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Regulation of Smad signaling through a differential recruitment of coactivators and corepressors by ZEB proteins

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Balancing signals derived from the TGFβ family is crucial for regulating cell proliferation and differentiation, and in establishing the embryonic axis during development. TGFβ/BMP signaling leads to the activation and nuclear translocation of Smad proteins, which activate transcription of specific target genes by recruiting P/CAF and p300. The two members of the ZEB family of zinc finger factors (ZEB-1/δEF1 and ZEB-2/SIP1) regulate TGFβ/BMP signaling in opposite ways: ZEB-1/δEF1 synergizes with Smad-mediated transcriptional activation, while ZEB-2/SIP1 represses it. Here we report that these antagonistic effects by the ZEB proteins arise from the differential recruitment of transcriptional coactivators (p300 and P/CAF) and corepressors (CtBP) to the Smads. Thus, while ZEB-1/δEF1 binds to p300 and promotes the formation of a p300–Smad transcriptional complex, ZEB-2/SIP1 acts as a repressor by recruiting CtBP. This model of regulation by ZEB proteins also functions *in vivo*, where they have opposing effects on the regulation of TGFβ family-dependent genes during *Xenopus* development.

Keywords: CtBP/p300-P/CAF/Smad proteins/TGFβ/BMP/ZEB proteins

Introduction

Members of the TGFβ family of growth factors regulate cell growth and differentiation through their ability to regulate transcription of various target genes. Binding of TGFβ factors to specific transmembrane II receptors leads to the phosphorylation and activation of type I receptors, which in turn phosphorylates and activates the R-Smad proteins (reviewed in Massagué and Chen, 2000; Massagué *et al*., 2000; ten Dijke *et al*., 2000; Moustakas *et al*., 2001; Dennler *et al*., 2002). Broadly, while Smad1, Smad5 and Smad8 are activated in response to BMP and GDF factors, Smad2 and Smad3 respond to TGFβ, activin and nodal. Once activated, R-Smads bind to Co-Smad (Smad4 in mammals or Smad4α and Smad4β in *Xenopus*) and the resulting complexes translocate to the nucleus where they activate transcription of downstream target genes. In addition, Smad proteins bind to a number of coregulators that either repress or activate Smad-mediated transcription (e.g. Ski, TGIF, SNIP1, and FAST, TFE-III, OAZ, Mixer/Mix, respectively) (reviewed in Massagué and Wotton, 2000; Miyazono, 2000; Miyazono *et al*., 2001). Experiments with *Xenopus* have shown that regulation of Smad activity by these factors is crucial for a proper tissue specification and patterning during embryonic development (reviewed in Hill, 2001; von Bubnoff and Cho, 2001).

Proteins encoded by the *Zfhx1a* and *Zfhx1b* genes (ZEB-1/δEF1 and ZEB-2/SIP1, respectively) repress transcription of a wide number of key regulatory genes involved in different differentiation and developmental events (Genetta *et al*., 1994; Postigo and Dean, 1997; Postigo *et al*., 1997, 1999; Sekido *et al*., 1994; Takagi *et al*., 1998; Grooteclaes and Frisch, 2000; Murray *et al*., 2000; Comijn *et al*., 2001; Fontemaggi *et al*., 2001). ZEB repression is mediated through several repressor motifs within the central region of these proteins (Figure 1A; located in between the N- and C-terminal DNA-binding zinc finger clusters) through a mechanism that, at least in part, involves the NAD^+-dependent corepressor CtBP (Postigo and Dean, 1999a,b, 2000; Zhang *et al*., 2002). CtBP also mediates the repressor activity of other key regulatory proteins including Snail, BKLF, TCF-3, Rb, BRCA-1 and Polycomb HPC2 (reviewed in Turner and Crossley, 2001; Chinnadurai, 2002). Interestingly, ZEB-1/δEF1 has also been shown to directly activate the transcription of the ovalbumin and vitamin D3 receptor genes, although the molecular mechanism behind these effects is unknown (Chamberlain and Sanders, 1999; Lazarova *et al*., 2001).

We have recently found that ZEB proteins bind to Smad proteins and antagonistically regulate their transcriptional activity (Postigo, 2003). Thus, while ZEB-1/δEF1 activates Smad-mediated transcription, ZEB-2/SIP1 represses it. ZEB proteins also have an antagonistic role in several TGFβ and BMP functions, the molecular mechanism of which was previously unknown.

Here, we provide evidence that the two ZEB proteins regulate TGFβ/BMP signaling through differential recruitment of coactivators (p300 and P/CAF) and corepressors (CtBP) to the Smad complex. p300 and P/CAF are acetyltransferases that activate transcription by acetylating histones and loosening chromatin structure (Bannister and Kouzarides, 1996; Ogryzko *et al*., 1996; Yang *et al*., 1996). As with many other DNA-binding factors, Smad proteins recruit p300 and P/CAF to mediate their transcriptional effects, and this interaction is dependent on the phosphorylation/activation of R-Smad induced by TGFβ signaling (Feng *et al*., 1998; Janknecht *et al*., 1998; Pouponnot *et al*., 1998; Itoh *et al*., 2000).

We found that the N-terminal region of ZEB-1/δEF1 (but not ZEB-2/SIP1) binds both p300 and P/CAF. Moreover, the interaction between R-Smad and ZEB-1/δEF1 forms a complex that recruits p300 much more

efficiently, thus accounting for their transcriptional synergy. In addition to acetylating histones, p300 and P/CAF acetylate a number of transcription factors (e.g. p53, E2F, Rb, etc.) to modulate their activity (Gu and Roeder, 1997; Martinez-Balbas *et al*., 2000; Chan *et al*., 2001; reviewed in Kouzarides, 2000). Binding of P/CAF to ZEB-1/δEF1 acetylates several lysine residues following the CtBP interaction domain (CID) of ZEB-1/δEF1, displacing CtBP and switching ZEB-1/δEF1 from a repressor to an activator. In contrast, ZEB-2/SIP1 is unable to interact with p300 or P/CAF and thus only serves as a transcriptional repressor by recruiting CtBP to the Smad complex. These opposing roles of ZEB-1/δEF1 and ZEB-2/SIP1 in cell culture systems were also found *in vivo*, where the ZEB proteins also show antagonistic roles in the regulation of TGFβ-dependent genes during *Xenopus* development.

