Mechanisms of Development 120 (2003) 629–637

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Tob proteins enhance inhibitory Smad-receptor interactions to repress BMP signaling

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Received 20 September 2002; received in revised form 27 January 2003; accepted 27 January 2003

Abstract

Tob inhibits bone morphogenetic protein (BMP) signaling by interacting with receptor-regulated Smads in osteoblasts. Here we provide evidence that Tob also interacts with the inhibitory Smads 6 and 7. A yeast two-hybrid screen identified Smad6 as a protein interacting with Tob. Tob co-localizes with Smad6 at the plasma membrane and enhances the interaction between Smad6 and activated BMP type I receptors. Furthermore, we have isolated *Xenopus Tob2*, and show that it cooperates with Smad6 in inducing secondary axes when expressed in early *Xenopus* embryos. Finally, Tob and Tob2 cooperate with Smad6 to inhibit endogenous BMP signaling in *Xenopus* embryonic explants and in cultured mammalian cells. Our results provide both in vitro and in vivo evidence that Tob inhibits endogenous BMP signaling by facilitating inhibitory Smad functions.

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Keywords: BMP signaling; Smad; TGF-β signaling; Tob2; *Xenopus*

1. Introduction

Bone morphogenetic proteins (BMPs) comprise a rapidly expanding subclass of the TGF-β superfamily of secreted signaling molecules. They were first identified as osteoinductive proteins and play many important roles in a variety of fundamental processes, including dorsal–ventral patterning during embryonic development, bone formation, neurogenesis, and decisions of left–right asymmetry (reviewed in Hogan, 1996; Massague, 2000; Miyazono et al., 2001). BMP signaling has been most intensely studied in the context of mesoderm induction and repression of neural fate during early amphibian development (Weinstein and Hemmati-Brivanlou, 1999). When BMP activity is blocked in ventral mesodermal cells of the *Xenopus* embryo, ventral fates are eliminated and a secondary dorsal axis forms. In addition, when BMP signaling is blocked in prospective epidermal (ventral) cells, these cells instead differentiate into neural (dorsal) tissue.

Signaling by BMPs, like other TGF-βs, requires the heteromeric interaction between distinct type I and II transmembrane serine/threonine kinase receptors (Massague, 2000; Miyazono et al., 2001). Upon BMP stimulation, type I receptors are phosphorylated and activated by type II receptor kinases. The type I receptor kinases then, in turn, transmit intracellular signals by phosphorylating Smad proteins. Smad proteins play a central role in mediating BMP-induced signals from the cell surface to the nucleus. Smads are subdivided into three classes: receptor-regulated Smads (R-Smads), a ‘common’ Smad (Co-Smad), and inhibitory Smads (I-Smads), each of which has a distinct function. The BMP pathway-specific R-Smads (Smad1, 5, and 8) are phosphorylated on a carboxy-terminal SSXS

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0925-4773/03/$ - see front matter © 2003 Elsevier Science Ireland Ltd. All rights reserved.
doi:10.1016/S0925-4773(03)00020-0

