International Immunology, Vol. 21, No. 3, pp. 227–235
doi:10.1093/intimm/dxn143

The transcription repressor, ZEB1, cooperates with CtBP2 and HDAC1 to suppress IL-2 gene activation in T cells

Jun Wang, Seungsoo Lee, Charis En-Yi Teh, Karen Bunting, Lina Ma and **M. Frances Shannon**

Division of Molecular Bioscience, John Curtin School of Medical Research, The Australian National University, Canberra, ACT 2601 Australia

Keywords: cytokines, T cells, transcriptional regulation

Abstract

Activation of T cells leads to the induction of many cytokine genes that are required for appropriate immune responses, including IL-2, a key cytokine for T cell proliferation and homeostasis. The activating transcription factors such as nuclear factor of activated T cells, nuclear factor κB/Rel and activated protein-1 family members that regulate inducible *IL-2* gene expression have been well documented. However, negative regulation of the *IL-2* gene is less studied. Here we examine the role of zinc finger E-box-binding protein (ZEB) 1, a homeodomain/Zn finger transcription factor, as a repressor of *IL-2* gene transcription. We show here that ZEB1 is expressed in non-stimulated and stimulated T cells and using chromatin immunoprecipitation assays we show that ZEB1 binds to the IL-2 promoter. Over-expression of ZEB1 can repress IL-2 promoter activity, as well as endogenous IL-2 mRNA production in EL-4 T cells, and this repression is dependent on the ZEB-binding site at −100. ZEB1 cooperates with the co-repressor C-terminal-binding protein (CtBP) 2 and with histone deacetylase 1 to repress the IL-2 promoter and this cooperation depends on the ZEB-binding site in the promoter as well as the Pro-X-Asp-Leu-Ser protein–protein interaction domain in CtBP2. Thus, ZEB1 may function to recruit a repressor complex to the IL-2 promoter.

Introduction

T cell activation, through the interaction of the TCR with an antigen–MHC class II complex on the surface of antigen-presenting cells (APCs) as well as a second signal provided by the engagement of B7 molecules on the APCs with the CD28 co-stimulatory molecule on T cells, leads to the production of many cytokines including IL-2. IL-2 plays an important role in the development, differentiation and homeostasis of T cells, and IL-2 expression is dysregulated in diseases such as leukaemia, autoimmunity and viral pathogenesis [reviewed in (1)]. Thus, it is important to understand the molecular mechanisms of the induction, maintenance and repression of *IL-2* gene expression in T cells.

Mechanisms of transcriptional regulation of the *IL-2* gene have been extensively studied where the 360-bp region immediately upstream of the transcription start site was defined as the critical proximal promoter, conferring the inducible expression features of the endogenous *IL-2* gene on a reporter gene (2–4). This region contains binding sites for many transcription factor families such as nuclear factor of activated T cells (NFAT), activated protein-1 (AP-1) and nuclear factor κB (4–6). The appropriate combination of these transcription factors and the assembly of activator complexes on the IL-2 promoter are essential for the expression of the *IL-2* gene (7). Studies of chromatin remodelling during *IL-2* gene activation revealed that the proximal promoter region became highly accessible to DNaseI, micrococcal nuclease and restriction enzyme (RE) digestion following T cell activation (8, 9). These changes in accessibility correlate well with nucleosome disassembly across the proximal promoter (10), thus allowing the recruitment of transcription factors and activator complexes to the promoter to initiate transcription.

Negative regulation of cytokine expression is important both for maintaining the gene in an inactive state in resting cells and for repressing the gene following activation, but the mechanisms of negative regulation of *IL-2* gene expression are much less studied. Glucocorticoids inhibit the transcription of the *IL-2* gene by interfering with the binding of AP-1 and NFAT to their respective sites on the IL-2 promoter (11). The cyclic adenosine 3′,5′-monophosphate-mediated

Correspondence to: M. F. Shannon; E-mail: frances.shannon@anu.edu.au

Received 19 September 2008, *accepted* 8 December 2008

Transmitting editor: A. Kelso

Advance Access publication 30 January 2009

suppression of IL-2 transcription involves the interaction of cAMP response element-binding protein with the IL-2 promoter, which interferes with the recruitment of CBP/p300 (12).

The δEF1/zinc finger E-box-binding protein (ZEB) 1 protein is a transcription repressor containing two C2H2 zinc finger clusters located close to the N- and C-termini of the protein as well as a central homeodomain and resembles a number of other repressors such as ZEB2 in mammals and Zfh-1 and snail in *Drosophila* (13). ZEB1 is expressed in many tissues including the immune system and plays an important role in regulating both muscle and lymphoid differentiation (14). ZEB1-deficient mice show a poorly developed thymus and significant decreases in the T cell population (15). This is due to the depletion of c-kit+ T cell precursors, the earliest intrathymic cells migrating from the bond marrow (15). ZEB1 has been implicated in the regulation of several genes important in the immune system, including the Ig heavy chain enhancer, CD4 (16), GATA-3 (17), α7-integrin (18) and IL-2 (19, 20). ZEB1 has also been shown to block the function of a range of transcription factors involved in regulation of immune genes (14) and has been shown to have two means of repression: direct competition for binding of activators to certain E-box sites (17) and trans-repression involving both an N-terminal region and the Pro-X-Asp-Leu-Ser (PXDLS) motif region of the protein (21).

A negative regulatory element (NRE-A) has previously been identified in the proximal promoter region of the *IL-2* gene which can bind ZEB-like activity from T cell nuclear extracts as well as recombinant glutathione-*S*-transferase-ZEB in *in vitro* binding assays (20). ZEB activity has been implicated in the repressor function of this site by over-expression of a partial cDNA encoding the C-terminal Zn finger region as well as using anti-sense ZEB expression constructs (19, 20). While studying RE accessibility on the IL-2 promoter, we found that the ZEB-binding site at −100 upstream from the transcription start site did not become accessible to RE digestion in response to weak signals (CD3/CD28) but become fully accessible when T cells were activated with phorbol 12-myristate 13-acetate (PMA)/ionophore (Ion)/CD28 (8, 22). We speculated that ZEB may help maintain a closed chromatin structure by targeting co-repressor complexes to the promoter.

In this study, we report that full-length ZEB1 can repress the *IL-2* gene promoter through the −100 NRE. In addition, ZEB1 can repress transcription from the endogenous *IL-2* gene and binds to the IL-2 promoter in T cells. Its repressive activity involves cooperation with C-terminal-binding protein (CtBP) 2 and histone deacetylase 1 (HDAC1), indicating the presence of a ZEB1 repressor complex in T cells.

Materials and methods

Cell culture

EL-4 thymoma cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 10 mM HEPES and antibiotics. Cells were stimulated at density of 1–1.5 × 10^6 cells per millilitres with PMA (10 ng ml^{-1}) and calcium Ion (1 μM).