Results

The CID of both ZEB-1/δEF1 and ZEB-2/SIP1 represses Smad activity

ZEB proteins repress transcription of a wide number of genes involved in differentiation and development. The central region (between the N- and C-terminal zinc fingers) of both ZEB-1/δEF1 and ZEB-2/SIP1 serves as a transcriptional repressor domain in part as a result of the recruitment of CtBP through a specific CID (Figure 1A) (Postigo and Dean, 1999a, 2000; Grooteclaes and Frisch, 2000; Zhang *et al*., 2002). We have previously found that ZEB proteins have opposing effects on TGFβ signaling, with ZEB-1/δEF1 synergizing with Smad-mediated transcription and ZEB-2/SIP1 repressing it (Postigo, 2003).

Smad3 tethered to the DNA as a Gal4 fusion protein mediates transcription when activated by the cotransfection of a vector encoding for the constitutively active type I receptor ALK 5 (T204D) (Figure 1B). We found that, as when using natural TGFβ- and BMP-dependent promoters, the transcriptional activity of Gal4–Smad3 was enhanced by cotransfection of ZEB-1/δEF1 and repressed by ZEB-2/SIP1 (Figure 1B). Next, we investigated the effect of the central region of both ZEB proteins (CR-ZEB-1 and CR-ZEB-2) on the transcriptional activity mediated by TGFβ1. This central region includes the Smad-interaction domain (SID) and the CID region. As expected, the central repressor region of ZEB-2/SIP1 (CR-ZEB-2) repressed Gal4–Smad3 (Figure 1B). Surprisingly, we found that the central repressor domain of ZEB-1/δEF1 (CR-ZEB-1) also inhibited Smad3 activity (Figure 1B).

Since both ZEB proteins repress transcription by mechanisms that involved, at least in part, the recruitment of CtBP, we tested whether CtBP is responsible for the repression of Smad3-mediated transcription by CR-ZEB-1 and CR-ZEB-2. To that effect, CtBP-1 itself or the CID regions of ZEB-1/δEF1 and ZEB-2/SIP1 were fused to the DNA-binding domain (DBD) of the bacterial protein LexA and tested for their ability to repress transcriptional activation mediated by Gal4–Smad3. We found that the CIDs of both ZEB proteins (as well as CtBP-1 itself) blocked transcriptional activation by Smad3 (Figure 1C).

These results indicated that in order for full-length ZEB-1/δEF1 to activate Smad-mediated transcription, ZEB-1/δEF1 has to have a domain outside the zinc finger clusters that is dominant over the repressor effect of the CID region. However, it remains unclear how full-length ZEB-1/δEF1 could augment Smad activity.

The N-terminal region of ZEB-1/δEF1 interacts with p300 and P/CAF to assemble a ZEB-1/δEF1–Smad activation complex

Smad proteins activate transcription of target genes through the recruitment of p300 and P/CAF coactivators (Feng *et al*., 1998; Janknecht *et al*., 1998; Pouponnot *et al*., 1998; Itoh *et al*., 2000). Therefore, we reasoned that ZEB-1/δEF1 might synergize with Smad proteins by promoting the recruitment of P/CAF–p300 to the complex. The adenovirus E1a protein, which binds and inactivates p300 (Chakravarti *et al*., 1999), has been shown to inhibit transcriptional activation of TGFβ-dependent reporters by TGFβ (Feng *et al*., 1998). As shown in Figure 2A, we found that E1a not only blocked the activation of the 3TP reporter by TGFβ but also blocked the synergy between ZEB-1/δEF1 and TGFβ.

The sensitivity of ZEB-1/δEF1–TGFβ synergy to E1a inhibition suggests that ZEB-1/δEF1 activity may depend on p300. Indeed, we found that full-length ZEB-1/δEF1, but not ZEB-2/SIP1, co-immunoprecipated with p300 (Figure 2B). From Figure 1B it appears that the region of ZEB-1/δEF1 responsible for synergy with Smads is outside the central region. Therefore, we investigated the N- and C-terminal regions of both ZEB proteins for binding to p300. As shown in Figure 2C, the N-terminal region of ZEB-1/δEF1 co-immunoprecipitated with p300, whereas the N-terminal region of ZEB-2/SIP1 or the C-terminal region of ZEB-1/δEF1 did not. Since Smad3 has also been shown to bind to P/CAF (Itoh *et al*., 2000), we investigated whether ZEB-1/δEF1 and ZEB-2/SIP1 could bind P/CAF. Indeed, we found that P/CAF also interacts with the N-terminal region of ZEB-1/δEF1 but not with ZEB-2/SIP1 (Figure 2D).

From Figure 2C and Postigo (2003), it seems that ZEB-1/δEF1 contains independent binding sites for P/CAF–p300 and R-Smads. It was then tempting to speculate whether ZEB-1/δEF1 might serve as a scaffold to facilitate the assembly of a Smad–p300 transcriptional complex. To test this possibility we examined the effect of coexpressing ZEB-1/δEF1 on the interaction between Smad3 and p300. As reported (Feng *et al*., 1998; Janknecht *et al*., 1998; Pouponnot *et al*., 1998; Itoh *et al*., 2000), in the absence of TGFβ signaling we did not detect any interaction between Smad3 and p300, but we did when the constitutively active type I receptor ALK5 (T204D) was coexpressed (Figure 3A). Interestingly, coexpression of ZEB-1/δEF1 led to a much stronger co-immunoprecipitation of p300 by Smad3 (Figure 3A), suggesting that indeed ZEB-1/δEF1 enhances the assembly of a Smad3–p300 complex. We confirmed this result using a p300–VP16 fusion protein in a two-hybrid approach. As shown in Figure 3B, expression of ZEB-1/δEF1 further increased the recruitment of p300–VP16 to Smad3.

The assembly of a ZEB-1/δEF1–Smad3–p300 complex still requires interaction between Smad3 and p300, as a Smad3 mutant (2SA) deficient for binding to p300 not only did not activate transcription (as reported in Feng *et al*.,

