes its binding ability on its target genes. (S1A) NE from COS-7 and CHO-K1 cells were incubated with [^{32}P] CD4 CD4 and were untreated (lanes 1, 3) or treated with CIP enzyme. **(S1B)** NE from COS-7 cells were incubated with [^{32}P] α4-integrin and either untreated (lane 1) or treated with CIP enzyme. The probe was competed by 20-50X excess of mutant α4-integrin cold oligonucleotide, 30X actin cold oligonucleotide or 30X p73E5 cold oligonucleotide. **(S1C)** NE from COS-7 cells were incubated with [^{32}P] ZEB1oligonucleotide and were untreated (lane 1) or treated with CIP enzyme (lanes 2, 4, 5). Alternatively, the cells were treated with PMA+IONO prior to making NE (lane 3). [^{32}P] ZEB1 probe was competed by 20X excess of mutant ZEB1oligonucleotide (4) or actin oligonucleotide (5). **(S1D)** COS-7 cells were incubated with DMSO (solvent) (1), and consecutively with Calphostin C (CalC) (2), PMA+Iono (3), and CalC-PMA+Iono (4) prior to making NE. NE from cells was incubated with [32P]α4-integrin for EMSA.

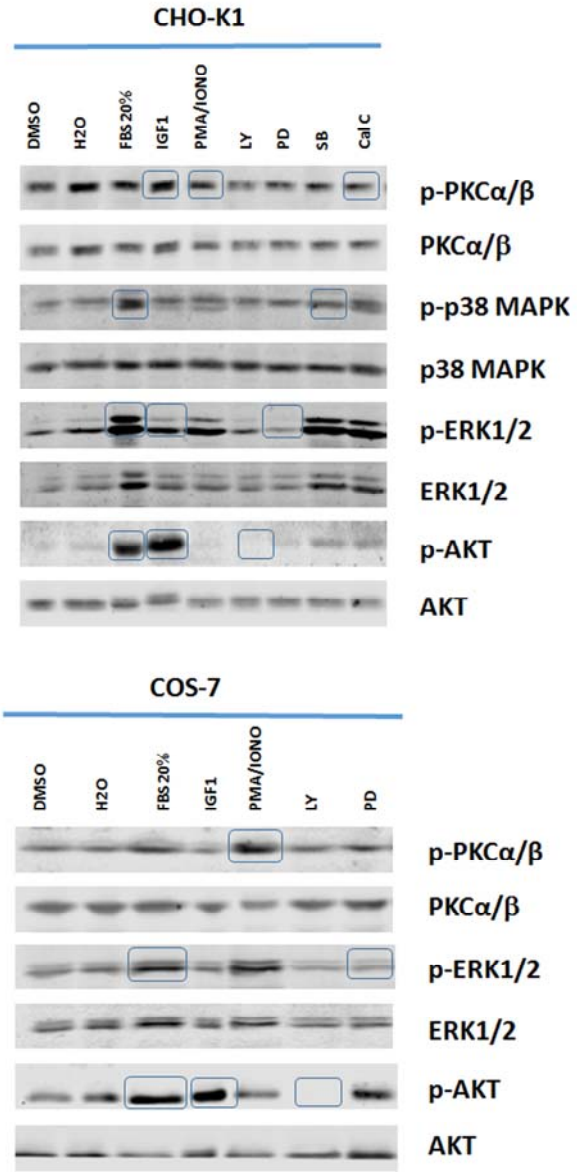
Table 1

Site-Directed Mutagenesis Oligonucleotides	
Oligo name	**Sequence (5' to 3')**
mZD2-1A forward	CAG GAT GAA AGA CAA GAC GCT GCC GCA GAA GGA GTC
mZD2-1A reverse	GTC TTG TCT TTC ATC CTG GTT TCC ATT TGG-3'
mZD2-1B forward	GCC GCA GAA GGA GTC GCC GTG GAG GAC CAG
mZD2-1B reverse	GAC TCC TTC TGC GGC AGC GTC TTG TCT TTC ATC
mZD2-2A forward	GAT ATT TCA GAA GAG CAG CGC CCT GCT GAG AC
mZD2-2A reverse	GCT GCT CTT CTG AAA TAT CTT GTC ACA CAG
mZD2-2B forward	TGT GGC AAG CGC TTC GAC TCG GGG GCT TAC TCT C
mZD2-2B reverse	GAA GCG CTT GCC ACA CTT GTC ACA TTG GTA GGG CTT
mZD2-2C forward	CAC ATG AAT CAC CGC TAC GCC TAC TGC AAC AG
mZD2-2C reverse	GTA GCG GTG ATT CAT GTG TTG AGA GTA TGC
ZEB1-T876A forward	CCA GAA TGA CTC TGA CTG CGC GCC ACC C
ZEB1-T876A reverse	G GGT GGC GCG CAG TCA GAG TCA TTC TGG
mZEB1 forward	CAC CGT CGA CAG TCA GTA GCG TTT ACC
mZEB1 reverse	GAA TAG GGC CCT CTA GAA CTA GTG GAT CC

