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Opposing functions of ZEB proteins in the regulation of the TGFβ/BMP signaling pathway

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Binding of TGFβ/BMP factors to their receptors leads to translocation of Smad proteins to the nucleus where they activate transcription of target genes. The two-handed zinc finger proteins encoded by *Zfhx1a* and *Zfhx1b*, ZEB-1/δEF1 and ZEB-2/SIP1, respectively, regulate gene expression and differentiation programs in a number of tissues. Here I demonstrate that ZEB proteins are also crucial regulators of TGFβ/BMP signaling with opposing effects on this pathway. Both ZEB proteins bind to Smads, but while ZEB-1/δEF1 synergizes with Smad proteins to activate transcription, promote osteoblastic differentiation and induce cell growth arrest, the highly related ZEB-2/SIP1 protein has the opposite effect. Finally, the ability of TGFβ to mediate transcription of TGFβ-dependent genes and induce growth arrest depends on the presence of endogenous ZEB-1/δEF1 protein.

Keywords: Smad proteins/TGFβ/BMP signaling/ transcriptional regulation/ZEB-1/δEF1/ZEB-2/SIP1

Introduction

The TGFβ family comprises over 30 different cytokines including TGFβs, BMPs, GDFs and activin and regulates a wide array of biological processes (Massagué and Chen, 2000; Massagué *et al*., 2000). Binding of these factors to transmembrane type II receptors leads to the phosphorylation of specific type I receptors (also known as activin-like kinases, ALKs), which in turn phosphorylate activating R-Smad proteins (receptor-regulated Smads) (Attisano and Wrana, 2000; Massagué and Wotton, 2000; ten Dijke *et al*., 2000; Miyazono *et al*., 2001). In vertebrates, R-Smads include Smad2 and Smad3 (which are activated in response to TGFβ and activins) and Smad1, Smad5 and Smad8 (which are specifically regulated by BMP and GDF factors) (Attisano and Wrana, 2000; ten Dijke *et al*., 2000; Miyazono *et al*., 2001). Once activated, R-Smads bind Smad4 and the complexes translocate to the nucleus where they bind to short Smad-binding elements in the promoters of responsive genes (Shi *et al*., 1998; Zawel *et al*., 1998). A third group of Smad proteins, I-Smads or inhibitory Smads, including Smad6 and Smad7, antagonize R-Smads (Christian and Nakayama, 1999).

TGFβ proteins are involved in a wide variety of functions during development and differentiation. Interestingly, TGFβ cytokines can both promote or inhibit cell growth, depending on the cell type and, while BMP-4 ventralizes *Xenopus* embryos, activin and BMP-3 have a dorsalizing effect (Koskinen *et al*., 1991; Jones *et al*., 1992; Daluiski *et al*. 2001). How can such a relatively simple signal transduction scheme account for these pleiotropic (and sometimes antagonistic) biological effects of the TGFβ family? In the last few years, several reports have begun to address this question. A number of proteins that interact with Smads and modulate their transcriptional activity have been identified (reviewed in Massagué and Wotton, 2000; Miyazono *et al*., 2001). Some of these factors (e.g. TGIF, Ski/SnoN, Evi-1 and SNIP1) inhibit Smad-mediated transcription, whereas others (e.g. FAST, c-jun, TFE-III Mixer/Mix and OAZ) synergize with Smads (Chen *et al*., 1997; Kurokawa *et al*., 1998; Labbe *et al*., 1998; Zhang *et al*., 1998; Hua *et al*., 1998; Luo *et al*., 1999; Sun *et al*., 1999; Wotton *et al*., 1999; Hata *et al*., 2000; Wang *et al*., 2000). These Smad regulatory factors vary in their scope of action; whereas OAZ specifically synergizes with BMP-2 to activate the *Xenopus Vent-2* gene (but not other BMP-responsive genes), Ski/SnoN block both TGFβ and BMP signaling pathways in a more general fashion (Luo *et al*., 1999; Sun *et al*., 1999; Hata *et al*., 2000; Wang *et al*., 2000).

Some Smad coactivators (e.g. FAST, Mix proteins, c-jun, TFE-III, OAZ, etc.) appear to mediate their regulatory activities through binding to specific DNA sites and, therefore, targeting a particular subset of TGFβ/BMP-responsive genes (Miyazono *et al*., 2001). In this report we provide evidence that the two vertebrate members of the zfh-1 family of two-handed zinc finger factors, ZEB-1/δEF1 (encoded by the *Zfhx1a* gene) and ZEB-2/SIP1 (encoded by the *Zfhx1b* gene), have opposing effects on TGFβ/BMP signaling. Both ZEB proteins share a central repressor domain located in between the N- and C-terminal DNA-binding zinc finger clusters (Figure 1A). The repressor domain of both ZEB proteins interacts with the corepressor CtBP (Postigo and Dean, 1999a, 2000), which is also essential for the activity of other key developmental regulatory proteins (reviewed in Chinnadurai, 2002). ZEB proteins have been shown to repress gene expression in several cell types: in hematopoietic cells, ZEB-1/δEF1 negatively regulates the expression of interleukin 2, immunoglobulin μ heavy chain, CD4, GATA-3 and α4 integrin (Williams *et al*., 1991; Genetta *et al*., 1994; Postigo and Dean, 1997, 1999b; Postigo *et al*., 1997; Brabletz *et al*., 1999; Gregoire and Romeo, 1999); in mesenchymal cells, ZEB-1/δEF1 inhibits p73 gene expression (Fontemaggi *et al*., 2001); in osteoblasts, ZEB-1/δEF1 protein represses type I and type II collagen expression (Murray *et al*., 2000; Terraz *et al*., 2001) and both ZEB proteins repress the E cadherin gene, promoting tumor invasion in epithelial tumors