Primary T cell preparation

Spleens were isolated from C57BL/6 mice (5–6 weeks old). The $CD4^+$ cells were purified using automated magnetic cell sorting $CD4^+$ (LT34) beads according to the manufacturer's guidelines (Miltenyi Biotec, Bergisch Gladbach, Germany). The isolated cells were stained and analysed by flow cytometry to determine purity. The isolated T cell populations were between 90 and 95% $CD4^+$ cells. The $CD4^+$ T cells (1 × 10^6 cells per m^{-1}) were stimulated for the indicated times with PMA (10 ng ml^{-1}) and Ion (1 μM).

Plasmid construction

The IL-2 Luc reporter construct contains 415 bp of the *IL-2* gene sequence encompassing 374 bp upstream of the transcription initiation site and 41 bp of the 5′ untranslated region. This fragment was amplified by PCR and cloned into a pXPG luciferase reporter construct. The mutation of the ZEB-binding site at −100 was generated in this construct using the Quick Change™ (Stratagene, La Jolla, CA, USA) kit. The constructs were verified by sequencing. Expression plasmids for AP-1 (c-Jun and c-Fos) (23), NFATc (24), ZEB1 (25) CtBP2 and its mutant (26) were previously described.

Real-time PCR analysis

Total RNA was prepared from stimulated and unstimulated T cells using TriReagent (Sigma–Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. RNA was treated with DNaseI and reverse transcribed using First-strand cDNA Synthesis (Marligen, Ljamsville, MD, USA) as detailed in the manufacturer's instructions. SYBR green real-time PCR reactions were performed with 50 ng of cDNA in a total volume of 25 μl on an ABI 7500 sequence detector (Applied Biosystems, Foster City, CA, USA) with primers for IL-2 (forward 5′-CCTGAGCAGGATGGAGAATTACA-3′, reverse 5′-TCCAGAACATGCCGCAGAG-3′) or ZEB1 (forward 5′-GCTTTCTGCCACACATTTAGACTCTTTGAAAA-3′, reverse 5′-CATATTTTCAAAGAGTCTAAATGTGTGGCAGA-3′).

The Ct values for IL-2 and ZEB1 were normalized to the housekeeping gene ubiquitin-conjugating enzyme E2D whose expression was not altered in response to PMA/Ion stimulation of EL-4 cells.

The following PCR conditions were used: stage1, 50°C for 2 min for one cycle; stage 2, 95°C for 10 min for one cycle and stage 3, 95°C for 15 s and 60°C for 1 min for 40 cycles. Since PCR amplification was monitored by SYBR green, the absence of non-specific amplification was determined by analysing the dissociation curve of the PCR amplification products.

Transient transfection and reporter gene assays

Transfections were performed either with the Lipofactamin 2000 Kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions or by electroporation. For electroporation, 5 × 10^6 cells were incubated with 30 μg of DNA (5 μg of IL-2 reporter plasmids, 5 μg of each of the expression plasmids or the empty expression plasmid to a total of 30 μg DNA) in 300 μl of growth medium supplemented with 20% FCS for 15 min at room temperature and subjected to electroporation at 270 V and 975 μF capacitance using

a GenePulser (Bio-Rad, Hercules, CA, USA). Control cells were transfected with appropriate amounts of IL-2 reporter plasmid and empty expression plasmid to a total amount of 30 μg. The transfected cells were transferred into fresh medium and left to recover for 20 h. The transfected cells were transferred into 96-well tissue culture plates (300 μl cells per well) and treated with or without PMA/Ion for 8 h. Luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA). The light signal was detected using the Reporter^{TM} Microplate Luminometer (Turner Biosystems, Sunnyvale, CA, USA).

The effect of ZEB1 over-expression on endogenous IL-2 mRNA expression was performed as previously described (24). IL-2 mRNA levels were analysed by real-time PCR as described above.

Chromatin immunoprecipitation analysis

chromatin immunoprecipitation (ChIP) analysis was performed according to the manufacturer's instructions with slight modifications (Upstate Biotechnology, Lake Placid, NY, USA). In brief, 3×10^7 stimulated and non-stimulated (NS) EL-4 T cells were treated with formaldehyde (1%) for 15 min at room temperature and the reaction terminated by the addition of 0.125 M glycine followed by 10 min of incubation. Cells were washed twice with ice-cold PBS. Nuclei were extracted by incubating cells for 10 min on ice in cell lysis buffer (20 mM Tris–HCl, pH 8.0, 85 mM KCl, 0.5% nonidet P40), with the addition of complete EDTA-free protease inhibitor cocktail tablets (Roche Molecular Biochemicals). Extracted nuclei were re-suspended in SDS lysis buffer (50 mM Tris–Cl, pH 8.0, 10 mM EDTA, 1% SDS) containing protease inhibitor and incubated for 10 min on ice. The chromatin in the lysates was sheared by sonication to an average size of 500 bp, as determined by 1% agarose gel electrophoresis. The lysates were pre-cleared with 60 μl of salmon sperm DNA–protein A agarose beads (Upstate) for 1 h of rotation at 4°C, followed by addition of ZEB1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody or no antibody as a control, with rotation overnight at 4°C. After washing with low-salt buffer, high-salt buffer, LiCl wash buffer and Tris–EDTA buffer (Upstate), the cross-links between DNA and proteins were eluted twice from the beads with 200 μl of fresh elution buffer (1% SDS and 0.1 M $NaHCO_3$). Twenty microlitres of 4 M NaCl was added to eluates, and the cross-links were then reversed at 65°C overnight. Samples were recovered by phenol–chloroform extraction twice followed by ethanol precipitation. DNA pellets were washed in 70% ethanol, air-dried and re-suspended in 50 μl MilliQ water for real-time PCR analysis. Using the conditions described above, SYBR green real-time PCR reactions were performed with IL-2 set B primers to amplify a ~100-bp region across the ZEB-binding site of the IL-2 proximal promoter region (forward 5′-CACAGGTAGACTCTTTGAAAATATGTGTAA-3′, reverse 5′-CATGGGAGGCAATTTATACTGTTAATG-3′) and the IL-2 set F primers located at ~2 kb upstream of the IL-2 transcription start site (forward 5′-CATGCAGAGAGTTTTTTGTTGTTGTTTTCTAG-3′, reverse 5′-GCCTAAAGTCTCTCACAAAGAACAGA-3′) and rhodopsin (Rho) primers (forward 5′-ATATCTCGCGGATGCTGAAT-3′, reverse 5′-GACAGAGACCAAGGCTGCTT-3′). The amount of precipitated target sequence was determined by comparison with the no antibody control and total input.