TABLE 2. Italic bold small characters indicate ZEB1 binding sites. Italic bold capital letters indicate mutated bases. Underlined bold letters indicate AP-2 binding site (one base –in italics- was changed to eliminate possible ZEB1 binding site). Capital letters are non genomic sequences, added for labeling purposes. With respect to the p73 gene, it is worth noting that Fontemaggi et al. (2001) identified five E-box /ZEB1 binding sites in the first intron of p73 gene. We designed two oligonucleotides for EMSAs that include E-box 5 (p73E5) and E-boxes 3 and 4 (p73 E3-4).

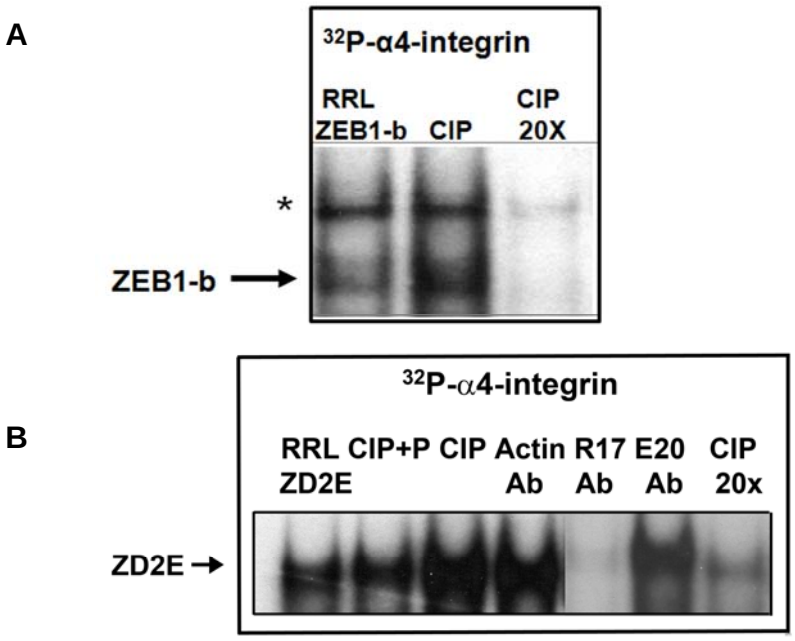
EMSA oligonucleotides			
Name of GENE	**Location in Promoter**	**Oligo name**	**Sequence (5' to 3')**
Human ZEB1	–197 to –236 of the ZEB1 promoter (Manavella et al. 2007)	BS2 U BS2 L	tctccccacca***cacctg***aggaaaactttttccctcgcccct attgaggggcgagggaaaagttttcct***caggtg***tggtggg
Human p73	intron 1(Fontemaggi et al. 2001)	p73E5 U p73E5 L	CCTTgcaaggcgggggg***cacctg***ctccagggatgc GGTTgcatccctggag***caggtg***cccccgccttgc
Human α4-integrin	1621 to 1650 in L26059 (Postigo et al. 1997)	A4int361 U A4int361 L	GATTcactacccag ttc***aggtgt***g ttgcgttgtg TGTGcacaacgcaaca***cacctg***aactgggtagtg
Mouse CD4	2222 to 2253 (AF 088189 genbank seq) (Brabletz et al. 1999)	CD4 U CD4 L	Tttctccaaagggtaa***caggtg***tcagctggctg ggttcagccagctga***cacctg***ttacccttgg
Human E-cadherin	-13 to -38 of human E cadherin promoter	Ecadh E3 U Ecadh E3 L	AGTTtccggggct***cacctg***gctacagtcac AGTTgtgactgtagc***caggtg***agccccgga
Mutated p73	intron 1	p73E5MutA p73E5MutB	CCTTgcaaggcgggggg***cTcGAg***ctccagggatgc GGTTgcatccctggag***cTCgAg***cccccgccttgc
Mutated α4-integrin	1621 to 1650 in L26059	A4int361MutA A4int361MutB	GATTcactacccag tt***caggtA***tg ttgcgttgtg TGTGcacaacgcaaca***Tacctg***aactgggtagtg
Mutated ZEB1	–197 to –236 of the ZEB1 promoter	BS2mut U BS2mut L	Tctccccacca***CAtaTG***aggaaaactttttccctcgcccct attgaggggcgagggaaaagttttcct***caggtg***tggtggg