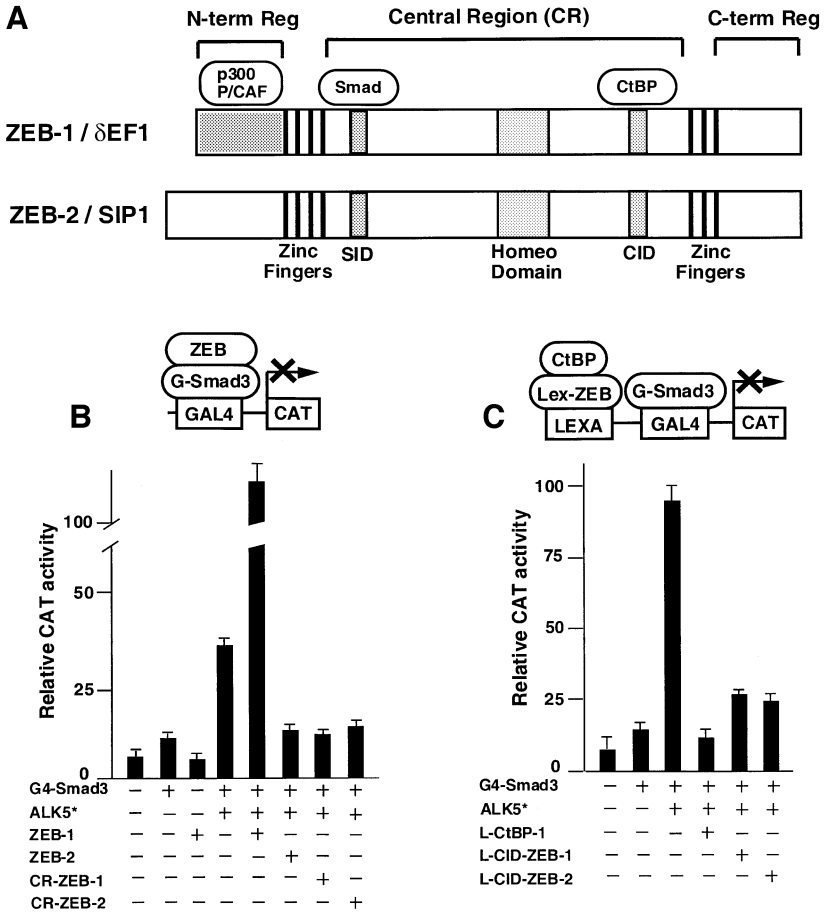


Fig. 1. (**A**) Schematic representation of the ZEB family (ZEB-1/δEF1 and ZEB-2/SIP1) of two-handed zinc finger factors. The central region (CR) in between both zinc finger clusters act as a repressor domain in part through the recruitment of the CtBP corepressor through a CtBP-interacting domain (CID). There is a region 3′ of the N-terminal zinc fingers that acts as a Smad-binding domain (SID). The N-terminal region of ZEB-1/δEF1 serves as a p300–p/CAF-binding domain (see Figure 2C). (**B**) The central repressor (CR) domain of both ZEB-1/δEF1 and ZEB-2/SIP1 inhibits the activity of Smad3 when brought directly to the promoter. 293T cells were transfected with 0.25 μg of a reporter construct (PG5-CAT) containing five Gal4 DNA-binding sites driving the CAT gene, 0.25 μg of Gal4–Smad3 and 0.1 μg of an expression vector for constitutively active ALK5 (T204D) (ALK5*) along with either 1 μg of expression vectors for either ZEB-1/δEF1 or ZEB-2/SIP1, 0.8 μg of either CR-ZEB-1 or CR-ZEB-2, or 0.7 μg of vector alone (CS2MT, not shown). (**C**) The CID of both ZEB proteins or CtBP-1 itself represses Smad3 transcriptional activation. 293T cells were cotransfected with 0.5 μg of a CAT reporter construct (PL6G5-CAT) containing both LexA and Gal4 DNA-binding sites along with 0.25 μg of Gal4–Smad3, 0.1 μg of an expression vector for constitutively active ALK5 (T204D) (ALK5*) and either 0.45 μg of LexA empty vector (PBXL3, not shown) or 0.5 μg of LexA-CtBP-1, LexA-CID-ZEB-1 or LexA-CID-ZEB-2 expression vectors. In all the above experiments, equal molar amounts of each empty vector were transfected as controls; 0.1 μg of SV40βgal was also cotransfected to control for transfection efficiency. Luciferase values were assessed as described in Material and methods.

1998) but also failed to synergize with ZEB-1/δEF1 (Figure 3C).

Together, these results suggested that the N-terminal domain of ZEB-1/δEF1 allows the assembly of a Smad–ZEB-1/δEF1–p300 activation complex. In contrast, ZEB-2/SIP1, which lacks the p300-binding site, cannot assemble this coactivator complex and is thus a constitutive repressor by recruiting CtBP to the Smads.

P/CAF recruitment leads to the displacement of CtBP-1 from ZEB-1/δEF1

The interaction between ZEB-1/δEF1 and p300 explains the transcriptional synergy between ZEB-1/δEF1 and R-Smads. However, the above results did not clarify the interplay between coactivator (P/CAF–p300) and corepressor (CtBP-1), and how the binding of p300 and P/CAF to ZEB-1/δEF1 could be dominant over the repressor domain (the CID region) to switch ZEB-1/δEF1 from a repressor to an activator. One possibility was that the binding of P/CAF–p300 to ZEB-1/δEF1 leads to displacement of CtBP-1. It was recently noted by Goodman's group that in many CtBP-binding proteins CID sites are followed by lysines and that P/CAF can acetylate these residues blocking CtBP binding (Zhang *et al*., 2000).

CtBP-binding sites in ZEB-1/δEF1 are also flanked by basic residues, and we found that ZEB-1/δEF1 was acetylated by P/CAF *in vitro* and that the mutation of the lysines following the CID sites to alanines inhibited acetylation (Figure 4A).

In immuno-coprecipitation assays we found that whereas ZEB-1/δEF1 interacts very efficiently with CtBP-1 (right panel in Figure 4B), the ZEB-1/δEF1 complex immunoprecipitated by P/CAF was completely devoid of CtBP-1 (left panel in Figure 4B). This experiment indicated that binding of P/CAF to ZEB-1/δEF1 displaces CtBP-1 from ZEB-1/δEF1. However, and as reported for the mutation of the lysines following the CID site in E1a (Zhang *et al*., 2000; Madison *et al*., 2002), the