Western blot analysis

Nuclear extracts were prepared according to the method by Schreiber (27). Protein concentrations were determined by the Bradford assay (Bio-Rad). Proteins (15 μg) were resolved by SDS–PAGE, transferred to nitrocellulose and subjected to western blot analysis using anti-ZEB1 and anti-Sp1 antibodies (Santa Cruz). Chemiluminescence was detected using ECL reagents (Amersham Biosciences) according to the manufacturer's instructions.

Results

Expression of ZEB1 mRNA and protein is increased in response to PMA/Ion stimulation of T cells

Since a ZEB-binding site in the *IL-2* gene promoter has previously been implicated in promoter activity as well as chromatin structure (8, 20), we examined the kinetics of ZEB1 expression in T cells. EL-4 T cells were treated with PMA/Ion for times ranging from 0 to 24 h, total RNA extracted, reverse transcribed and the accumulation of ZEB1 mRNA was measured by quantitative PCR. The level of ZEB1 mRNA increased ~3-fold peaking at 8 h and then declined towards baseline by 24 h (Fig. 1A). In agreement with previous observations (8), the expression of IL-2 mRNA also reached a peak at 6–8 h of PMA/Ionorphore activation (Fig. 1B). In $CD4^+$ T cells isolated from mouse spleen and treated with PMA/Ion for times ranging from 0 to 24 h, ZEB1 mRNA was also increased ~3-fold but with a peak seen earlier between 2 and 4 h (Fig. 1C). As expected, the kinetics of IL-2 accumulation in the $CD4^+$ cells were similar to that of EL-4 cells (Fig. 1D). These results show that ZEB1 mRNA is produced in NS cells and levels increase in response to activation in both the EL-4 cell line and primary T cells.

To investigate ZEB1 protein expression in EL-4 cells, total nuclear protein prepared from PMA/Ion-treated EL-4 cells was subjected to western blot analysis using an antibody against ZEB1 (Santa Cruz, E20). As shown in Fig. 1(E), ZEB1 protein was detected in unstimulated T cells, and following stimulation, a small (~1.5-fold) but consistent (in at least four independent experiments) increase in ZEB1 protein levels was observed between 1.5 and 8 h. No ZEB1 protein was detected in the cytoplasm in any of the tested conditions showing that ZEB1 is a constitutive nuclear protein (data not shown). The constitutively expressed nuclear transcription factor, Sp1, was used to monitor equal protein loading in all lanes.

The data show that there is an increase in ZEB1 mRNA levels following activation and that ZEB1 protein is present in the nucleus of resting EL-4 T cells with a small increase following activation.

The repressive activity of ZEB1 on the IL-2 promoter is mediated by the negative regulatory region at −100

Previous studies have shown that expression of a partial ZEB1 cDNA (referred to as Nil-2-A) encoding the C-terminal

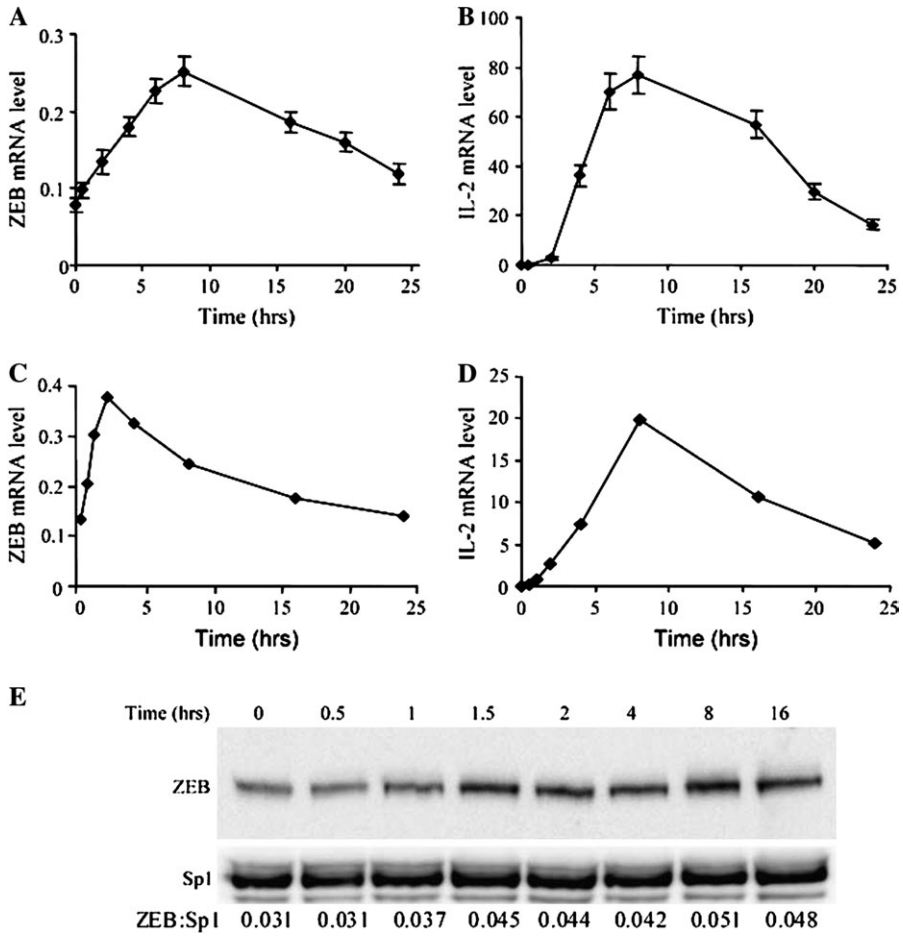

Fig. 1. Expression of ZEB1 mRNA and protein in T cells. Total RNA was prepared from EL-4 and primary T cells either unstimulated or stimulated with PMA/Ion for the indicated times. After reverse transcription, quantitative PCR analysis was performed on cDNA with primers designed to detect ZEB1 and IL-2. (A and C) Expression of the ZEB1 mRNA in EL-4 T cells (A) and primary T cells (C) was normalized to ubiquitin-conjugating enzyme (UBC) housekeeping gene. (B and D) Expression of IL-2 mRNA in EL-4 T cells (B) and primary T cells (D) is presented as fold change relative to unstimulated cells using UBC expression to normalize samples. The data in (A and B) are the means ± standard errors of three independent experiments. (C and D) show the data from a representative experiment. (E) Nuclear extracts were prepared from EL-4 T cells stimulated with PMA/Ion or unstimulated for the indicated times. Fifteen micrograms of nuclear extract of each sample was subjected to PAGE and western blotting analysis using anti-ZEB1 (upper panel) and anti-Sp1 (lower panel) antibodies. The molecular weight of ZEB1 is 170 KDa. Protein levels of both ZEB1 and Sp1 were quantified using Multi Gauga v3.0 software (Fujifilm). The ratios of ZEB1 to Sp1 are indicated on the bottom of panel (E).