S2



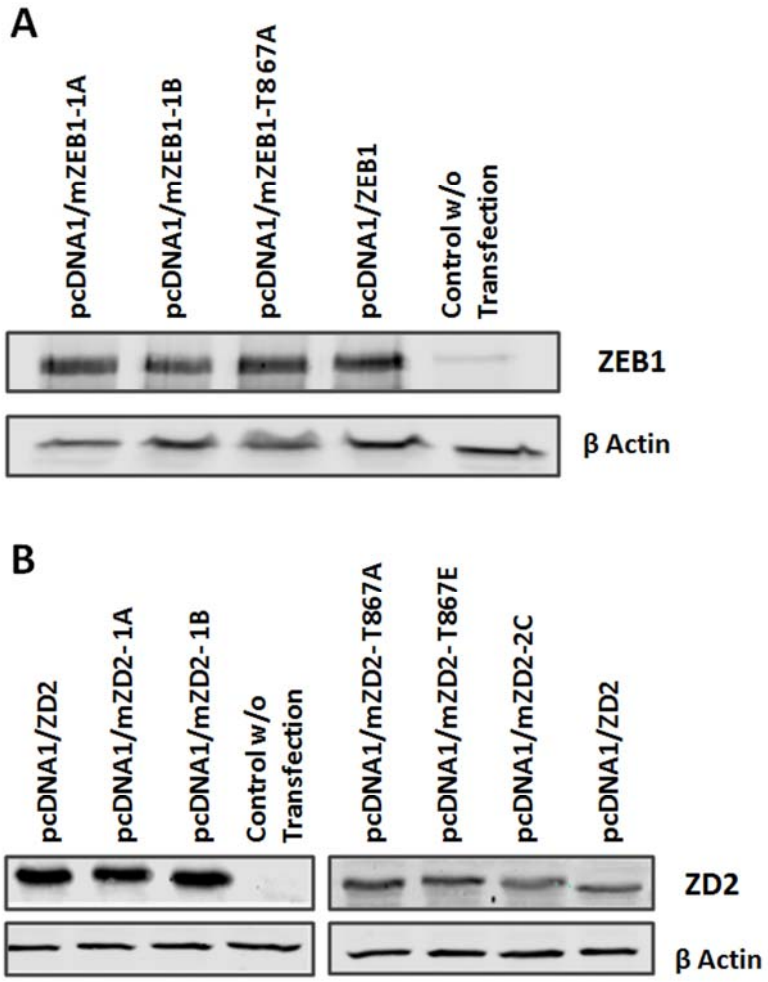
SUPPLEMENTAL FIGURE 2. Effect of inhibitors and activators of intermediate signaling components on the activity of signaling pathways. Representative immunoblot from two independent experiments showing phospho and total PKC α/β, phospho and total p38 MAPK, phospho and total ERK1/2 and phospho and total AKT levels in CHO-K1 (upper blot) and COS-7 (lower blot) cells. Cells were pre-incubated with DMSO (solvent) or water (IGF-1 solvent), 20% Fetal Bovine Serum (FBS), 10 nM IGF-1, 40 ng/ml PMA plus 1 µg/ml ionomycin (IONO); 10 µM LY294002 (LY); 10 µM PD98059 (PD); 10 µM SB20358 (SB) and 50 nM Calphostin C (Cal C) as indicated in M&M. The expected specific responses are pointed out by a frame.