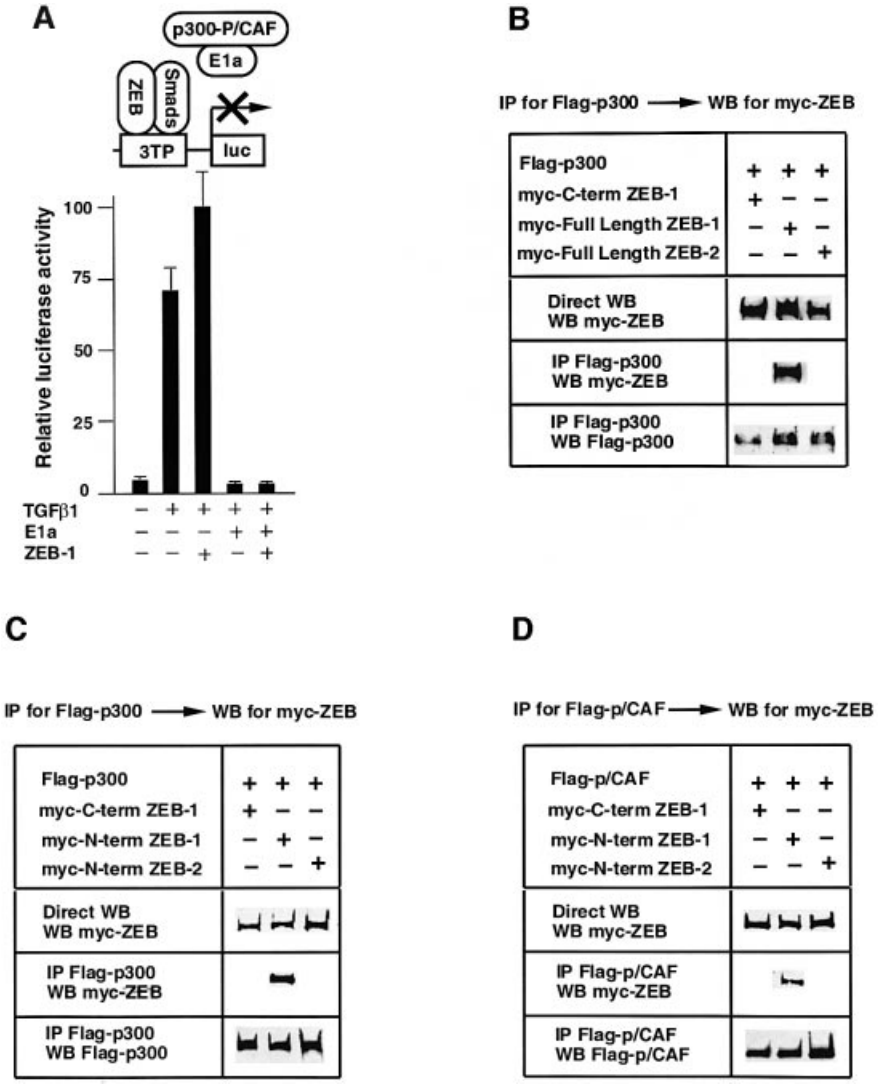


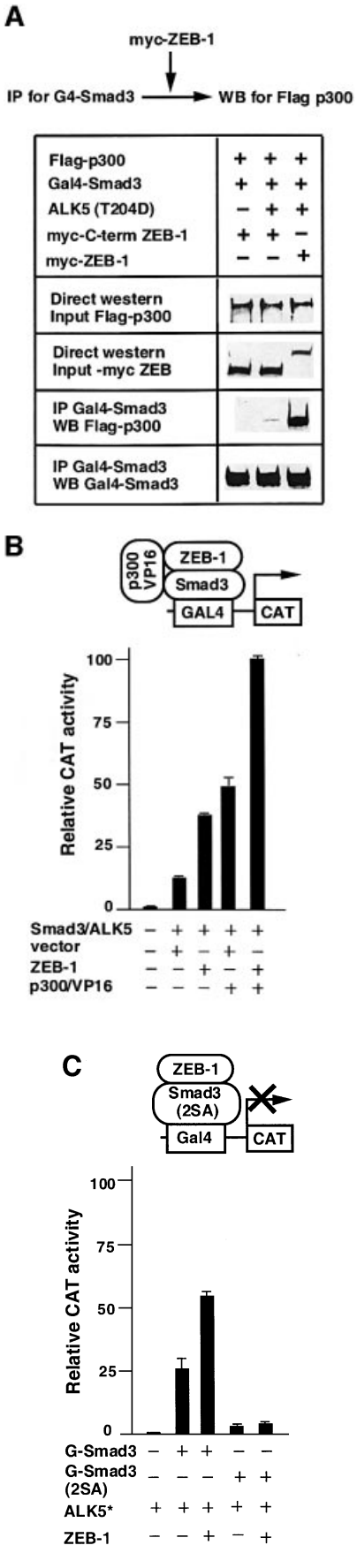
Fig. 2. The N-terminal domain of ZEB-1/δEF1 interacts with coactivators p300 and P/CAF. (**A**) ZEB-1/δEF1 synergy with TGFβ requires the p300 coactivator. 3TP-luc reporter (0.3 μg) was transfected in Mv1Lu along with 0.3 μg of an expression vector for E1A 12S and either 0.48 μg of CS2MT empty vector (not shown) or 0.7 μg of CS2MT-ZEB-1 either in the absence or presence of 25 pM of TGFβ1. SV40βgal (0.1 μg) was cotransfected in all points to control for transfection efficiency. (**B**) Full-length ZEB-1/δEF1, but not ZEB-2/SIP1, interacts with p300. 293T cells were cotransfected with 10 μg of a Flag-tagged p300 expression vector and 20 μg of either myc-tagged ZEB-1/δEF1 or myc-tagged ZEB-2/SIP1 expression vectors. After 48 h, cells lysates were immunoprecipitated (IP) for Flag-p300 and the binding to ZEB-1/δEF1 (but not to ZEB-2/SIP1) determined by western blotting (WB) with an anti-myc 9E10 mAb. (**C**) The N-terminal domain of ZEB-1/δEF1 interacts with p300. 293T cells were cotransfected with 10 μg of Flag-tagged p300 expression vector and 5 μg of the indicated myc-tagged ZEB expression vectors. Cell lysates were immunoprecipitated for Flag-p300 and binding to the different regions of ZEB-1/δEF1 and ZEB-2/SIP1 determined by western blotting with an anti-myc 9E10 mAb. (**D**) P/CAF interacts with the N-terminal region of ZEB-1/δEF1 but not ZEB-2/SIP1. Experiments were performed similarly to those in (C) except that P/CAF was expressed instead of p300.

mutation of these residues in ZEB-1/δEF1 also had only a partial effect on the ability of ZEB-1/δEF1 to repress transcription through CtBP (data not shown). It is possible that *in vivo* acetylation of lysine residues by P/CAF is having a more dramatic effect on CtBP-1 binding than mutation. Alternatively, P/CAF may displace CtBP-1 from binding to CtBP-binding proteins by additional mechanisms such as a steric block.

Antagonistic roles of ZEB-1/δEF1 and ZEB-2/SIP1 in the regulation of activin- and BMP-dependent genes during Xenopus embryogenesis

The results described here and in Postigo (2003) indicate that ZEB proteins have opposing effects on TGFβ/BMP signaling. Regulation of activin and BMP signaling is crucial for establishing the dorso-ventral axis in vertebrate embryogenesis (Candia *et al*., 1997; reviewed in Dale and Wardle, 1999; McDowell and Gurdon, 1999; Hill, 2001; Muñoz-Sanjuan and Brinvalou, 2001; von Bubnoff and Cho, 2001). In view of our results in cell culture systems, we wondered whether ZEB proteins could also have opposing roles in the expression of genes targeted by activin and BMP signaling during *Xenopus* embryogenesis.