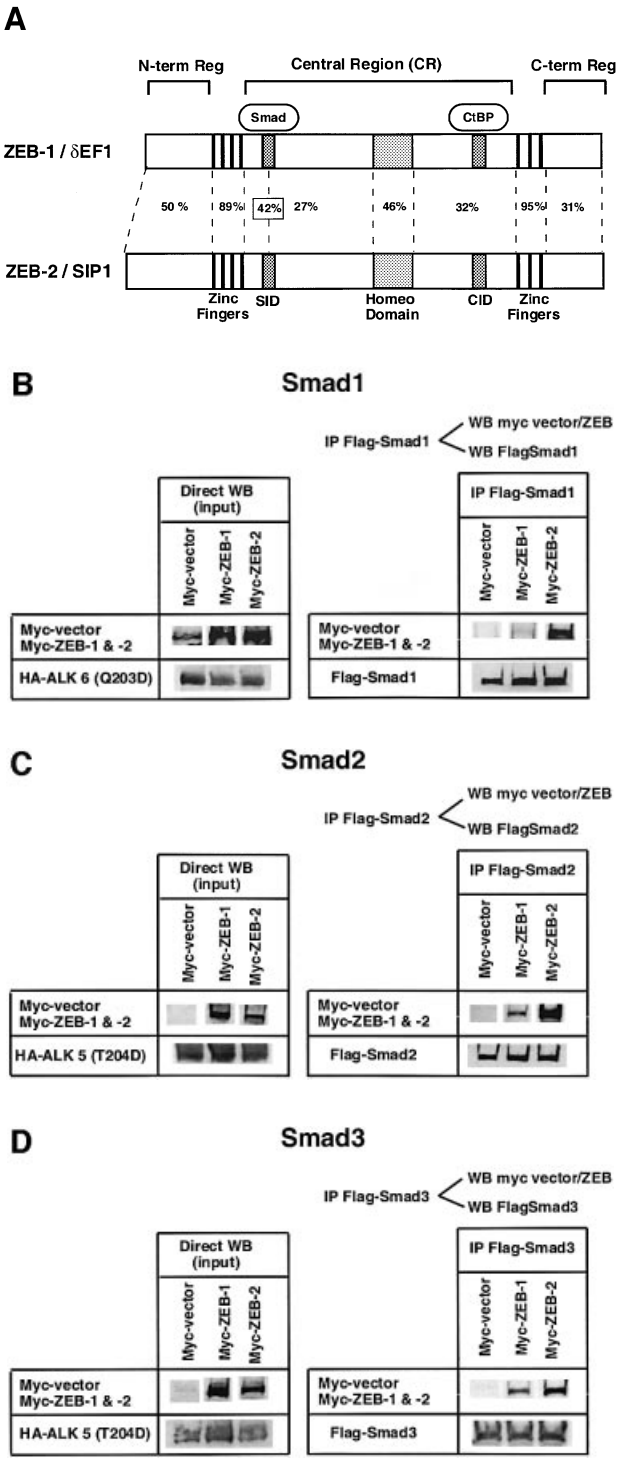

Fig. 1. (**A**) Schematic representation of the ZEB family (ZEB-1/δEF1 and ZEB-2/SIP1) of two-handed zinc finger factors. The central region (CR) in between both zinc finger clusters act as a repressor domain in part through the recruitment of the CtBP corepressor through a CID. 3′ of the N-terminal zinc fingers there is a region that acts as a SID. (B, C and D) ZEB-1/δEF1 and ZEB-2/SIP1 bind to activated R-Smads. 293T cells were cotransfected with the indicated expression vectors: 3 μg of either Flag-tagged Smad1, Smad2 or Smad3, 4 μg of either myc-tagged full-length human ZEB-1/δEF1 or ZEB-2/SIP1 and 0.8 μg of the corresponding constitutively active hemagglutinin-tagged constitutively active ALKs ALK6 (Q203D) for Smad1 (**B**) and ALK5 (T204D) for Smad2 and Smad3 (**C** and **D**). After 48 h, cells lysates were immunoprecipitated (IP) for Flag-Smads and the co-immunoprecipitated ZEB-1/δEF1 and ZEB-2/SIP1 was detected by western blotting (WB) with 9E10 myc antibody. The input represents 15% of the lysate.

(Grooteclaes and Frisch, 2000; Comijn *et al*., 2001). Interestingly, ZEB-1/δEF1 protein has also been shown to activate the expression of the ovalbumin and vitamin D3 receptor genes in some cell types, although the molecular mechanism behind this activation effect is unknown (Chamberlain and Sanders, 1999; Lazarova *et al*., 2001; Dillner and Sanders, 2002).

Interestingly, ZEB-2/SIP1 has been shown to interact with R-Smad (Verschueren *et al*., 1999) and its expression in *Xenopus* induces neuralization of the embryos and blocks the expression of the activin-dependent *Brachyury* gene (Remacle *et al*., 1999; Verschueren *et al*., 1999; Eisakei *et al*., 2000; Lerchner *et al*., 2000). However, no additional information is available regarding the role of ZEB-2/SIP1 in TGFβ/BMP signaling or its mechanism of action. On the other hand, mice carrying a targeted deletion of the ZEB-1/δEF-1 gene present a number of skeletal defects that show a striking parallel with the phenotype of knock out animals for TGFβ gene family members (e.g. BMP-5 and GDF-5), or genes regulated by this family (Msx.1 and Hoxa-13), suggesting that ZEB-1/δEF1 may also play a role in this signaling pathway (Takagi *et al*., 1998). Patients with heterozygous mutations of the ZEB-2/SIP1 gene present a Mowat–Wilson syndrome with mental retardation, multiple congenital anomalies and, in most cases, Hirschsprung disease (Cacheux *et al*., 2001; Wakamatsu *et al*., 2001; Zweier *et al*., 2002).

All the above results suggest that both ZEB proteins may have important roles in the regulation of Smad signaling. Here, I provide evidence that indeed both proteins bind Smads and regulate TGFβ/BMP signaling in opposing ways. Whereas ZEB-1/δEF1 synergizes with Smads to regulate a number of functions (transcription, differentiation and cell growth arrest), ZEB-2/SIP1 represses Smad functions. Moreover, we provide evidence that TGFβ depends on endogenous ZEB-1/δEF1 to mediate some of its actions.

Results

ZEB-1/δEF1 and ZEB-2/SIP1 interact with ligand-activated Smads

The ability of ZEB-2/SIP1 to interact with activated R-Smads (Verschueren *et al*., 1999) and the phenotypic resemblance of mice mutant for ZEB-1/δEF1 with those mutant for some TGFβ family members (or their target genes) (Takagi *et al*., 1998) suggested that ZEB proteins could be involved in regulating TGFβ signaling. Further supporting this hypothesis, we found that several genes regulated by TGFβ/BMP have ZEB-binding sites in close proximity to the Smad sites (unpublished observations).

I decided to investigate whether ZEB-1/δEF1 could also bind R-Smads. In co-immunoprecipitation experiments ZEB-1/δEF1 did indeed interact with Smad1, 2 and 3 (Figure 1B–D). It is of note that for all three Smads, ZEB-2/SIP1 seemed to bind more efficiently than ZEB-1/δEF1. This interaction requires the phosphorylation of R-Smad as no binding of either ZEB-1/δEF1 or ZEB-2/SIP1 was detected in the absence of the constitutively active type I receptors (Figure 2A–C).