Zn finger region repressed IL-2 promoter activity in Jurkat T cells (19, 20). This activity was abrogated by a mutation in NRE-A site (19). We asked if over-expression of full-length ZEB1 could repress transcriptional activation of the IL-2 promoter in EL-4 cells through the NRE-A site. A DNA fragment containing −378 to +40 of the mouse IL-2 promoter was cloned into the pXPG luciferase reporter construct (Fig. 2A). This wild-type IL-2 construct was transfected into EL-4 cells, and luciferase activity was determined after stimulation with or without PMA/Ion. As expected, a significant increase in IL-2 promoter activity was detected following treatment by PMA/Ion (Fig. 2B). When the IL-2 luciferase reporter gene construct was co-transfected with a ZEB1 expression plasmid controlled by the cytomegalovirus promoter, the transcriptional activity of the IL-2 promoter was suppressed (Fig. 2B). *P*-values calculated using paired *T* tests indicate significant decreases in IL-2 transcriptional activity upon addition of 1.5 μg ($P = 0.013$) and 3 μg ($P = 0.017$) of ZEB1 expression plasmid (Fig. 2B). The amount of full-length ZEB1 protein (170 kDa) was increased ~3-fold in the transfected cells as detected by western blots with a ZEB1 antibody (Fig. 2D).

To assess the role of the −100 NRE-A in the repression of the IL-2 promoter by ZEB1, a mutation of the NRE-A, previously shown to abolish ZEB binding *in vitro* (20), was introduced into the IL-2 promoter reporter construct (Fig. 2A). Wild-type and mutant IL-2 luciferase reporter constructs were transfected into EL-4 cells, respectively, and transfectants treated with PMA/Ion. Mutation of the ZEB-binding site resulted in an elevation of IL-2 promoter activity in both PMA/Ion-stimulated ($P = 0.026$) and unstimulated cells verifying this site as a NRE in EL-4 T cells (Fig. 2C). Overexpression of ZEB1 repressed the PMA/Ion-mediated response of the wild-type construct by ~2-fold ($P = 0.02$) but was unable to repress the PMA/Ion-induced activity of the mutant construct ($P = 0.48$) (Fig. 2C). These experiments show that full-length ZEB1 is a repressor of IL-2 promoter

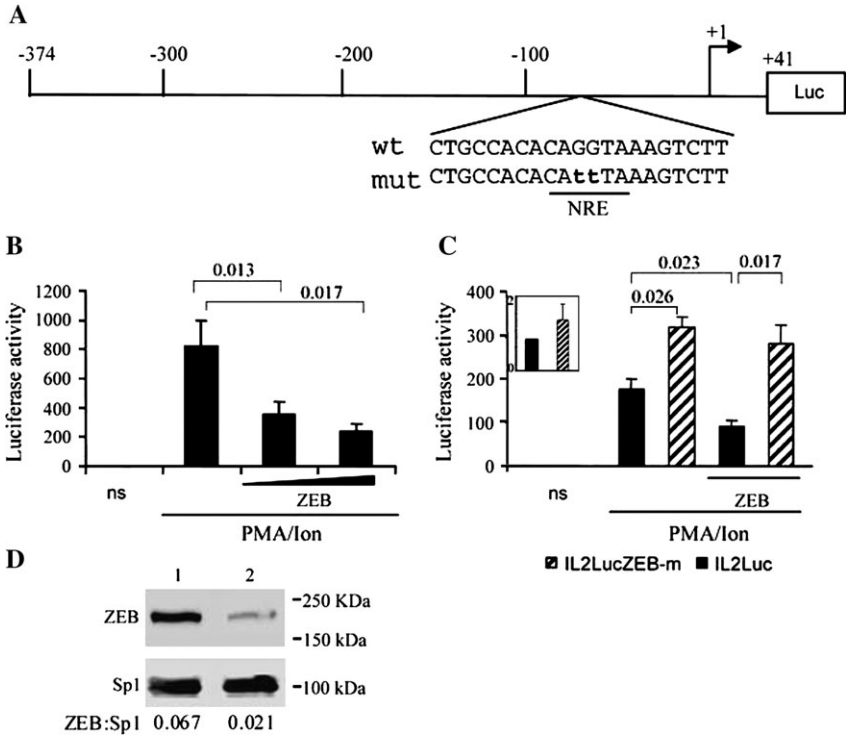

Fig. 2. Suppression of IL-2 promoter activity by ZEB1 through its binding site at −100. (A) Diagram of the proximal IL-2 promoter region showing the region cloned into the luciferase reporter plasmid. Wild-type and ZEB site mutant sequences are shown and the ZEB consensus binding site (NRE) is indicated. (B) The wild-type IL-2 luciferase reporter construct (5 μg) was transiently transfected into EL-4 T cells with two different concentrations (1.5 or 3 μg) of a plasmid expressing ZEB1 or empty expression vector to maintain total DNA transfected at 30 μg. Luciferase activity was measured 8 h following stimulation with PMA/Ion and is presented as fold change in reporter gene activity relative to a NS sample transfected with the appropriate control plasmids and the values represent the mean ± standard error of three separate experiments. Paired Student's *T* tests was used to calculate significance and those with *P*-values <0.05 are indicated on the top of panel (B). (C) Plasmids containing the wild-type (filled bars) or ZEB-binding site mutant (hashed bars) IL-2 luciferase reporter construct (5 μg) were co-transfected with the ZEB1 expression plasmid (5 μg) into EL-4 T cells. Total transfected DNA was maintained at 30 μg by addition of appropriate amounts of empty expression plasmid. Luciferase activity was calculated and presented as in panel (B). The inset figure is the enlarged picture of unstimulated EL-4 T cells transfected with the plasmids containing the wild-type (filled bars) or ZEB-binding site mutant (hashed bars) IL-2 luciferase reporter construct. *P*-values were calculated and are indicated as in (B). (D) A plasmid expressing ZEB1 (5 μg, lane 1) or empty expression vector (5 μg, lane 2) was transfected into EL-4 T cells. After overnight incubation at 37°C without stimulation, nuclear extracts were prepared. Fifteen micrograms of nuclear extract of each sample was subjected to PAGE and western blotting analysis using anti-ZEB1 (upper panel) and anti-Sp1 (lower panel) antibodies. The molecular weight of ZEB1 is 170 KDa. Protein levels of both ZEB1 and Sp1 were quantified using MultiGauga v3.0 software (Fujifilm). The ratios of ZEB1 to Sp1 are indicated on the bottom of panel (D).

function in activated EL-4 T cells and that it functions through the NRE-A at −100.