Injection of ZEB-2/SIP1 mRNA into *Xenopus* embryos has been examined previously (Remacle *et al*., 1999; Eisaki *et al*., 2000; Lerchner *et al*., 2000; Papin *et al*., 2002). Such ectopic expression of ZEB-2/SIP1 led to head



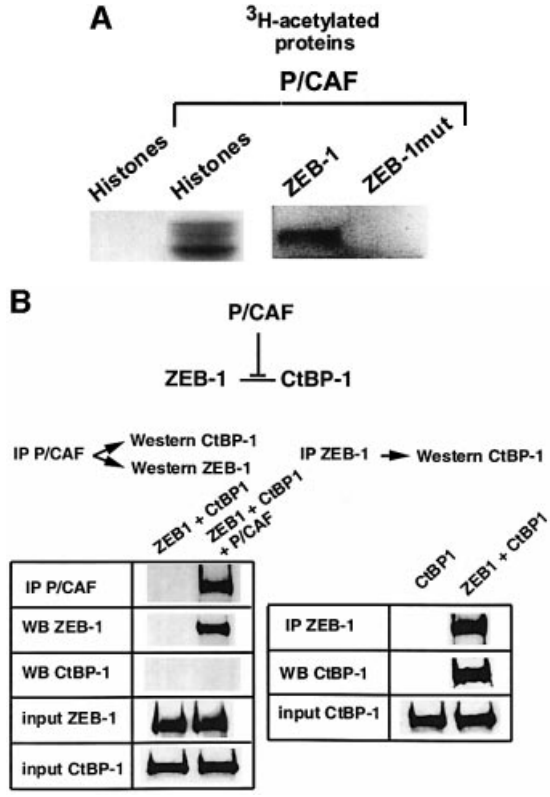


Fig. 4. P/CAF acetylates the lysine residues flanking the CID in ZEB-1/δEF1 and displaces CtBP. (**A**) Purified recombinant P/CAF was used in an *in vitro* assay to acetylate core histones or agarose beads coupled to either GST–CID-ZEB-1 (wild type) or GST–CID-ZEB-1 mutant (K741A, K774A, K775A). (**B**) Expression of P/CAF leads to displacement of CtBP-1 from ZEB-1/δEF1. The indicated tagged expression vectors (Flag-P/CAF, Gal4-ZEB-1/δEF1 and myc-CtBP) were transfected into 293T cells as in previous experiments and cell extracts were immunoprecipitated with antibodies to either Gal4-ZEB-1/δEF1 or Flag-P/CAF. Note that the experiments were designed such that a similar amount of ZEB-1/δEF1 was immunoprecipitated directly (anti-Gal4-ZEB-1/δEF1) or co-immunoprecipitated by P/CAF (compare the left and right panels). No CtBP-1 was associated with ZEB-1/δEF1 when it was bound to P/CAF.

Fig. 3. ZEB-1/δEF1 promotes the assembly of a Smad3–p300 complex. (**A**) 293T cells were cotransfected with 5 μg of the indicated expression vectors and 0.8 μg of the constitutively active ALK5 (T204D). ZEB-1 contains the sequence of ZEB-1/δEF1 from the N-terminal region to the SID. After 48 h, cell lysates were immunoprecipitated (IP) for Smad3 and associated p300 was determined by western blotting (WB). The input (indicated as direct western) represents 15% of the lysate. (**B**) ZEB-1/δEF1 recruits p300 to Smad3. The PG5-CAT reporter containing five Gal4 sites fused to CAT (0.6 μg) was transfected in C33a cells along with the following expression vectors: 0.2 μg of Gal4–Smad3, 0.3 μg of constitutively active ALK5, either 0.48 μg of empty vector CS2MT or 0.7 μg of CS2MT-ZEB-1 and 0.25 μg of a p300–VP16 fusion protein. One-tenth of 1 μg of SV40βgal was cotransfected to control for transfection efficiency. CAT assays were performed as described in Materials and methods. (**C**) Formation of a p300–Smad3–ZEB-1/δEF1 complex requires the binding of p300 to Smad3. C33a cells were transfected with the same CAT reporter as in (B) along with either 0.2 μg of Gal4–Smad3 or Gal–Smad3 (2SA), 0.3 μg of constitutively active ALK5 (T204D) (ALK5*) and either 0.48 μg of empty vector CS2MT (not shown) or 0.7 μg of CS2MT-ZEB-1. One tenth of 1 μg of SV40βgal was cotransfected to control for transfection efficiency.

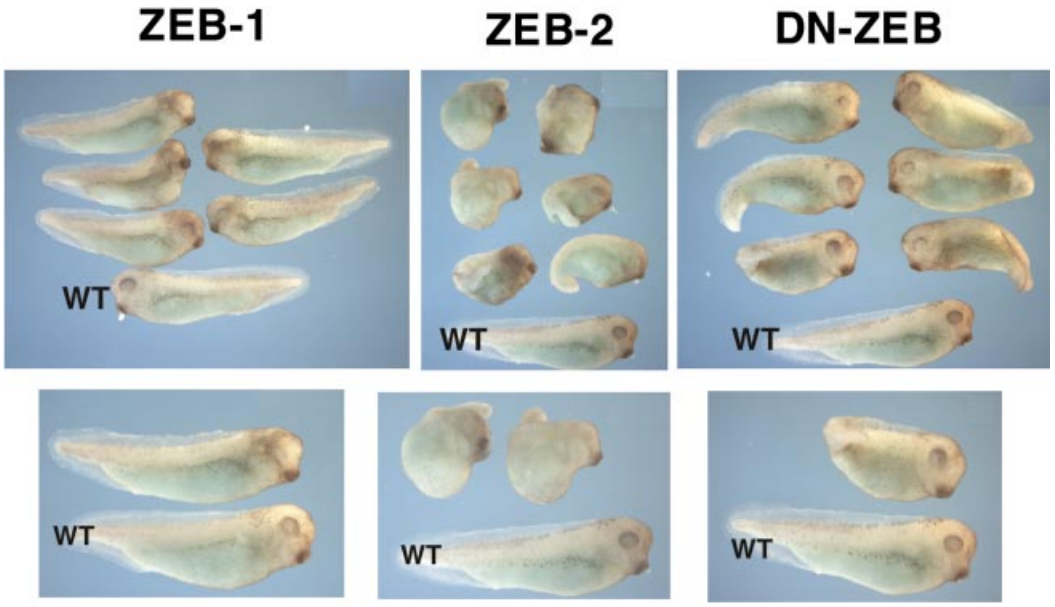


Fig. 5. Regulation of *Xenopus* development by ZEB-1/δEF1 and ZEB-2/SIP1 *in vivo*. Phenotype of *Xenopus* embryos injected at the two-cell stage with RNA for ZEB-1/δEF1, ZEB-2/SIP1 or DN-ZEB. Embryos were allowed to develop until tailbud (stage 29–30). Embryos injected with ZEB-2/SIP1 or DN-ZEB mRNA were significantly dorsalized. In contrast, ZEB-1/δEF1 showed no dorsalizing effect, and there was disruption of eye development in the injected side. In each panel, a wild-type embryo is shown at the bottom (indicated as WT).