The interaction between ZEB-1/δEF1 and R-Smads was also confirmed at the endogenous level. Endogenous

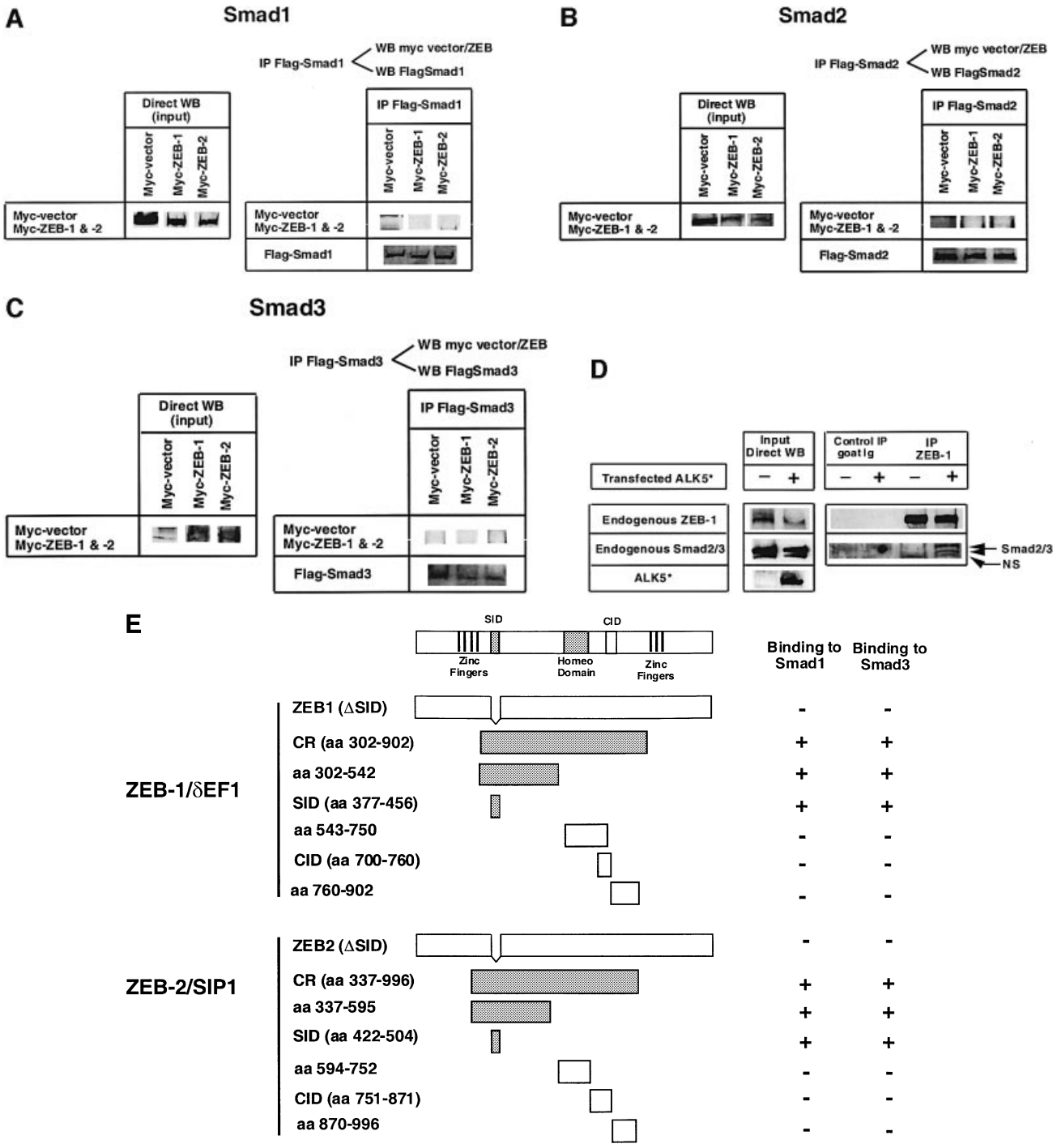

Fig. 2. (**A**–**C**) Interaction of ZEB proteins with Smad1, Smad2 and Smad3 requires TGFβ signaling. Experiments were performed as in Figure 1B–D, but in the absence of the corresponding constitutively active ALKs ALK5 (T204D) and ALK6 (Q203D). (**D**) Interaction between endogenous ZEB-1/δEF1 and Smad2 and Smad3. Cell lysates from 293T cells immunoprecipitated with either an antibody against ZEB-1/δEF1 or a control antibody (goat-Ig) in the presence or absence of 20 μg of an expression vector for ALK5 (T204D). Proteins immunoprecipitated by ZEB-1/δEF1 were then western blotted for associated endogenous Smad2 and Smad3. NS indicates a non-specific band. (**E**) The same region in ZEB-1/δEF1 and ZEB-2/SIP1 (Smad-interacting region, SID) mediates their binding to activated Smad1 and Smad3. The indicated myc-tagged constructs for ZEB-1/δEF1 and ZEB-2/SIP1 (see Materials and methods for further details) were tested for their ability to interact with Smad1 and Smad3 in the presence of either constitutively active ALK6 (Q203D) and ALK5 (T204D), respectively.

ZEB-1/δEF1 was able to interact with Smad2/Smad3, but only when TGFβ signaling was activated by cotransfection of constitutively active TGFβ-RI [(ALK5 (T204D)] (Figure 2D).

I proceeded to identify the region within ZEB-1/δEF1 responsible for the interaction. As shown in Figure 2E, a region located downstream of the N-terminal zinc fingers cluster acted as the Smad-interacting domain (SID) (Verschueren *et al.*, 1999) and its deletion abolished binding of both ZEB proteins to Smad1 and Smad3. The SID is highly conserved across species within both ZEB-1/δEF1 and ZEB-2/SIP1. The homology between the human

clones of both ZEB proteins is 42%. Similar levels of expression for wild-type ZEB proteins and their deletion mutants were detected (data not shown).

ZEB-1/δEF1 and ZEB-2/SIP1 have opposite effects on TGFβ/BMP-mediated transcription

Studies from several groups have shown that both ZEB proteins are active transcriptional repressors (Postigo *et al*., 1997; Sekido *et al*., 1997; Remacle *et al*., 1999; Postigo and Dean, 2000). I decided to investigate whether the interaction of ZEB proteins with R-Smads could repress TGFβ-mediated transcription. Indeed, it was found that ZEB-2/SIP1 inhibited activation by TGFβ of the 3TP, p21, p15 and c-jun promoter reporters (Figure 3A and B and data not shown). Surprisingly, ZEB-1/δEF1 synergized with TGFβ1 to activate transcription of these same reporters (Figure 3A and B; data not shown). Both effects were dependent on TGFβ signaling, as neither ZEB-1/δEF1or ZEB-2/SIP1 significantly affected the basal transcription of these reporters in the absence of TGFβ.