ZEB1 represses AP-1- and NFAT-mediated transactivation of the IL-2 promoter

The 300-bp proximal promoter region of the *IL-2* gene contains a variety of binding sites for known PMA/Ion inducible transcription factors, among which AP-1 and NFAT are well-documented activating factors (28). Since ZEB1 has been shown to repress individual transcription factors in the synthetic LexA/Gal4 system (21, 29), we wished to determine if ZEB1 could counteract the activating function of transcription factors in the more native context of the IL-2 promoter. To examine if ZEB1 could repress AP-1- and NFAT-mediated IL-2 promoter activity, transactivation experiments were performed using expression constructs encoding AP-1 (c-Fos and c-Jun) and NFATc. These constructs were transiently co-transfected together with the IL-2 luciferase reporter construct in the presence or absence of the ZEB1 expression construct. Both AP-1 and NFAT can transactivate the IL-2 promoter and also cooperate to generate high levels of promoter activity (Fig. 3A and B). Activation by the single or combined factors was counteracted by over-expression of ZEB1 (Fig. 3A and B). These data show that ZEB1 acts as an inhibitor of the IL-2 promoter not only in response to PMA/Ion but also when activated by specific activating transcription factors.

ZEB1 represses and binds directly to the endogenous IL-2 gene

To verify the biological significance of ZEB1 inhibition of IL-2 promoter activity, the effect of ZEB1 on expression of the endogenous *IL-2* gene was investigated. To this end, EL-4 cells were transiently transfected with a plasmid expressing either green fluorescent protein (GFP) alone or ZEB1/GFP. The GFP-positive cells, representing the transfected cells, were isolated by FACS sorting. As shown in Fig. 4(A), IL-2 mRNA levels were significantly decreased in cells

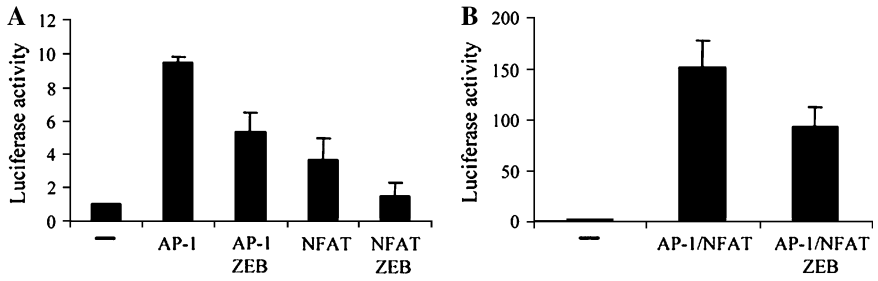

Fig. 3. ZEB1 represses transcription factor-mediated IL-2 promoter activity. EL-4 T cells were co-transfected with the IL-2 luciferase reporter plasmid (5 μg) together with 5 μg of expression plasmids for AP-1 (c-Fos + c-Jun) or NFATc (A) or AP-1 and NFATc together (B) with or without the ZEB1 expression plasmid. Total transfected DNA was maintained at 30 μg by addition of appropriate amounts of empty expression plasmid. The values represent the mean ± standard error of three separate experiments.

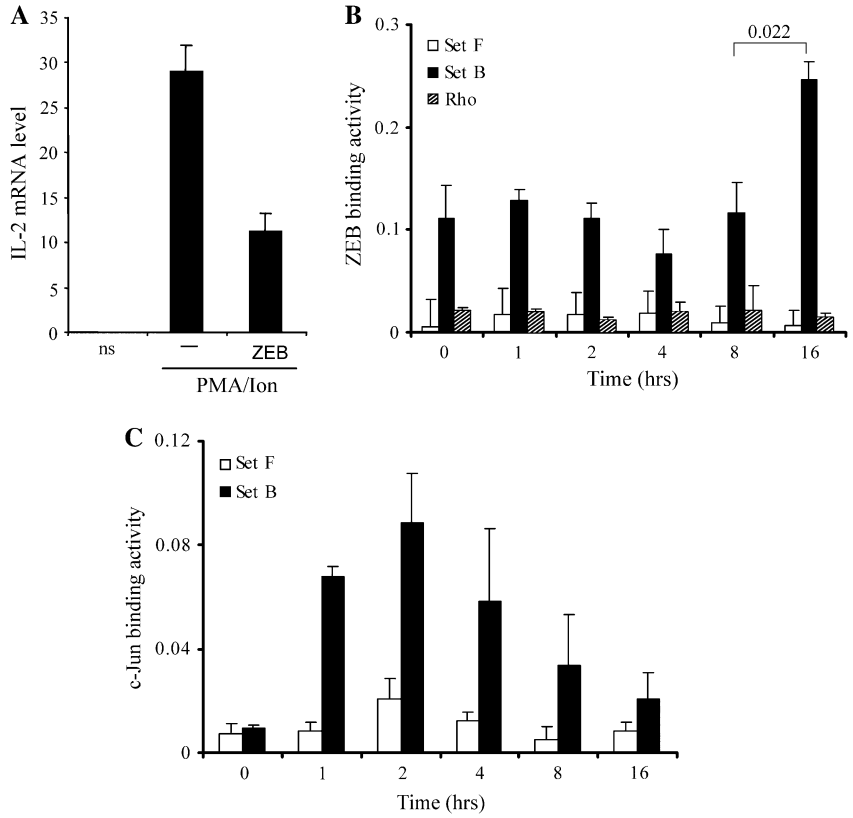

Fig. 4. ZEB1 represses endogenous IL-2 mRNA and binds to the promoter in cells. (A) EL-4 T cells were transfected with ZEB1 (5 μg) and GFP (1 μg) expression plasmids at a 5 to 1 ratio or with the GFP plasmid alone (1 μg). The cells were incubated for 20 h and subjected to FACS sorting. The GFP-positive cells were treated or untreated with PMA/Ion for 4 h. IL-2 mRNA levels were calculated and normalized to the ubiquitin-conjugating enzyme housekeeping gene and the data presented as mean ± standard error (SE) of three independent experiments. (B) ChIP assays were performed using anti-ZEB1 antibodies. The binding activity of ZEB1 was determined using real-time PCR with primer set B at the IL-2 proximal promoter region and primer set F located at −2-kb region. Rho primers were used as a control. Significant *P*-values calculated using paired *T* tests are indicated on the top of panel (C). (C) ChIP assays were performed using anti-c-Jun antibodies. The binding activity of c-Jun was determined using real-time PCR with primer set B at the IL-2 proximal promoter region and primer set F located at −2-kb region. The amount of precipitated target sequence was determined by comparing with no antibody and total input. The data are presented as mean ± SE of three separate experiments. Time refers to hours following stimulation with PMA/Ion.

transfected with the ZEB1/GFP construct compared with the cells expressing GFP alone.