S3



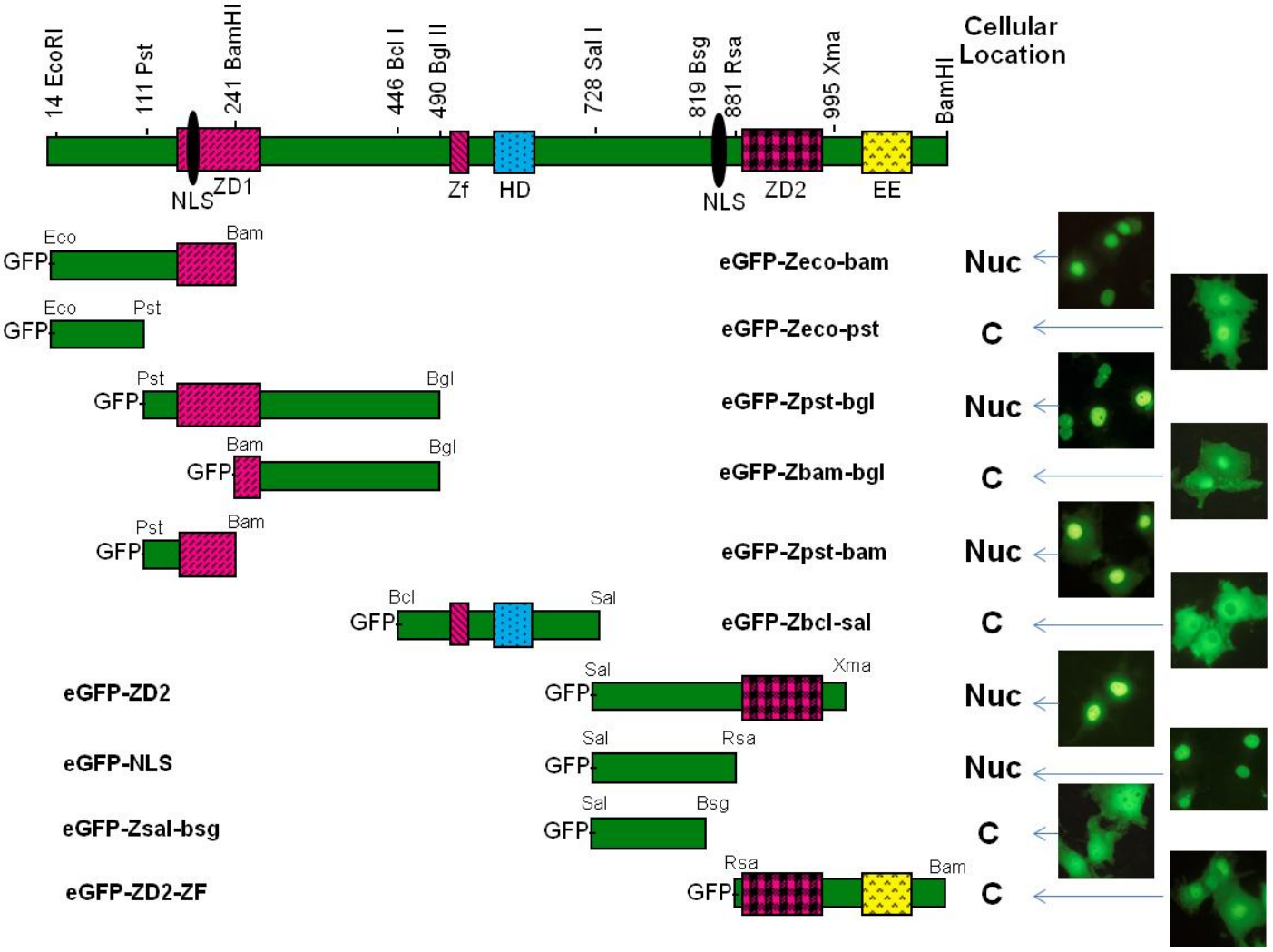
SUPPLEMENTAL FIGURE 3. CIP treatment increases DNA binding of the ZEB1 deletion mutants ZEB1-b, or ZD2E. EMSAs were performed with RRL programmed for ZEB1-b (**S3A**), or ZD2E (**S3B**) and were untreated (first lane on the left) or treated with CIP enzyme for 30 minutes. RRLs were incubated with [^{32}P]α4-integrin, and competed by 20X excess of cold α4-integrin. ZD2E bands were supershifted by N-terminus ZEB1 antibody (E20), C-terminus ZEB1 antibody (R17) or the unrelated anti-actin antibody. As expected, E20 and anti-actin Ab were not able to shift ZD2E band. * non-specific band present in unprogrammed RRL.