Then, I investigated whether a similar pattern of transcriptional regulation existed for BMP-2 signaling. This is important because the BMP subfamily uses a different set of R-Smads (Smad1, Smad5 and Smad8) than the TGFβ/activin subfamily to mediate its effects and also because of the similarity in the phenotype of ZEB-1/δEF1(–/–) mice to BMP-5 (–/–) and GDF-5 (–/–) mice. As shown in Figure 3C, ZEB proteins also showed opposing effects on the BMP-2-mediated activation of a reporter containing the promoter of the *Xenopus Vent-2* gene. Together, these results indicate that ZEB-1/δEF1 can augment transcriptional activation by both TGFβ1 and BMP-2, whereas ZEB-2/SIP1 has the opposite effect.

Regulation of TGFβ-mediated transcription by ZEB proteins requires an active R-Smad–Smad4 complex

The Smad3 (D470E) mutant has been shown to act as a Smad3 dominant-negative and to block TGFβ-mediated transcription by displacing endogenous (wild-type) Smad3 from its DNA-binding sites (Goto *et al*., 1998). Expression of Smad3 (D470E) also prevented the synergistic effect of ZEB-1/δEF1 in the activation of a TGFβ-dependent promoter (Figure 3D). Since Smad3 (D470E) completely blocked TGFβ-mediated transcription, it was not possible to assess any further effect by ZEB-2/SIP1. This result indicates that the ability of ZEB-1/δEF1 to modulate TGFβ/BMP-mediated transcription depends on functionally active R-Smads. The effect of both ZEB proteins was found to be independent of the way both Smads and ZEB proteins were brought to the promoter, as the transcriptional activity of Smad3 tethered directly to DNA as a Gal4 fusion protein was also augmented by ZEB-1/δEF1

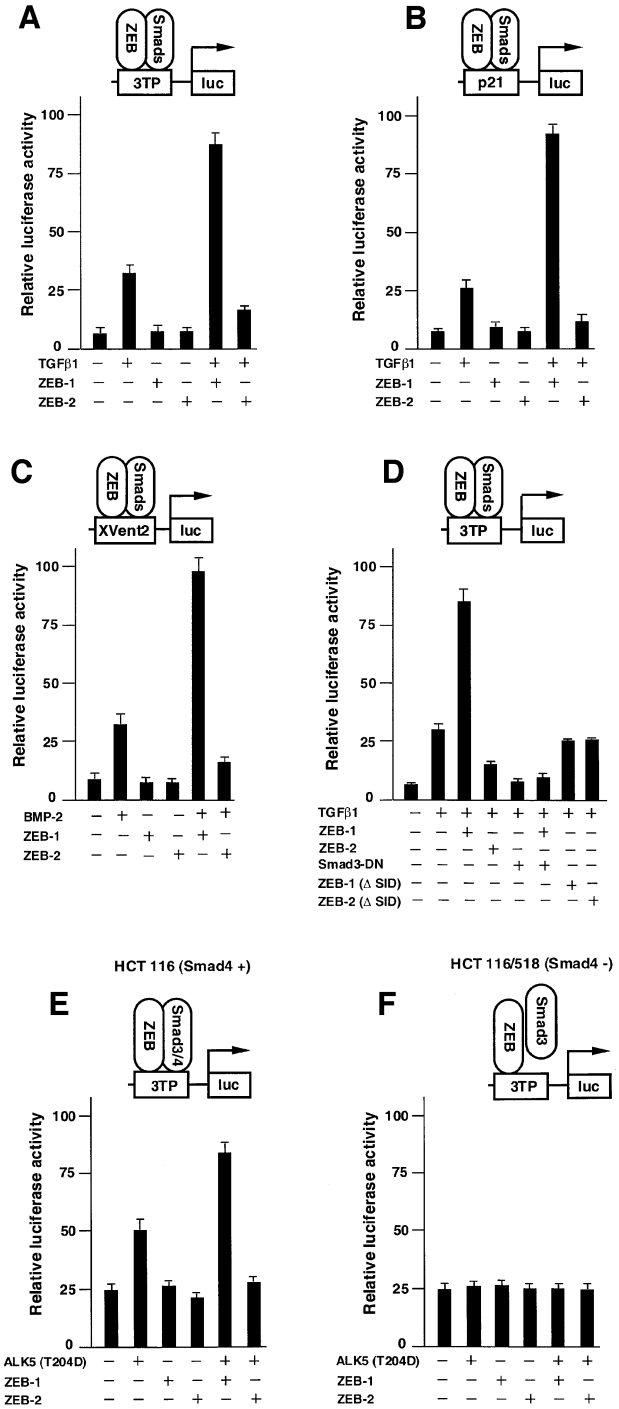

Fig. 3. ZEB-1/δEF1 synergizes with TGFβ/BMP in transcriptional activation, whereas ZEB-2/SIP1 represses. (**A** and **B**) Mv1Lu or HaCAT cells (similar results were obtained in both cell types) were cotransfected with 0.3 μg of a firefly luciferase reporter containing the indicated promoter along with either 0.48 μg of the empty vector (CS2MT, not shown) or 0.7 μg of either ZEB-1/δEF1 or ZEB-2/SIP1 expression vectors and stimulated with TGFβ1 (25 pM for Mv1Lu or 100 pM for HaCAT). (**C**) C2C12 cells were transfected as in (A) and (B) with a firefly luciferase reporter containing the BMP-2-responsive Xvent2 promoter. Where indicated, cells were treated with 100 ng/ml BMP-2. (**D**) Regulation of TGFβ-mediated transcription by ZEB proteins is dependent on the transcriptional activity of R-Smads and requires the SID. Mv1Lu or HaCAT cells were transfected with 0.3 μg of 3TPluc and either 0.48 μg of vector (CS2MT, not shown), 0.6 μg of ZEB-1 (ΔSID) or ZEB-2 (ΔSID) (lacking the SID) or 0.7 μg of full-length ZEB-1 or ZEB-2. Where indicated, an expression vector for Smad3-dominant negative (Smad3-DN, D470E) was cotransfected. (**E** and **F**) Regulation of TGFβ-mediated transcription by ZEB proteins requires the presence of Smad4. Smad4 (+/+) HCT 116 cells (E) and Smad 4 (–/–) HCT 116/518 cells (F) were cotransfected as in (A) with 3TP-luc, either ZEB-1 or ZEB-2 and ALK5 (T204D) to activate the TGFβ signaling pathway.

and repressed by ZEB-2/SIP1 (data not shown; Postigo *et al*., 2003).

Moreover, the regulatory effects of ZEB-1/δEF1 and ZEB-2/SIP1 require the formation of a R-Smad–Smad4 complex because neither ZEB protein had any effect on the activation by TGFβ of the 3TP promoter in cells that are mutant for the Smad4(–) gene (Figure 3E and F).

Finally, a direct interaction between ZEB proteins and R-Smads is needed for ZEB proteins to mediate their transcriptional effects because deletional mutants of both ZEB-1/δEF1 and ZEB-2/SIP1 lacking the SID failed to affect TGFβ1-dependent transcription (Figure 3D).