To determine whether the effect of ZEB1 on IL-2 mRNA accumulation was mediated by direct binding of ZEB1 to the promoter, we examined ZEB1 binding using ChIP assays. We found that ZEB1 bound to the IL-2 proximal promoter (Set B) in resting T cells (Fig. 4B) but not to a region of the *IL-2* gene located 2 kb upstream from the promoter (Set F) nor to the promoter region of the non-expressed Rho gene (Fig. 4B). The level of ZEB1 binding remained relatively constant at both the promoter and the −2-kb region following stimulation for up to 8 h with PMA/Ion and then doubled

at 16 h after activation (P = 0.02) (Fig. 4B). This trend was observed neither at −2 kb nor at a control *Rho* gene (Fig. 4B). This pattern of binding is in contrast to the binding of an activator, c-Jun, which showed a sharp increase at 1–2 h followed by a decrease in binding across the time course measured (Fig. 4C).

Taken together, these data show that ZEB1 directly binds to and represses the endogenous *IL-2* gene in EL-4 T cells.

ZEB1 cooperates with CtBP2 and HDAC1 to repress IL-2 promoter activity

Recently, ZEB1 has been shown to interact strongly with the co-repressor CtBP and to cooperate with CtBP to repress activity in reporter constructs containing multiple ZEB-binding E boxes or where ZEB1 is recruited as a Gal4 fusion protein (21, 30). In addition, a CtBP-containing repressor complex has been isolated from HeLa cells and shown to contain ZEB1 and ZEB2, the histone-modifying enzyme HDAC1, the histone methyltransferase EuHMTase1 and the chromodomain-containing protein CoREST (31). This led us to speculate that ZEB1 may also interact with components of this complex in EL-4 T cells to repress *IL-2* gene transcription. Transfection of CtBP2 or HDAC1 alone into EL-4 cells repressed the PMA/Ion-induced IL-2 promoter activity (Fig. 5A). Co-transfection of ZEB1 with CtBP2 increased the repressive effect of each factor alone but interestingly ZEB1 and HDAC1 co-expression did not lead to a cooperative effect (Fig. 5A). Co-transfection of HDAC1 and CtBP2 together had a stronger inhibitory effect that either factor alone and when combined with ZEB1 led to an even stronger repression of promoter activity (Fig. 5A).

We also tested the effects of these co-repressors on the IL-2 promoter containing the mutant ZEB site (Fig. 2A). Even though ZEB1 could not repress the activity of this mutant construct, as shown above, CtBP2 and HDAC1 over-expression could inhibit PMA/Ion-mediated transcription activation of this IL-2 mutant plasmid (Fig. 5B). However, there was little or no evidence for cooperative repression activity of these proteins with ZEB1 on the mutant construct (Fig. 5A), indicating that the CtBP and HDAC co-repressor complexes can be recruited to the IL-2 promoter via other DNA-bound

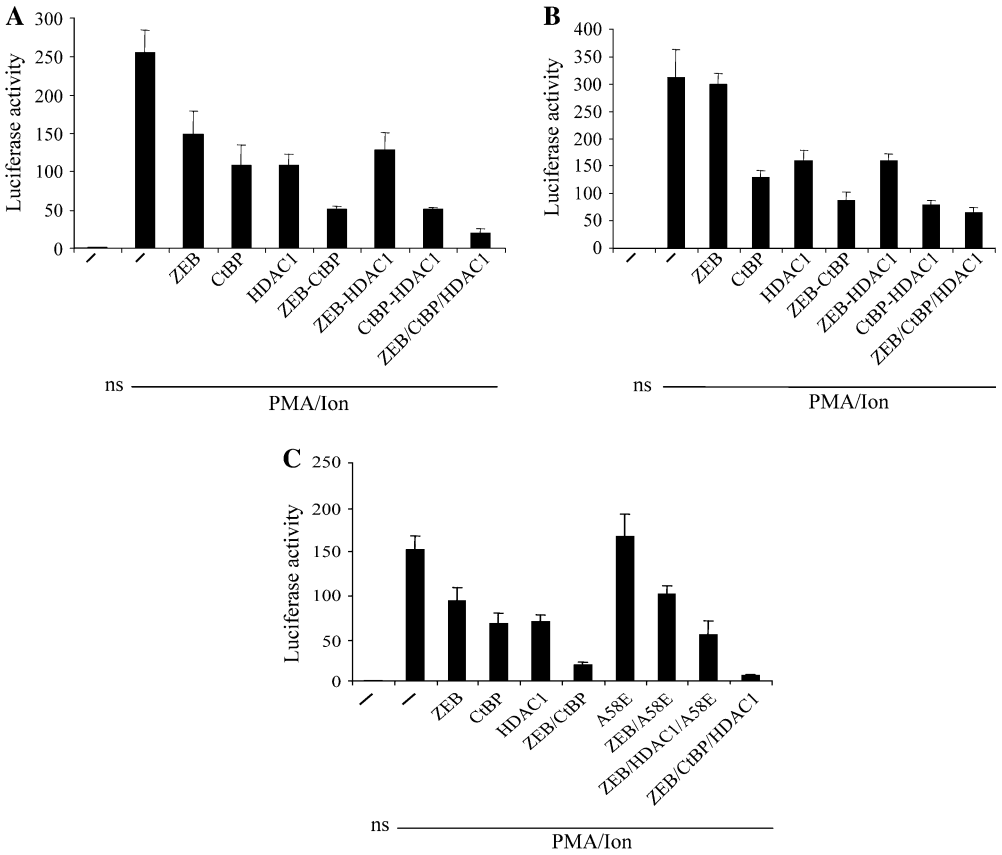

Fig. 5. Cooperation between ZEB1, CtBP and HDAC1 in repression of the IL-2 promoter. (A) The wild-type IL-2 luciferase reporter plasmid (5 μg) was transiently transfected together with ZEB1, CtBP or HDAC1 expression plasmids (5 μg each) or different combinations of these factors into EL-4 T cells. Total transfected DNA was maintained at 30 μg by addition of appropriate amounts of empty expression plasmid. The transfected cells were incubated for 20 h at 37°C followed by stimulation with PMA/Ion for 8 h. Luciferase activity is presented as fold change relative to the reporter gene co-transfected with the empty expression plasmid and the values represent the mean ± standard error of three separate experiments. (B). The ZEB site mutant reporter plasmid (5 μg) was co-transfected together with ZEB1, CtBP or HDAC1 (5 μg each) or different combinations of these factors into EL-2 T cells and treated as described in (A). The data are calculated and presented as in panel (A). (C) The wild-type IL-2 luciferase reporter construct (5 μg) was co-transfected with 5 μg each of the indicated expression plasmids including wild-type CtBP and CtBP mutant (A58E) into EL-4 T cells. The data are calculated and presented as in panel (A).

transcription factors but that the functional interaction of ZEB1 with CtBP2 and/or HDAC1 requires an intact ZEB-binding site.

These results demonstrate that ZEB1-mediated repression of the IL-2 promoter may involve the interaction of ZEB1 with CtBP2 and HDAC1.