abnormalities and thickening of the neural tube, induction of the neural marker NCAM and repression of the activin-dependent *Brachyury* gene (Eisaki *et al*., 2000; Lerchner *et al*., 2000). We found that injection of ZEB-2/SIP1 mRNA into two-cell stage *Xenopus* embryos, in addition to blocking *Brachyury* (arrow in Figure 6b), also inhibited expression of the BMP-regulated msx-1 gene (Figure 7) and induced a neuralization and dorsalization of the embryo (Figure 5). This was shown by ectopic expression of markers such as Sox2, Six3, Xnot and NCAM, and accompanying loss of epidermal keratin (Figures 6e, h and k and 7; data not shown).

BMP-4 signaling is known to inhibit neural induction in *Xenopus* (Jones *et al*., 1992; reviewed in Dale and Wardle, 1999). Interestingly, the BMP-4 promoter contains ZEB sites and is itself BMP dependent (Vainio *et al*., 1993; Ebara *et al*., 1997; Kawasaki *et al*., 1999). As shown in Figure 7, we found that BMP-4 expression was inhibited by ZEB-2/SIP1, which could explain the neuralization/dorsalization effect of ZEB-2/SIP1. This *in vivo* repression of Smad-dependent genes by ZEB-2/SIP1 is also consistent with the inhibition of Smad signaling by ZEB-2/SIP1 that we observed in cell line systems, as described in this manuscript and in Postigo (2003).

Next, we examined the effect of ZEB-1/δEF1 RNA injection into *Xenopus* embryos. Consistent with our results in cultured cell lines showing a synergy between TGFβ family members and ZEB-1/δEF1, we found that microinjection of ZEB-1/δEF1 also had an opposite effect to ZEB-2/SIP1 *in vivo*, as it induced the ectopic expression of the activin-dependent *Brachyury* gene (arrow in Figure 6a).

From these results, it seems that ZEB-1/δEF1 and ZEB-2/SIP1 can regulate activin/BMP signaling *in vivo*. However, the above studies relied on ectopic overexpression of ZEB-1/δEF1 and ZEB-2/SIP1 in *Xenopus* embryos. Therefore, it was still unclear whether endogenous ZEB proteins do play a role. To address this we decided to use a dominant-negative approach to displace ZEB proteins from binding to their DNA-binding sites in activin/BMP-dependent promoters. ZEB-1/δEF1 and ZEB-2/SIP1 share high homology in their zinc finger clusters that serve as DBDs (Genetta *et al*., 1994; Sekido *et al*., 1997). We and others have shown that the C-terminal zinc finger region has higher affinity for DNA than the full-length proteins, displaces endogenous ZEB proteins from DNA-binding sites and serves as an efficient dominant negative for both ZEB proteins (Postigo and Dean, 1997; Remacle *et al*., 1999; Grooteclaes and Frisch, 2000; Fontemaggi *et al*., 2001). Therefore, we reasoned that this dominant negative (DN-ZEB) could be used to test the role of endogenous ZEB proteins in activin and BMP signaling in *Xenopus* embryogenesis.

As in other ZEB-dependent genes, the particular configuration and arrangement of the ZEB DNA-binding sites in the promoter of the *Xenopus Brachyury* gene is important to determine the specificity of these sites for ZEB regulation (Remacle *et al*., 1999; Lerchner *et al*., 2000). We examined the effect of injecting DN-ZEB mRNA on the expression of *Brachyury*. Interestingly, we found that DN-ZEB had the same effect as ZEB-2/SIP1, namely it inhibited the expression of the activin-dependent *Brachyury* gene (arrow in Figure 6c). Likewise, DN-ZEB also induced dorsalization of the embryo, ectopic neural differentiation and expression of neural markers (Figures 5 and 6f, i and l).

Because ZEB-2/SIP1 and DN-ZEB led to a similar phenotype, we suggest that DN-ZEB and ZEB-2/SIP1 are displacing an activator from the *Brachyury* promoter. This activator is likely to be ZEB-1/δEF1 (i) because of the already mentioned specificity of the ZEB sites in the *Brachyury* gene (Remacle *et al*., 1999; Lerchner *et al*.,