The results presented above indicate that ZEB proteins could regulate TGFβ/BMP-mediated transcription in opposing ways. I next wondered whether ZEB proteins could modulate physiological functions mediated by TGFβ and BMP. To address this question, I explored the ability of ZEB-1/δEF1 and ZEB-2/SIP1 to regulate the osteogenic differentiation activity of BMP-2 and the growth suppressor effect of TGFβ1.

ZEB-1/δEF1 synergizes with BMP-2 in the induction of alkaline phosphatase

BMP proteins regulate the formation of bone and cartilage both *in vivo* and *in vitro* (reviewed in Kingsley, 2001). The ability of ZEB-1/δEF1 to synergize with BMP-2 in transcriptional activation suggested that it might functionally augment BMP signaling. Treatment of mesenchymal C2C12 cells with BMP-2 triggers osteogenic differentiation and expression of osteblastic markers [e.g. alkaline phosphatase (ALP), osteopontin, osteonectin, etc.] (Katagiri *et al*., 1994). As shown in Figure 4, ZEB-1/δEF1 was able to synergize with BMP-2 to induce ALP. This ability to cooperate with BMP-2 is consistent with the skeletal defects observed in ZEB-1/δEF-1 (–/–) mice (Takagi *et al*., 1998). In contrast, ZEB-2/SIP1 partially inhibited the ability of BMP-2 to induce ALP activity (Figure 4; Tylzanowsky *et al*., 2001). The functional effect of ZEB-1/δEF1 and ZEB-2/SIP1 in this system required the interaction with Smads as (i) both ZEB proteins failed to show any effect in the absence of BMP-2 and (ii) ZEB mutants with a deletion in the SID region failed to regulate ALP activity (Figure 4).

ZEB-1/δEF1 augments the anti-proliferative signal from TGFβ

TGFβ blocks proliferation of epithelial, endothelial, lymphoid and neural cells by inducing growth arrest in the G_1 phase of the cell cycle (reviewed in de Caestecker *et al*., 2000; Massagué *et al*., 2000). Therefore, we asked whether ZEB-1/δEF1 might also synergize with TGFβ in growth arrest. In the absence of TGFβ, expression of ZEB-1/δEF1 did not significantly affect the cell cycle profile of the Mv1Lu epithelial cells (Figure 5A; data not shown). However its expression along with sub-optimal levels of TGFβ (which do not induce a full growth arrest) led to an increased accumulation of cells in the G_1 phase of the cell cycle (compare c and d in Figure 5A), implying that ZEB-1/δEF1 is synergizing with TGFβ to arrest cells. Since Mv1Lu cells have a very large number of cells in G_1 and in order to demonstrate that the expression of ZEB-1/δEF1 arrested the cells at G_1, cells were treated with nocodazole, an inhibitor of microtubule formation that arrests cells in G_2/M (Nusse and Egner, 1984; Zhang *et al*., 2000). Cells would normally progress around the cell cycle and arrest at G_2/M due to the nocodazole block. However, if the cells were indeed arrested in a ZEB-1/δEF1- and TGFβ-dependent fashion, they should arrest in G_1, unable to progress to G_2/M. Indeed, there was a ZEB-1/δEF1–TGFβ-dependent arrest of cells in G_1 (Figure 5B, compare c and d, and Figure 5C).

This synergistic effect of ZEB-1/δEF1 with TGFβ depended on the ability of ZEB-1/δEF1 to interact with Smads because the ZEB-1/δEF1 mutant lacking the SID region (ZEB-1/δEF1 ΔSID) did not synergize with TGFβ in G_1 arrest (Figure 5C).

To further examine the effects of the ZEB proteins on TGFβ signaling, stable clones of mink lung epithelial cells expressing both ZEB proteins were generated, and tested to different doses of TGFβ1. As expected, increasing amounts of TGFβ1 induced a dose-dependent inhibition in cell proliferation, as measured by the incorporation of [^{3}H]thymidine (Figure 6). It was found that ZEB-1/δEF1 synergized with TGFβ as its stable overexpression significantly lowered the threshold of TGFβ1 required for growth arrest (Figure 6). In contrast, ZEB-2/SIP1 clones required higher concentrations of TGFβ1 for growth arrest. Taken together, the above results provide evidence that ZEB-1/δEF1 and ZEB-2/SIP1 can have opposing effects on the TGFβ signaling pathway, which leads to growth arrest.

Endogenous ZEB-1/δEF1 in TGFβ signaling

The overexpression of ZEB-1/δEF1 in the above experiments provides strong evidence for a role of ZEB-1/δEF1 in regulating TGFβ family signaling. However, it was still unclear whether endogenous ZEB-1/δEF1 indeed contributes to TGFβ signaling in the absence of overexpression. To address this, antisense oligos for ZEB-1/δEF1 mRNA were tested for their ability to affect TGFβ functions, namely the ability to activate transcription and induce cell growth arrest (Figure 7A and B). These antisense oligos

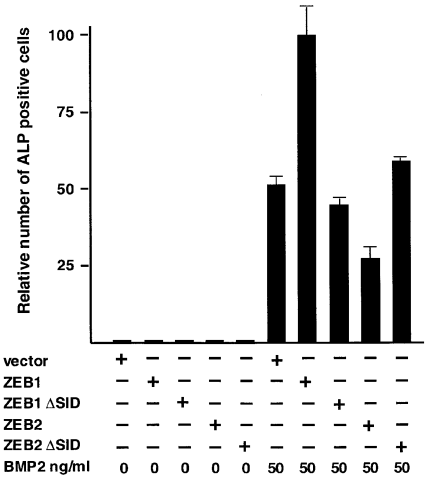

Fig. 4. ZEB-1/δEF1 augments BMP-2-dependent osteogenic differentiation of mesenchymal cells. C2C12 were cotransfected with 8 μg of either vector alone (CS2MT) or expression vectors for ZEB-1, ZEB-2 or their ΔSID deletional mutants (ZEB-1 ΔSID and ZEB2 ΔSID) and treated with the indicated amount of BMP-2. After 4 days, cells were assessed for ALP activity as described in Materials and methods.