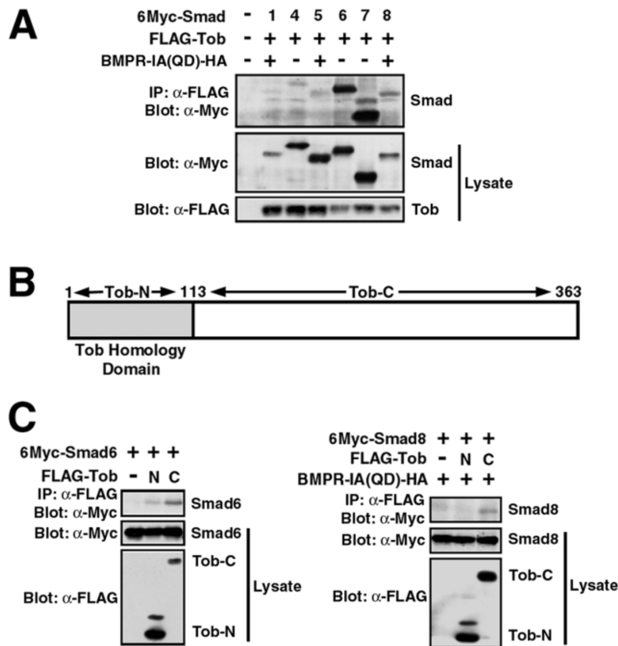

Fig. 1. Interaction of mouse Tob with Smads. (A and C) COS7 cells were transfected with Flag-Tob and 6Myc-Smad expression vectors. The proteins in the cell lysates were subjected to immunoprecipitation (IP) followed by immunoblotting (Blot). BMPR-IA(QD) is a constitutively active form of the mouse BMP type IA receptor and is tagged at the C-terminus with the HA epitope. (A) Full length mouse Tob interacts strongly with inhibitory Smads 6 and 7. The top panel shows the interactions, and the lower two panels show expression of each indicated protein. Levels of Smads and Tob in the transfected cell lysates were examined with anti-Myc and anti-Flag antibodies. (B) Deletion constructs used for immunoprecipitation assays in (C). Tob-N is the N-terminal region of mouse Tob (a.a. 1–113), which includes the 110 a.a. long Tob homology domain. Tob-C is the C-terminal region of mouse Tob (a.a. 114 –363). Both proteins are tagged at the C-terminus with the Flag epitope. (C) Smad6 and Smad8 interaction with Tob-N- and Tob-C.

motif by type I BMP receptors. Once phosphorylated, R-Smads dissociate from the receptor, bind to the Co-Smad (Smad4), and translocate to the nucleus. In the nucleus, heteromeric R-Smad/Smad4 complexes function as effectors of BMP signaling by regulating the transcription of specific target genes; they specifically recognize target promoters by associating with other DNA-binding coactivators. In contrast, the I-Smads Smad6 and 7 antagonize the BMP/TGF-β signaling pathway at several levels by different mechanisms (reviewed in von Bubnoff and Cho, 2001). First, I-Smads prevent phosphorylation of R-Smads by interacting with the activated type I receptors. Second, Smad6 has been shown to interact with phosphorylated Smad1, thus preventing complex formation between Smad1 and Smad4 (Hata et al., 1998). Third, Smad6 appears to block an unconventional BMP pathway by binding to and inhibiting TAK1, a MAP Kinase Kinase Kinase (Kimura et al., 2000). In addition, Smad6 is localized in the nucleus and functions as a transcriptional corepressor by interacting with histone deacetylases (Bai and Cao, 2002). And finally, Smad7 induces degradation of the TGF-β type I receptor through recruitment of Smurfs, which are members of the HECT family of E3 ubiquitin ligases, to the receptor (Ebisawa et al., 2001; Kavsak et al., 2000).

Tob is a member of a new anti-proliferative protein family consisting, in humans, of Tob, Tob2, ANA, BTG1, BTG2 (PC3 in rat, and TIS21 in mouse), BTG3 in mouse, (Bradbury et al., 1991; Fletcher et al., 1991; Rouault et al., 1992; Matsuda et al., 1996; Rouault et al., 1996; Guehenneux et al., 1997; Yoshida et al., 1998; Ikematsu et al., 1999). Tob/BTG family proteins have been shown to suppress proliferation when over-expressed in NIH3T3 cells (Rouault et al., 1992; Matsuda et al., 1996; Montagnoli et al., 1996; Yoshida et al., 1998; Ikematsu et al., 1999), but the biological importance of these proteins in vivo is poorly understood. Recent studies in mice carrying a targeted deletion of the *Tob* gene indicate that *Tob* is a negative regulator of osteogenesis (Yoshida et al., 2000). A previous analysis of Tob protein behavior has revealed that Tob can directly interact with BMP receptor-regulated Smads1, 5 and 8, and with Smad4, and that Tob represses a BMP-specific reporter activated in the presence of excess Smad1 and 5 (Yoshida et al., 2000). These results are consistent with the notion that Tob functions as a negative regulator of BMP signaling by sequestering Smad1 and 5. In addition, Tob has been implicated as a negative regulator of T-cell proliferation and shown to associate with Smad2 and 4 (Tzachanis et al., 2001).