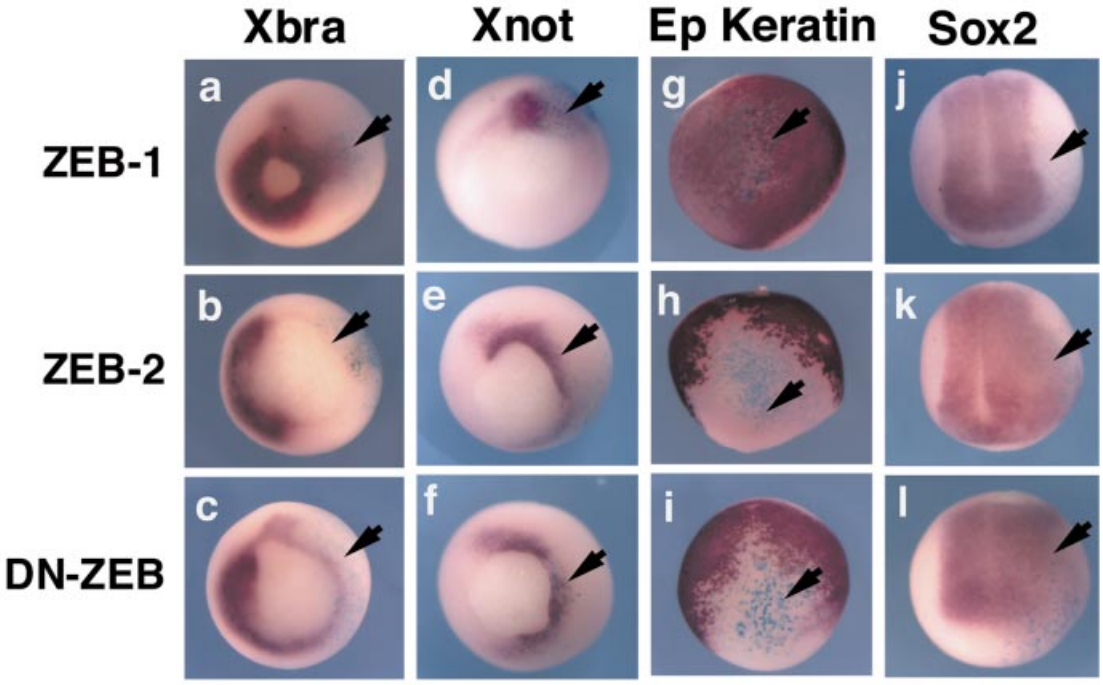


Fig. 6. Regulation of activin/BMP-dependent genes and neural genes by ZEB-1/δEF1 and ZEB-2/SIP1. *In situ* hybridization for the indicated genes in embryos injected with ZEB-1/δEF1, ZEB-2/SIP1 or DN-ZEB RNAs along with mRNA encoding β-galactosidase (to mark the side of the injection) and allowed to develop to stage 11.5 (for expression of Xbrachyury, Xnot and epidermal keratin) or stage 13 (for Sox2). Whereas ZEB-1/δEF1 induced ectopic expression of *Brachyury*, ZEB-2/SIP1and DN-ZEB inhibited *Brachyury* expression. There was no effect on the control uninjected side (left side of each embryo).

2000) and (ii) because if DN-ZEB and ZEB-2/SIP1 were displacing an activator other than ZEB-1/δEF1, injection of ZEB-1/δEF1 should have also displaced this activator and had an effect similar to ZEB-2/SIP1 and DN-ZEB (namely the repression of *Brachyury*); instead, injection of ZEB-1/δEF1 induced ectopic expression of *Brachyury* (arrow in Figure 6a).

The effect of injecting ZEB-2/SIP1 RNA appears to be somewhat more dramatic than that caused by DN-ZEB injection, perhaps reflecting the importance of active repression versus simple displacement. Both ZEB-2/SIP1 and DN-ZEB would displace ZEB-1/δEF1 from the *Brachyury* promoter, but ZEB-2/SIP1 has an active transcriptional repressor domain that DN-ZEB does not have. Thus, expression of ZEB-2/SIP1 is likely to replace ZEB-1/δEF (associated with Smads and p300) with an active repressor complex containing ZEB-2/SIP1 and CtBP. Interestingly, ZEB-2/SIP1 expression is induced by TGFβ (Comijn *et al*., 2001; our own data not shown), suggesting the possibility of a feedback mechanism between both ZEB proteins (see Discussion in Postigo, 2003).

Taken altogether, the above results indicate that ZEB-1/δEF1 and ZEB-2/SIP1 not only antagonize in their regulation of Smad-mediated functions in tissue culture systems, but they also have opposing roles in modulating TGFβ family signaling *in vivo*.

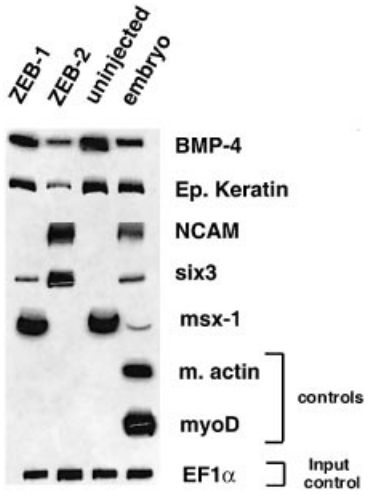


Fig. 7. Differential effect of ZEB-1/δEF1 versus ZEB-2/SIP1 on gene expression. mRNA isolated from animal caps of *Xenopus* embryos (either uninjected or injected with mRNA for ZEB-1/δEF1 or ZEB-2/SIP1) was used for RT–PCR analysis of the indicated genes (see Materials and methods for details).

Discussion

ZEB-1/δEF1 and ZEB-2/SIP1 are structurally quite similar. Both contain N- and C-terminal zinc fingers, which serve as DBDs, and their central region interacts with the corepressor CtBP (Genetta *et al*., 1994; Sekido *et al*., 1994, 1997; Postigo and Dean, 1999a, 2000; Verschueren *et al*., 1999). Although ZEB proteins have been classically described as transcriptional repressors, they function antagonistically in regulating TGFβ/BMP signaling (Postigo, 2003). In this paper, we present evidence indicating that this antagonism results from the differential recruitment of coactivators (p300 and P/CAF)

and corepressors (CtBP) by ZEB-1/δEF1 and ZEB-2/SIP1. TGIF also recruits CtBP to repress Smads but it does not require direct binding to DNA to mediate its repressor effects (Melhuish and Wotton, 2000). In contrast, regulation of TGFβ signaling by ZEB proteins is unique not only because of the specificity dictated by their DNA-binding sites in TGFβ-dependent promoters, but also because of their mechanism of action. Whereas ZEB-2/SIP1 only binds CtBP and acts as a Smad repressor, ZEB-1/δEF1 recruits p300 and P/CAF to the Smads to form a complex that not only activates transcription of TGFβ-dependent genes more efficiently but also displaces CtBP from ZEB-1/δEF1. p300 has also been shown to act as a bridge between Smad1 and Stat3 in the developing brain (Nakashima *et al*., 1999). ZEB-1/δEF1 activates the vitamin D3 receptor and the estrogen-responsive ovalbumin gene in a cell-specific manner (Chamberlain and Sanders, 1999; Lazarova *et al*., 2001; Dillner and Sanders, 2002). Although the molecular mechanism remains to be determined, it is tempting to speculate that the activation of these two genes by ZEB-1/δEF1 also involves recruitment of p300 and P/CAF and displacement of CtBP.

In the last few years, there have been a number of reports indicating that p300 and P/CAF acetylate proteins other than histones, including many transcription factors (reviewed in Kouzarides, 2000). The effect of acetylation on transcription factors varies from protein to protein (Gu and Roeder, 1997; Martinez-Balbas *et al*., 2000; reviewed in Kouzarides, 2000). However, and even for p53 (where the effect of acetylation has been extensively studied), there is still controversy in the literature about the consequences of its acetylation that could reflect specific functions for the acetylation of individual lysines or different experimental conditions (Nakamura *et al*., 2000; Barlev *et al*., 2001; Espinosa and Emerson, 2001; Prives and Manley, 2001). In general, acetylation of transcription factors has been associated with stimulation of transcription. In this line, our results indicated that P/CAF switches ZEB-1/δEF1 to an activator. When the N-terminal p300–P/CAF binding site in ZEB-1/δEF1 was deleted (as in the case of CR-ZEB-1 in Figure 1A), the truncated ZEB-1/δEF1 behaved in the same fashion as ZEB-2/SIP1: it became a repressor of Smad activity.