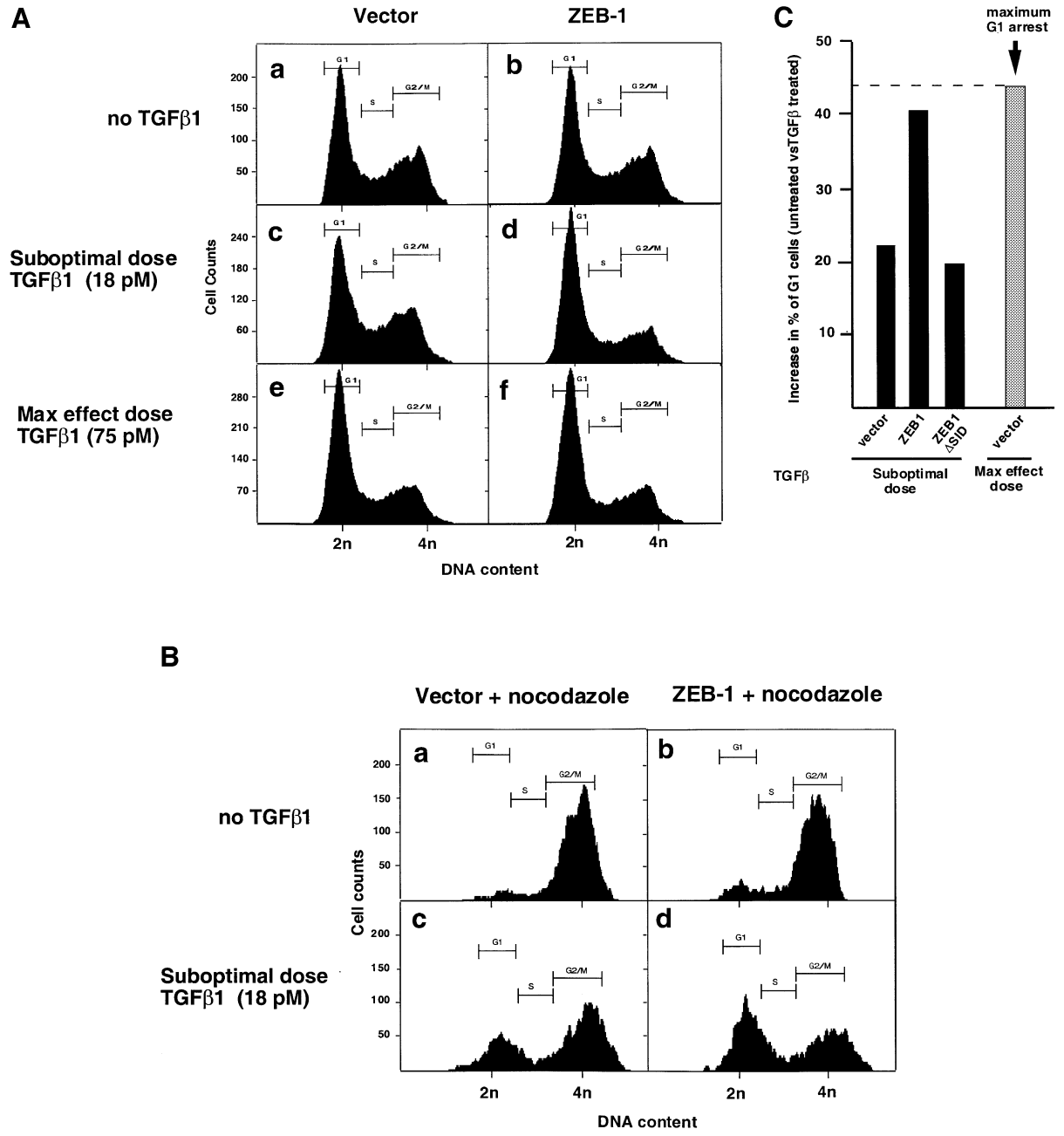

Fig. 5. ZEB-1/δEF1 synergizes with TGFβ to mediate G_1 cell cycle arrest. (**A**) Mv1Lu cells were transfected with 20 μg of the empty vector or ZEB-1/δEF1 along with 1 μg of an expression vector for EGFP (as a marker for sorting transfected cells) and, where indicated, treated with either 18 pM (suboptimal dose) or 75 pM (maximum growth arrest dose) TGFβ1. The cell cycle profile of EGFP-positive cells was analyzed by FACS analysis. (**B**) As in (A), but cells were preincubated for 48 h with 75 ng/ml nocodazole to induce G_2/M arrest (see Materials and methods and Zhang *et al.*, 2000). (**C**) Mv1Lu cells were transfected with 20 μg of either empty vector, full-length ZEB-1/δEF1 or a mutant of ZEB-1/δEF1 with a deletion of the SID (ZEB-1/δEF1 ΔSID) and EGFP in the presence of nocodazole as in (B). The percentage of cells in G_1 phase was determined by FACS and their value compared in the absence or presence of TGFβ1 (both suboptimal and maximum effect doses).

partially blocked activation of the 3TP reporter by TGFβ, whereas a scrambled control oligo did not, suggesting that TGFβ-mediated transcription is dependent to some extent upon endogenous ZEB-1/δEF1 (Figure 7A).

Likewise, antisense oligos to ZEB-1/δEF1 also significantly reversed the anti-proliferative effect of TGFβ, suggesting that the ability of TGFβ to arrest epithelial cells is dependent, at least in part, on the presence of endogenous ZEB-1/δEF1 protein (Figure 7B).

Discussion

ZEB proteins are members of a large family of zinc finger proteins known as zfh, which was first identified in *Drosophila* (Fortini *et al.*, 1991; Lai *et al.*, 1991). The genes encoding ZEB-1/δEF1 and ZEB-2/SIP1 proteins (*Zfhx1* and *Zfhx1b*, respectively) appear to have evolved from a single *Drosophila* gene named *zfh-1* (Fortini *et al.*, 1991; Lai *et al.*, 1991; Postigo *et al.*, 1999). *zfh-1* is crucial

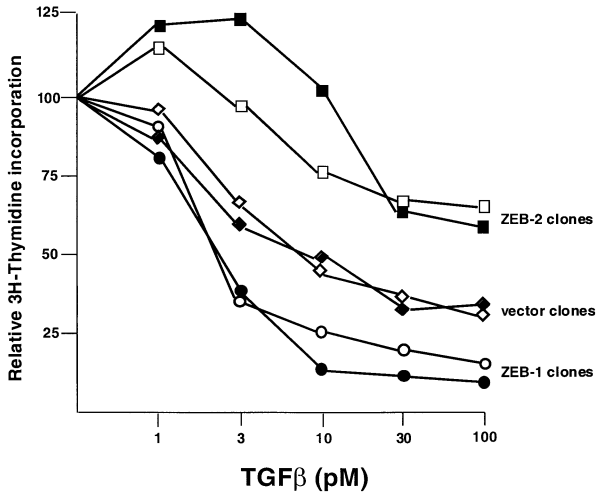

Fig. 6. ZEB proteins regulate TGFβ-dependent growth suppression. Mv1Lu cells were stably transfected with either the empty vector, ZEB-1/δEF1 or ZEB-2/SIP1. Stable clones were treated with different doses of TGFβ1 and assessed for cell proliferation by measuring their [^{3}H]thymidine uptake. Two representative clones for each construct are shown.