In this study, we identify an additional mechanism whereby Tob can inhibit BMP signaling: by interacting with and modulating the function of I-Smads. A yeast two-hybrid screen identified Smad6 as a protein interacting with Tob, and we show that Tob interacts more strongly with the inhibitory Smads 6 and 7 than with any other Smad. Furthermore, Tob co-localizes with Smad6 in the plasma membrane, and enhances the interaction between Smad6 and activated BMP type I receptors. Tob and Tob2 can both cooperate with Smad6 in inhibiting BMP signaling, as assayed by measuring the activity of endogenous BMP signaling in *Xenopus* animal caps and in P19 cells. In addition, Tob2 cooperates with Smad6 to induce secondary axes when over-expressed ventrally in early *Xenopus* embryos. Thus, our results suggest a novel mechanism whereby Tob inhibits BMP signaling, by enhancing the interaction of Smad6 with the activated BMP receptor.

2. Results

2.1. Tob and Tob2 preferentially interact with inhibitory Smads

In order to better understand the molecular action of Tob, we searched for proteins that associate with Tob using the yeast two-hybrid screen. As bait, we used the N-terminal 236 amino acids of mouse Tob, which include the ~110 amino acid long N-terminal Tob homology domain

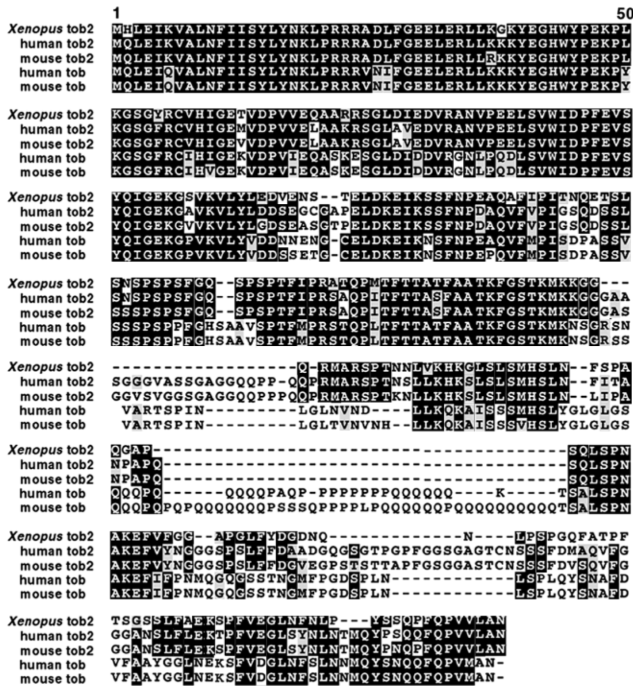

Fig. 2. Amino acid sequence alignments of *Xenopus*, human and mouse Tob2, and human and mouse Tob. Identical residues are indicated in black, and conservative changes are indicated in gray. The *Xenopus* Tob2 gene is 62 and 78% identical to human Tob and Tob2, respectively.

(Yoshida et al., 1998; Matsuda et al., 2001). We isolated Smad6 (data not shown), which has been implicated as a negative regulator of BMP signaling (Imamura et al., 1997; Hata et al., 1998). One of the isolated cDNA clones encoded the carboxy-terminal region of the Smad6 protein (amino acid positions 192–496), which includes a C-terminal portion of the MH1 domain, the linker region and the entire MH2 domain (Hata et al., 1998).

In order to determine the specificity of the interaction between Tob and Smad6, we transfected COS7 cells with an expression vector encoding Flag-tagged full-length mouse Tob together with various expression plasmids encoding Myc-tagged Smads 1, 4–8. Tob was immunoprecipitated from cell lysates using anti-Flag antibody and the bound protein was probed for Smads by immunoblotting with anti-Myc antibodies. Tob showed the strongest interaction with inhibitory Smads 6 and 7 (Fig. 1A). However, after longer exposures, we also observed interactions of Tob with Smads 1, 4, 5 and 8, as has been reported before (data not shown; see Yoshida et al., 2000). These results, together with the previous finding that Tob weakly interacts with Smads 2 and 3 (Yoshida et al., 2000), suggest that inhibitory Smads 6 and 7 are the preferred binding partners of Tob among all the Smad proteins. A similar experiment using Tob2, a closely related Tob gene we previously identified (Ikematsu et al., 1999), gave essentially identical results (data not shown), indicating that the activity of Tob and Tob2 is indistinguishable using the present assay.