How is the switch of ZEB-1/δEF1 from a transcriptional repressor to a transcriptional activator regulated? P/CAF and p300 are crucial for the activity of differentiation-promoting transcription factors such as the retinoic acid receptor and the myogenic factor myoD (Puri *et al*., 1997; Kawasaki *et al*., 1998; reviewed in Goodman and Smolik, 2000). At least in the case of myogenesis, it has been demonstrated that P/CAF–p300 is unable to associate with myoD until myoblasts receive a signal to differentiate into myotubes. It is then possible that endogenous ZEB-1/δEF1 does not associate with endogenous P/CAF–p300 at significant levels until such differentiation signals occur. This would allow the ZEB-1/δEF1–CtBP complex (which would be predominant in the absence of P/CAF–p300 binding) to actively repress transcription of target genes prior to the onset of differentiation and TGFβ/BMP signals. This model of regulation would be somewhat analogous to cell cycle control, where other transcription factors, E2F proteins, oscillate between activation and repression of target genes through differential interaction with P/CAF–p300 and the retinoblastoma protein Rb (along with other corepressors), respectively (reviewed in Harbour and Dean, 2000).

Altogether, the results presented here indicate that ZEB proteins play an important role in the regulation of the TGFβ family activities during cell differentiation and embryonic development. Moreover, their intricate mechanism of action also offers a unique model to study the transcriptional regulation of Smad-mediated signaling.

Materials and methods

Cells and cell culture

C33a and Mv1Lu cells were obtained from the American Type Culture Collection (Manassas, VA). 293T cells were obtained from Dr S.J.Korsmeyer (Dana Farber Cancer Institute, Boston, MA). Cell lines were maintained in Dulbecco's modified Eagle's medium containing 12% fetal calf serum (Gibco-Life Technologies, Grand Island, NY).

Plasmid construction

The 3TP-lux reporter was obtained from Dr J.Massagué (Sloan Kettering Cancer Center, New York, NY). A constitutively active form for activin-like kinases ALK5 (T204D) was obtained from Dr K.Miyazono (Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan).

The different myc-tagged versions of ZEB proteins have been described (Postigo, 2003). Mutations of individual amino acids in the context of either full-length ZEB-1/δEF1 or the CID region were generated using the QuikChange XL Directed-Mutagenesis kit (Stratagene, La Jolla, CA). PG5-CAT containing five Gal4 DNA-binding sites upstream of the fibronectin TATA box and fused to the chloramphenicol acetyl transferase (CAT) gene and PL6G5-CAT (as PG5-CAT but containing also six Lex DNA-binding sites) reporters have been described previously (Postigo *et al*., 1997). Gal4–Smad3 was constructed by cloning the Smad3 cDNA in-frame with the DBD of the Gal4 protein in the PM1 vector. Gal4–Smad3 (2SA) was obtained from Dr X.H.Feng (Baylor College, Houston, TX) (Feng *et al*., 1998). LexA-CtBP-1, LexA-CID-ZEB-1 and LexA-CID-ZEB-2 were described previously (Postigo and Dean, 2000). pCI-neo-Flag-p300 and pCI-neo-Flag P/CAF were obtained from Dr R.L.Schiltz (NIH, Bethesda). CMV-p300/VP16 was obtained from Dr D.Livingston (Dana Farber Cancer Institute, Boston, MA).

Transfections

Cells were transfected by the calcium phosphate method or with Superfectin reagent (Qiagen, Hilden, Germany) as described previously (Postigo and Dean, 2000). For luciferase assays, 5–12 h after transfection, cells were washed and activated with the indicated amounts of TGFβ-1. 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), the values of which are determined using a luminescent β-galactosidase detection Kit II® (Clontech Laboratories, Palo Alto, CA). Transcriptional assays using a CAT reporter gene were performed as described previously (Postigo and Dean, 2000).

In vitro acetylation assays

Purified core chicken histones (Upstate Biotechnologies, UBI) or either GST–CID-ZEB-1 and GST–CID-ZEB-1 (K745A; KK774/775AA) bound to beads were incubated with recombinant P/CAF (UBI) in a 30 μl reaction containing acetylation buffer (10 mM Tris–HCl pH 8.0, 10% glycerol, 1 mM EDTA, 10 mM sodium butyrate, 1 mM DTT) and [^{3}H]acetyl coA (Amersham Pharmacia Biotech) and incubated for 30 min at 30°C. Reactions were loaded in a 10% polyacrylamide gel and analyzed by fluorography.

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 analysis. 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).

Xenopus embryo culture and in situ hybridization analysis

Xenopus laevis embryos were obtained and cultured as described previously (Peng, 1991). CS2MT, CS2MT-ZEB-1, CS2MT-ZEB-2 and CS2MT-DN-ZEB plasmids were linearized with *Not*I and used to produced capped RNA with a SP6-Message Machine Kit® (Ambion). A total of 250–500 pg of each RNA was dissolved in water and injected into one blastomere at the two-cell stage along with β-galactoside mRNA to trace the site of injection. Embryos were cultured in 0.2× Marc's Modified Ringer's solution (MMR) containing 4% Ficoll and 50 μg/ml gentamycin. *In situ* hybridization assays were carried out as described previously (Kroll *et al*., 1998). Embryos were injected in both cells at the two-cell stage for animal cap isolations. Animal cap explants were made at stage 8 and raised to stage 18 in 0.7× MMR supplemented with 50 μg/ml gentamycin and 1 mg/ml bovine serum albumin.

RT–PCR analysis of animal caps

To analyze gene expression in animal caps, we extracted RNA at stage 18 (late neurula) from 10 animal caps and synthesized cDNA using an Omni-Script kit (Qiagen). The resulting cDNA was amplified by RT–PCR as described previously (Kroll *et al*., 1998). Primers for BMP4, six3 and EF-1α have been reported previously (Rao, 1994; Wilson and Melton, 1994; Kroll *et al*., 1998). Other primers for PCR included: epidermal keratin (5′-CACCAGAACACAGAGTAC-3′ and 5′-CAACCTTCCCATCAACCA-3′), NCAM (5-CACAGTTCCACCAAATGC-3′ and 5′-GGAATCA-AGCGGTACAGA-3′), muscle actin (5′-GCTGACAGAATGCAG-AAG-3′ and 5′-TTGCTTGGAGGAGTGTGT-3′), myoD (5′-GTGTTG-TTGTCCATCCATGAC-3′ and 5′-AGGTCCAACTGCTCCGACGG-CATGAA-3′) and msx.1 (5′-GGCAGTCTGCAACTACTA-3′ and 5′-CTATGTCATGTCACCCTC-3′). For each primer set, we determined whether amplification was within a linear range by quantification with a PhosphorImager (Molecular Dynamics) using serial dilutions of cDNA from embryonic samples.

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