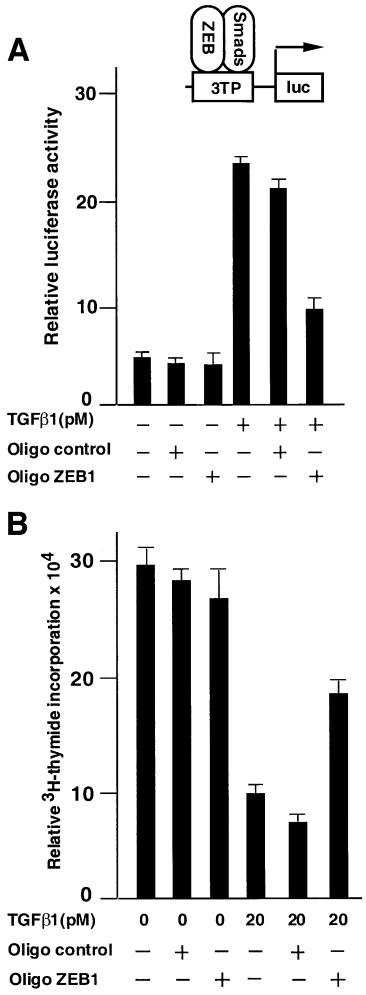

Fig. 7. Endogenous ZEB/δEF1 mediates TGFβ functions. (**A**) Endogenous ZEB/δEF1 is important for TGFβ-dependent transcriptional activation. Mv1Lu cells were transfected with 0.3 μg of the TGFβ-responsive 3TP promoter and transcriptional activity assessed as in Figure 3. Where indicated, either antisense oligos to ZEB-1/δEF1 (Oligo ZEB1) or a scrambled control oligo (Oligo control) were added (see Materials and methods). One-tenth of a microgram of SV40βgal was cotransfected to control for transfection efficiency. (**B**) Endogenous ZEB/δEF1 is important for TGFβ-mediated growth suppression. Mv1Lu cells were transfected with antisense oligos and 0.2 μg of puroBabe, and treated with 1 mg/ml puromycin and 20 pM TGFβ1 for 36 h before the proliferation was assessed by the incorporation of [^{3}H]thymidine.

for mesodermal (gonadal, skeletal and cardiac muscle) and neural differentiation in flies (Lai *et al*., 1991, 1993; Broihier *et al*., 1998; Postigo *et al*., 1999). The human orthologue of *Drosophila* zfh-2 seems to be ATBF-1, which has two isoforms: ATBF-1A and ATBF-1B (Miura *et al*., 1995; Kaspar *et al*., 1999; Berry *et al*., 2001). As in the case of ZEB proteins, a recent report demonstrated that ATBF-1A and ATBF-1B have opposing effects on the regulation of muscle differentiation (Berry *et al*., 2001). These results raise the interesting possibility that the *Drosophila* zfh family of zinc finger proteins may have evolved in vertebrates into proteins with opposing activities to balance signaling pathways during tissue differentiation and embryonic development (Postigo *et al*., 2003). ZEB-1/δEF1 and ZEB-2/SIP1 are structurally quite similar and both repress transcription of a number of genes involved in differentiation and development. However, the results presented here indicate that ZEB proteins function antagonistically in the regulation of TGFβ/BMP signaling.

Consensus binding sites for the ZEB proteins are evident in the promoters of several target genes for the TGFβ and BMP signaling pathways. Although in overexpression experiments the regulation of Smad-mediated transcription by ZEB proteins could be achieved through direct recruitment of ZEB proteins by Smads (e.g. in the absence of DNA-binding sites for ZEB in the reporter; Postigo *et al*., 2003), the presence of ZEB-binding sites in TGFβ/BMP-dependent genes could be crucial to concentrate endogenous ZEB and Smad proteins at target genes. It is therefore likely that, as with other proteins that augment Smad activity (e.g. OAZ, FAST), ZEB/δEF1 protein may only target a subset of genes in a fashion dictated by the distribution of their binding sites. For some Smad regulatory proteins, such as OAZ, the target is quite specific and may be restricted to the inhibitors of neural differentiation, *Xenopus Vent-1* and *Vent-2* genes (Hata *et al*., 2000).

In contrast, the several factors that inhibit Smad signaling (e.g. TGIF, Ski/SnoN, Evi-1) seem to do so in a less specific fashion—they do not seem to require direct binding to DNA to mediate their repressor effects, but rather they are recruited to target genes through their interaction with Smads (Kurokawa *et al*., 1998; Luo *et al*., 1999; Wotton *et al*., 1999; Izutsu *et al*., 2001). The exception to this pattern is ZEB-2/SIP1, which recognizes the same DNA-binding site as ZEB-1/δEF1 and would only block the activation of a subset of TGFβ/BMP-dependent genes—those containing ZEB-binding sites.

ZEB-2/SIP1 appears to bind ZEB sites with higher affinity than ZEB-1/δEF1, and it is also induced in response to TGFβ (Comijn *et al*., 2001; data not shown).

It is tempting to speculate about the existence of a feedback mechanism between both ZEB proteins to limit the extent/duration of TGFβ signaling. In this model, induction of ZEB-2/SIP1 in response to TGFβ would in turn displace ZEB-1/δEF1 from the DNA sites and block transcription of TGFβ-responsive genes, shutting off the amplification loop created by ZEB-1/δEF1 and TGFβ. This mechanism would only target genes containing both ZEB and Smad-binding sites. The existence of such feedback is currently under investigation.

Materials and methods

Cells and cell culture

Mv1Lu cells were obtained from the American Type Culture Collection (Manassas, VA). The following cell lines were obtained directly from investigators: 293T cells from Dr S.Korsmeyer (Dana Farber Cancer Institute, Boston, MA), HaCAT from Dr S.Dowdy (University of California, San Diego, CA) and HCT116 and HCT116/518 cells from Dr B.Vogelstein (Johns Hopkins University, Baltimore, MD). Cell lines were maintained in Dulbecco's modified Eagle's medium containing 12% fetal calf serum (Gibco-Life Technologies, Grand Island, NY). In selected experiments, cells were treated with the indicated amounts of recombinant TGFβ1 (R&D Systems, Minneapolis, MN) or BMP-2 (kind gift of Dr T.Celeste, Genetics Institute, Cambridge, MA and R&D Systems). Stable clones in Mv1Lu cells were obtained by cotransfection of either pCMV-MCS, pCMV-MCS-EGFP-ZEB-1 or pCMV-EGFP-ZEB-2 (see below) (Clontech Labs) along with a plasmid encoding the neomycin resistance (pCIneo; Promega, Madison, WI).