S4



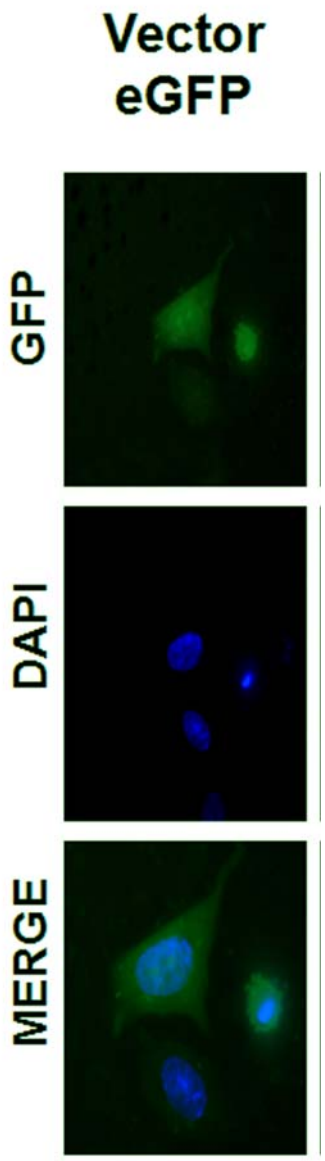
SUPPLEMENTAL FIGURE 4. Immunoblots confirm similar levels of protein expression in wild type and mutant transfected clones. **(S4A)** CHO-K1 cells were transfected with wild type (pCDNA1/ZEB1) or mutant (pCDNA1/mZEB1-1A, pCDNA1/mZEB1-1B, pCDNA1/mZEB1-T867A) clones as indicated in M&M. After 48 h, cells were analyzed by 8% SDS-PAGE and immunoblotting with antibodies against ZEB1. **(S4B)** CHO-K1 cells were transfected with wild type (pCDNA1/ZD2) or mutant (pCDNA1/mZD2-1A, pCDNA1/ mZD2-1B, pCDNA1/ mZD2-T867A, pCDNA1/mZD2-T867E and pCDNA1/mZD2-2C) clones as indicated in M&M. After 48 h, cells were analyzed by 12% SDS-PAGE and immunoblotting with antibodies against ZEB1.