Mutation of the PXDLS-binding cleft in CtBP abrogates its repression of the IL-2 promoter

CtBP consists of three domains: the substrate-binding domain comprising the PXDLS-binding cleft; the central domain required for nicotinamide adenine dinucleotide binding and a C-terminal region (22). CtBP acts as a co-repressor by interacting with sequence-specific DNA-binding repressor proteins which contain PXDLS motifs. This motif is present in many transcription factors including ZEB1 (26). Since ZEB1 can cooperate with CtBP to inhibit IL-2 promoter activity, we tested whether this required the PXDLS-binding pocket in CtBP and involve a direct protein–protein interaction. The IL-2 reporter construct was co-transfected with wild-type and cleft mutant CtBP2 (A58E) together with ZEB1. As shown in Fig. 5(C), the CtBP2 cleft mutant could not function as a repressor and could not cooperate with ZEB1 to repress IL-2 promoter activity. In addition, this CtBP2 mutant did not cooperate in a complex with ZEB1 and HDAC1 to repress *IL-2* gene activation (Fig. 5C).

These results suggest that the PXDLS-binding cleft of CtBP2 is critical for ZEB1-mediated *IL-2* gene repression and suggests that direct protein–protein interaction between ZEB1 and CtBP is required. In addition, it appears that HDAC1 cooperation with ZEB1 requires this ZEB1–CtBP interaction.

Discussion

Here we report that the Zn finger homeodomain transcription factor, ZEB1, can cooperate with CtBP2 and HDAC1 to repress IL-2 expression in T cells through a specific repressor element in the IL-2 promoter.

It was previously demonstrated that a NRE at −100 in the IL-2 proximal promoter could bind ZEB1 protein in *in vitro* binding assays and it was shown that expression of antisense ZEB1 led to increased promoter activity (20). Expression of a truncated cDNA encoding the C-terminal end of the protein was able to repress the IL-2 promoter in a manner dependent on the NRE-A implying that the C-terminal Zn fingers could bind to the NRE-A (19). We confirmed here that the NRE-A acts as a repressor element in the EL-4 mouse T cell line and in addition showed that the ability of full-length ZEB1 to repress IL-2 promoter activity also requires the intact NRE-A. ZEB1 has also been shown to counteract the activating function of a range of transcription factors in a LexA/Gal4 reporter system (21, 29, 32). We demonstrate here that this is also true in the context of the intact IL-2 promoter where AP-1 and NFAT activity was inhibited by over-expression of ZEB1. These proteins bind to sites both upstream and downstream of the NRE-A (3, 4) and inhibition by ZEB1 suggests a trans-repression model rather than a direct competition model.

Most importantly, we have demonstrated that ZEB1 can repress inducible expression from the endogenous *IL-2* gene and that ZEB1 binds to the IL-2 promoter in EL-4 T cells. The presence of ZEB1 protein in the nucleus and its association with the *IL-2* gene in resting T cells suggest that ZEB1 may play a role in maintaining the gene in a repressed state in these cells. We have previously shown that the ZEB1-binding site is inaccessible to RE digestion in resting T cells and the strength of the activating signal affected the level of RE accessibility at this site, leading to the speculation that a repressor complex resides in this region (8). In the EL-4 cell context, ZEB1 binds to the promoter in NS T cells, decreases slightly following activation and increases at later times, a pattern of binding in contrast to an activator, c-Jun, suggesting a role in repression. It would be of interest to examine ZEB1 binding following different strength signals in T cells.

We have demonstrated here that ZEB1 can function with CtBP and with HDAC1 to repress IL-2 promoter activity. The cooperation of ZEB1 with HDAC1 requires the co-expression of CtBP2 leading to the speculation that CtBP2 may behave as a scaffold protein between ZEB1 and HDAC1. There is evidence that CtBP acts as a scaffold protein in repressor complexes (26). The cooperation between ZEB1, CtBP2 and HDAC1 requires an intact NRE-A site, indicating that ZEB1 binding to its cognate site in the promoter is required for these functional interactions. However, CtBP and HDAC1 alone can still repress the NRE-A mutant construct, indicating that these proteins can be recruited to the IL-2 promoter via several mechanisms.

ZEB1 has been shown to function as a repressor by either direct competition with transcription activators for DNA binding or interaction with and recruitment of co-repressor complexes (17, 30). The former activity depends on its zinc finger clusters (32) while the latter also requires the PXDLS motif known to interact with a specific PXDLS-binding cleft in CtBP (21). We demonstrate here that the ability of ZEB1 to recruit co-repressor complexes is important for repression of the IL-2 promoter since a CtBP2 protein with a mutation in the PXDLS-binding cleft (A58E) was unable to cooperate with ZEB1 to repress IL-2 promoter activity. The CtBP2 mutant (A58E) also failed to cooperate in the three-ways ZEB1, CtBP2 and HDAC1 repression. Thus, the function of ZEB1 at the IL-2 promoter may be to recruit CtBP through interactions with the PXDLS motif and CtBP in turn may recruit other repressor proteins.

ZEB1-deficient mice have two major defects: in thymocyte development and in skeletal development. ZEB1-null mutant mice exhibit both defects (25), whereas mice lacking the C-terminal Zn finger domain, showed only the thymocyte defect (15). The ability of ZEB1 to repress *IL-2* gene transcription may relate to one of the major defects seen in ZEB1-deficient mice since IL-2 plays a role in thymocyte development (15). ZEB1, however, has also been identified as a transcription repressor for other genes involved in T cell function such as CD4, GATA-3 and α7-integrin (18) and it remains to be determined which activities of ZEB1 are critical for correct thymocyte development. ZEB1 may also play a role in the skewed expression of IL-2 in T_h1 cells

compared with T_h2 cells since ZEB1-binding activity is higher in T_h2 cells than that in T_h1 cells (20).

In summary, ZEB1 acts as a specific repressor of *IL-2* gene transcription and functions in cooperation with the CtBP2 and HDAC1 co-repressors. It will be of interest to examine the function of these complexes during T cell development and in T cell subsets such as anergic and regulatory T cells where IL-2 is specifically repressed.

Funding

National Health and Medical Research Council project grant; Australia Research Council Discovery grant to M.F.S.

Acknowledgements

We thank Y. Higashi for the ZEB plasmid, M. Crossley for the CtBP2 and CtBP2 mutant constructs, S. Ghosh for HDAC1 plasmid, S. Palmer for technical assistance and Jun Fan for help in the preparation of the manuscript.

Abbreviations

AP-1	activated protein-1
APC	antigen-presenting cell
ChIP	chromatin immunoprecipitation
CtBP	C-terminal-binding protein
GFP	green fluorescent protein
HDAC1	histone deacetylase 1
Ion	ionorphore
NFAT	nuclear factor of activated T cells
NRE-A	negative regulatory element-A
NS	non-stimulated
PMA	phorbol 12-myristate 13-acetate
PXDLS	Pro-X-Asp-Leu-Ser
RE	restriction enzyme
Rho	rhodopsin
ZEB	zinc finger E-box-binding protein

References

1 Gesbert, F., Delespine-Carmagnat, M. and Bertoglio, J. 1998. Recent advances in the understanding of interleukin-2 signal transduction. *J. Clin. Immunol.* 18:307.