Plasmid construction

3TP-lux, CMV5-Flag-Smad1, CS2-Flag-Smad2 and CMV-Flag-Smad3 were obtained from Dr J.Massagué (Sloan Kettering Cancer Center, New York, NY). p21, p15 and c-jun reporters, containing the TGFβ-responsive elements in these promoters fused to firefly luciferase, were obtained from Dr X.C.Wang (Duke University Medical Center, Durham, NC). *Xenopus* Vent-2-luciferase reporter was obtained from Dr C.Niehrs (Deutsches Krebforschungs zentrum, Heidelberg, Germany). A dominant-negative form of Smad3 (D470E) and constitutively active forms for ALK5 (T204D) and ALK6 (Q203D) were obtained from Dr K.Miyazono (Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan).

The different myc-tagged versions of ZEB proteins were constructed as follows. A *Xho*I–*Xba*I PCR fragment corresponding to the full-length human cDNA for ZEB-1/δEF1 was cloned into the corresponding sites of the CS2MT vector. A *Xba*I–*Eco*RV PCR fragment corresponding to the full-length human cDNA for ZEB-2/SIP1 was cloned into the *Xba*I–*Sna*BI sites of CS2MT. CS2MT-ZEB-1 (ΔSID) was cloned in two steps. First, a *Stu*I–*Xba*I fragment corresponding to the amino acids 1–377 of ZEB-1/δEF1 was cloned into the corresponding sites of CS2MT. The resulting plasmid was cut with *Xba*I–*Sna*BI and a PCR fragment encoding from amino acid 456 to the stop codon at 1125 of human ZEB-1/δEF1 with identical sites was cloned. CS2MT-ZEB-2 (ΔSID) was also constructed in two steps. First, a *Xba*I–*Eco*RV PCR fragment encoding amino acids 1–422 of human ZEB-2/SIP1 was cloned into the *Xba*I/*Sna*BI sites of CS2MT. The 3′ primer also contained *Mlu*I/*Blg*II sites where a *Mlu*I–*Bgl*II PCR fragment encoding amino acids 504 to the stop codon at 1215 of ZEB-2/SIP1 were cloned. The remainder of the constructs shown in Figure 2E correspond to the regions 1, 2, CtBP-interacting domain (CID) and 3 as described in Postigo and Dean (1999a, 2000). From the original Flag-tagged ZEB constructs described in those references, CS2MT derivatives were made as follows. For ZEB-1/δEF1 myc-tagged constructs, the different regions were amplified by PCR with primers containing *Xho*I/*Eco*RV ends and cloned into the *Xho*I/*Sna*BI sites of CS2MT. In the case of ZEB-2/SIP1 regions, we used oligos containing *Xba*I/*Eco*RV sites and the corresponding PCR fragments were then cloned into the *Xba*I/*Sna*BI sites of CS2MT. pCMV-MCS-EGFP-ZEB-1 and pCMV-EGFP-ZEB-2 were obtained by cloning a *Xho*I–*Not* fragment from the corresponding pCI-Flag-neo version of ZEB-1/δEF1 and ZEB-2/SIP1 (Postigo and Dean, 1997, 2000) into the corresponding sites of pCMV-MCS-EGFP (Clontech Lab).

Transfections

Cells were transfected by the calcium phosphate method or with Superfectin reagent (Qiagen, Hilden, Germany) as described (Postigo and Dean, 2000). For luciferase assays, 5–12 h after transfection, cells were washed and activated with the indicated amounts of TGFβ1 or BMP-2. Twenty-four hours later, luciferase activity was assessed. As an internal control for transfection, cells were cotransfected with SV40 β-gal (Promega Corp., Madison, WI), and values were determined using a luminescent β-galactosidase detection Kit II® (Clontech Laboratories, Palo Alto, CA). Transcriptional assays using a chloramphenicol acetyl transferase (CAT) reporter gene were performed as described previously (Postigo and Dean, 2000). In the experiments where antisense oligonucleotides were used, cells were transfected with 1 μM each oligo using Effectene (Qiagen) according to the manufacturer's recommendations. After 18 h, cells were treated with the indicated amount of TGFβ. Control oligo: TCTGCGGCCTGTGTCAAGCTA; antisense ZEB-1 (oligo A) CAAGGGTTCTTCCTGGACAG; antisense ZEB-1 (oligo B): CCCCYTCAAAGCTTTTGTCC.

Protein co-immunoprecipitations and western blot assays

Immunoprecipitation and western blots were performed as described previously (Postigo and Dean, 2000). Where indicated, lysates were immunoprecipitated with agarose-conjugated anti-Flag M2 (Sigma Chemicals, St Louis, MO), anti-Gal4-DBD, anti-ZEB-1 (ZEB C-20) and anti-myc 9E10 (Santa Cruz Biotechnologies, Santa Cruz, CA) antibodies. Also where indicated, 15% of the lysate was directly loaded into the gel as input control for direct western blotting. The following HRP-conjugated antibodies were used for western blotting: Gal4-DBD polyclonal antibody (Santa Cruz Biotechnologies), anti-LexA polyclonal antibody (Santa Cruz Biotechnologies), anti-Flag M2 mAb (Sigma Chemicals) or anti-myc 9E10 mAb (Santa Cruz Biotechnologies). Anti-Smad2/Smad3 antibody was purchased from Upstate Biotechnology.

Osteogenic differentiation assays

Twenty thousand C2C12 cells were transfected in a 6-well plate with 5 μg of empty vector, ZEB-1, ZEB-2 or their corresponding ΔSID mutants using Effectene (Qiagen). After 5 h, cells were washed and treated with the indicated amount of BMP-2 (Genetics Institute) and cultured for 3–5 days. Histochemical analysis of ALP activity was assessed using a kit from Sigma. After the reaction had developed, the number of ALP-positive cells was counted using a phase contrast microscope.

Flow cytometry analysis

Mv1Lu cells were cotransfected with 20 μg of either vector alone or either CS2MT-ZEB-1 or CS2MT-ZEB-1 (ΔSID) and 1 μg of a EGFP expression vector and treated with the indicated amounts of TGFβ1. The microtubule inhibitor nocodazole (Sigma Chemicals) was added at 75 ng/ml for 24 h prior to the experiment to induce a G_2/M arrest. Forty-eight hours after transfection, at least 10 000 EGFP-positive cells were collected, and the cell cycle profile was determined as described previously (Zhang *et al*., 2000).

Proliferation assays

Stable clones derived from Mv1Lu cells were plated at a density of 1×10^4 in 12-well culture plates, treated with the indicated amount of TGFβ1 and incubated for 36 h. During the last 8–12 h, cells were incubated with 1 μCi/well [^{3}H]thymidine (ICN Pharmaceuticals). After that period, cells were washed extensively with phosphate-buffered saline, fixed with 10% Tris–HCl for 1 h and solubilized in 0.5 M NaOH for 30 min. Lysates were then neutralized with HCl, and [^{3}H]thymidine uptake quantified in a liquid scintillation β-counter.

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