To further define the domains involved in the interaction between the Tob and Smad6 proteins, two deletion mutants of mouse Tob were generated as shown in Fig. 1B. Smad6 interacted with both the C-terminal and N-terminal regions of Tob, although the interaction with the C-terminal region was somewhat stronger (Fig. 1C, left). In contrast, both human Smads 1 and 8 associated only with the C-terminal fragment of Tob (Fig. 1C right, and data not shown). Thus, our results demonstrate that Smad6 interacts with both the C- and N-terminal (Tob/BTG homology) domains whereas the pathway-specific Smads 1 and 8 only interact with the C-terminal domain of Tob. This difference may contribute to the preferred interaction between Tob/Tob2 and inhibitory Smads over the BMP pathway specific R-Smads.

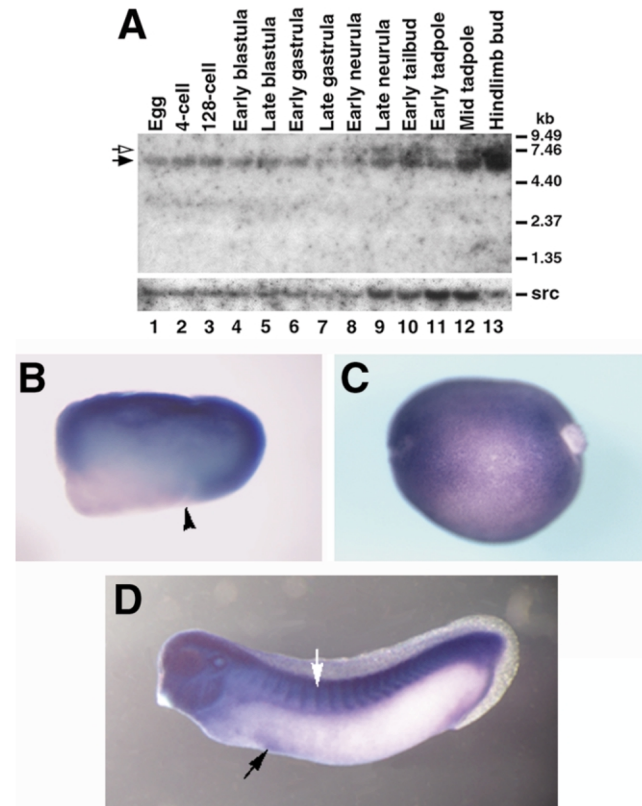

Fig. 3. Expression of *Xenopus Tob2* in early *Xenopus* embryos. (A) Northern blot analysis of *XTob2* expression in normal *Xenopus* embryos. There was an *XTob2* transcript of about 5.5 kb expressed maternally and throughout early *Xenopus* development (closed arrow). An additional, larger transcript of about 7.5 kb was detected by the late gastrula stage (open arrow). Src is used as a loading control. (B–D) Whole mount in situ hybridization analysis of *XTob2* expression in early *Xenopus* embryos. (B) Lateral view of a gastrula (stage 10.5) embryo; the animal pole is up. XTob2 is expressed ubiquitously in the animal region of the embryo. Arrowhead, dorsal blastopore lip. (C) Dorsolateral view of a late gastrula (Stage 12.5) embryo; anterior is to the left, dorsal is up. (D) Lateral view of a tailbud stage embryo (stage 30/31). Note that there is elevated expression in several areas: the anterior ventral blood islands (black arrow), somites (white arrow), otic vesicle, eyes, brain, branchial arches and the heart anlage.