S5

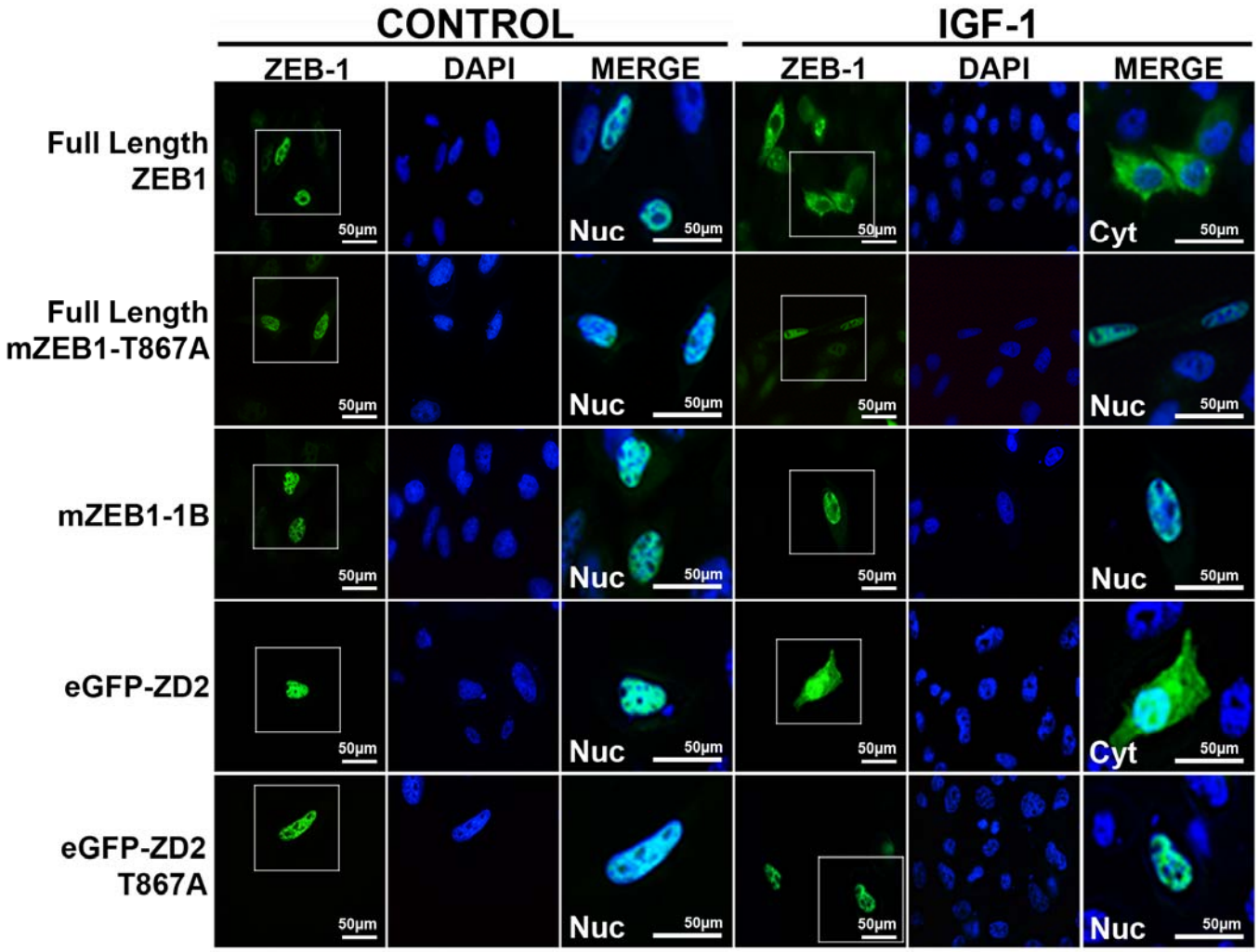


SUPPLEMENTARY FIGURE 5. Representation of GFP-ZEB1 fusion proteins and two independent nuclear localization signals. The major domains of rat ZEB1 are shown with a series of fusion proteins containing GFP and a portion of ZEB1. These clones were transfected into COS7 cells to test predicted NLS sequences. This identified a cryptic NLS within the first zinc finger domain, as well as a strong NLS immediately before the second zinc finger domain.

S6A



S6B



SUPPLEMENTARY FIGURE 6. Immunofluorescence of cells transfected with ZEB1 mutants and GFP-ZD2 clones. (S6A) CHO-K1 cells were transfected with eGFP Vector as a control. A merged figure is shown at the bottom panel. eGFP Vector shows fluorescence distribution at the citosol. **(S6B)** Cells were treated with 10 nM rhIGF-1 for 1 h, and the localization of ZEB1 (green) was examined by immunofluorescence imaging. Cells transfected with GFP clones show the signal GFP as green. Specimens were counterstained with DAPI (blue) to visualize the cell nuclei. A merged figure is shown at the right panel of each treatment.