2 Rothenberg, E. V. and Ward, S. B. 1996. A dynamic assembly of diverse transcription factors integrates activation and cell-type information for interleukin 2 gene regulation. *Proc. Natl Acad. Sci. USA* 93:9358.

3 Jain, J., Loh, C. and Rao, A. 1995. Transcriptional regulation of the IL-2 gene. *Curr. Opin. Immunol.* 7:333.

4 Serfling, E., Avots, A. and Neumann, M. 1995. The architecture of the interleukin-2 promoter: a reflection of T lymphocyte activation. *Biochim. Biophys. Acta* 1263:181.

5 Jain, J., Valge-Archer, V. E. and Rao, A. 1992. Analysis of the AP-1 sites in the IL-2 promoter. *J. Immunol.* 148:1240.

6 Jain, J., Valge-Archer, V. E., Sinskey, A. J. and Rao, A. 1992. The AP-1 site at -150 bp, but not the NF-kappa B site, is likely to represent the major target of protein kinase C in the interleukin 2 promoter. *J. Exp. Med.* 175:853.

7 Rooney, J. W., Sun, Y. L., Glimcher, L. H. and Hoey, T. 1995. Novel NFAT sites that mediate activation of the interleukin-2 promoter in response to T-cell receptor stimulation. *Mol. Cell. Biol.* 15:6299.

8 Rao, S., Gerondakis, S., Woltring, D. and Shannon, M. F. 2003. c-Rel is required for chromatin remodeling across the IL-2 gene promoter. *J. Immunol.* 170:3724.

9 Rao, S., Procko, E. and Shannon, M. F. 2001. Chromatin remodeling, measured by a novel real-time polymerase chain reaction assay, across the proximal promoter region of the IL-2 gene. *J. Immunol.* 167:4494.

10 Chen, X., Wang, J., Woltring, D., Gerondakis, S. and Shannon, M. F. 2005. Histone dynamics on the interleukin-2 gene in response to T-cell activation. *Mol. Cell. Biol.* 25:3209.

11 Schule, R., Rangarajan, P., Kliewer, S. *et al.* 1990. Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. *Cell* 62:1217.

12 Butscher, W. G., Haggerty, C. M., Chaudhry, S. and Gardner, K. 2001. Targeting of p300 to the interleukin-2 promoter via CREB-Rel cross-talk during mitogen and oncogenic molecular signaling in activated T-cells. *J. Biol. Chem.* 276:27647.

13 Postigo, A. A., Ward, E., Skeath, J. B. and Dean, D. C. 1999. zfh-1, the Drosophila homologue of ZEB, is a transcriptional repressor that regulates somatic myogenesis. *Mol. Cell. Biol.* 19:7255.

14 Postigo, A. A. and Dean, D. C. 1999. Independent repressor domains in ZEB regulate muscle and T-cell differentiation. *Mol. Cell. Biol.* 19:7961.

15 Higashi, Y., Moribe, H., Takagi, T. *et al.* 1997. Impairment of T cell development in deltaEF1 mutant mice. *J. Exp. Med.* 185:1467.

16 Brabletz, T., Jung, A., Hlubek, F. *et al.* 1999. Negative regulation of CD4 expression in T cells by the transcriptional repressor ZEB. *Int. Immunol.* 11:1701.

17 Gregoire, J. M. and Romeo, P. H. 1999. T-cell expression of the human GATA-3 gene is regulated by a non-lineage-specific silencer. *J. Biol. Chem.* 274:6567.

18 Jethanandani, P. and Kramer, R. H. 2005. Alpha7 integrin expression is negatively regulated by deltaEF1 during skeletal myogenesis. *J. Biol. Chem.* 280:36037.

19 Williams, T. M., Moolten, D., Burlein, J. *et al.* 1991. Identification of a zinc finger protein that inhibits IL-2 gene expression. *Science* 254:1791.

20 Yasui, D. H., Genetta, T., Kadesch, T. *et al.* 1998. Transcriptional repression of the IL-2 gene in Th cells by ZEB. *J. Immunol.* 160:4433.

21 Furusawa, T., Moribe, H., Kondoh, H. and Higashi, Y. 1999. Identification of CtBP1 and CtBP2 as corepressors of zinc finger-homeodomain factor deltaEF1. *Mol. Cell. Biol.* 19:8581.

22 Bodor, J., Feigenbaum, L., Bodorova, J., Bare, C., Reitz, M. S. Jr and Gress, R. E. 2001. Suppression of T-cell responsiveness by inducible cAMP early repressor (ICER). *J. Leukoc. Biol.* 69:1053.

23 Thomas, R. S., Tymms, M. J., McKinlay, L. H., Shannon, M. F., Seth, A. and Kola, I. 1997. ETS1, NFkappaB and AP1 synergistically transactivate the human GM-CSF promoter. *Oncogene* 14:2845.

24 Wang, J., Shannon, M. F. and Young, I. G. 2006. A role for Ets1, synergizing with AP-1 and GATA-3 in the regulation of IL-5 transcription in mouse Th2 lymphocytes. *Int. Immunol.* 18:313.

25 Takagi, T., Moribe, H., Kondoh, H. and Higashi, Y. 1998. DeltaEF1, a zinc finger and homeodomain transcription factor, is required for skeleton patterning in multiple lineages. *Development* 125:21.

26 Quinlan, K. G., Verger, A., Kwok, A. *et al.* 2006. Role of the C-terminal binding protein PXDLS motif binding cleft in protein interactions and transcriptional repression. *Mol. Cell. Biol.* 26:8202.

27 Schreiber, E., Matthias, P., Muller, M. M. and Schaffner, W. 1989. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res.* 17:6419.

28 Bunting, K., Wang, J. and Shannon, M. F. 2006. Control of interleukin-2 gene transcription: a paradigm for inducible, tissue-specific gene expression. *Vitam. Horm.* 74:105.

29 Postigo, A. A., Depp, J. L., Taylor, J. J. and Kroll, K. L. 2003. Regulation of Smad signaling through a differential recruitment of coactivators and corepressors by ZEB proteins. *EMBO J.* 22:2453.

30 Postigo, A. A. and Dean, D. C. 1999. ZEB represses transcription through interaction with the corepressor CtBP. *Proc. Natl Acad. Sci. USA* 96:6683.

31 Shi, Y., Sawada, J., Sui, G. *et al.* 2003. Coordinated histone modifications mediated by a CtBP co-repressor complex. *Nature* 422:735.

32 Postigo, A. A. 2003. Opposing functions of ZEB proteins in the regulation of the TGFbeta/BMP signaling pathway. *EMBO J* 22:2443.