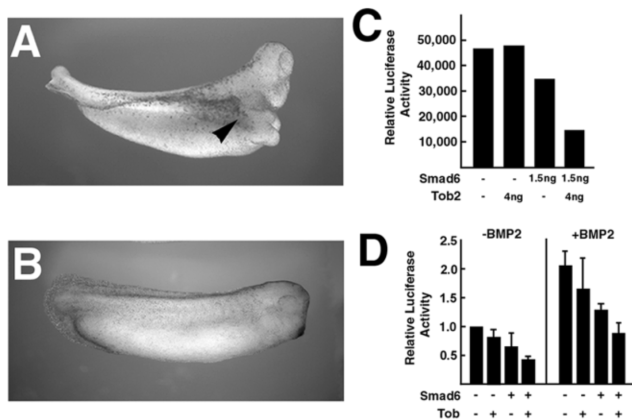

Fig. 4. Tob and XTob2 cooperate with Smad6 to inhibit BMP signaling in *Xenopus* embryos and in cultured cells. (A,B) *XTob2* mRNA was injected alone or together with mouse *Smad6* mRNA into the ventral marginal zone of four-cell stage embryos. Coinjection of 1.75 ng of mSmad6 and 4 ng of *XTob2* in *Xenopus* embryos efficiently induced secondary axes in *Xenopus* embryos (Panel A, 48%, $n = 21$), whereas up to 5 ng of *XTob2* alone did not induce axes (Panel B, 0%, $n = 46$). At a higher dose of Smad6 (3.5 ng), secondary axes were observed in 19% of the embryos ($n = 26$), and coinjection with *XTob2* mRNA resulted in the majority of the embryos showing secondary axes (64%, $n = 25$). (C) −385*Xvent2*/luc was injected into the animal pole region of four-cell stage embryos together with the indicated mRNAs. At the blastula stage, animal caps were dissected manually, cultured for 3 h, homogenized, and subjected to luciferase assays. Injection of *XTob2* together with *mSmad6* led to a stronger repression of endogenous BMP signaling as measured by luciferase activity than injection of mSmad6 alone. Note that in some experiments, injection of XTob2 mRNA alone led to a slight repression of reporter gene activity, but never as strong as when *mSmad6* mRNA was coinjected (data not shown). (D) P19 cells were transfected with −385*Xvent2*/luc together with Flag-Smad6 and/or Flag-Tob (mouse) expression constructs. After incubation for 24 h in the presence or absence of 300 ng/ml rhBMP2, luciferase activities were determined. Mean luciferase activity of the cells transfected with the empty plasmid and that received no BMP2 stimulation was set to 1. Shown are mean values from three experiments.

2.2. Identification and expression of Xenopus Tob2

To characterize the functional interaction between Tob and Smad6 in vivo, we decided to examine the action of Tobs in early *Xenopus* embryos, where the mechanisms of BMP signaling have been extensively studied (Cho and Blitz, 1998; von Bubnoff and Cho, 2001). We identified a *Xenopus Tob*-related gene, most closely related to *Tob2* (62 and 78% a.a identity to human Tob and Tob2, respectively; see Fig. 2). Therefore, this *Xenopus Tob* gene is referred to as *XTob2*.

Northern blot analysis of *XTob2* transcripts revealed a ~5.5 kb transcript that is expressed maternally and throughout early *Xenopus* development (Fig. 3A, closed arrow). Interestingly, by late gastrula stage, an additional larger *XTob2* transcript of ~7.5 kb was detected (Fig. 3A, open arrow).

Whole-mount in situ hybridization analysis revealed a widespread distribution of *XTob2* from early gastrula through tailbud stages (Fig. 3B–D). Around stage 23, we observed increased expression in the head region including the branchial arches, the cement gland, the eyes and the brain, and in the somites (data not shown). By the tailbud stage (Fig. 3D), there was still elevated expression in the branchial arches, the eyes, the brain and the somites. In addition, we observed elevated expression in the otic vesicle, the anterior-ventral blood islands and the heart anlage. Expression was absent in the region of the cement gland by the tailbud stage (Fig. 3D).

Our expression data are consistent with a potential interaction between XTob2 and inhibitory Smads in early *Xenopus* embryos, as the expression pattern of *XTob2* overlaps with that of *XSmad6* and *XSmad7* (Casellas and Brivanlou, 1998; Nakayama et al., 1998a,b). Like *XTob2*, *XSmad6* and *7* are expressed ubiquitously in the animal half of early gastrula embryos, and at the neurula stage, *XSmad6*, *7* and *XTob2* are expressed in the lateral edges of the anterior neural plate. At the tailbud stage, both *XSmad6* and *7* are expressed, like *XTob2*, in the eyes, the otic vesicles, the olfactory placodes, the brain, and the heart anlage.

2.3. Both Tob and XTob2 cooperate with Smad6 to inhibit BMP signaling

Our finding that Tob displays the highest affinity towards I-Smads (Smad6 and 7) raises the interesting possibility that Tob's in vivo function is to modify the activity of inhibitory Smads. Since Tob can interact with Smad6, and since Tob has been shown to inhibit BMP signaling in over-expression assays (Yoshida et al., 2000), we wanted to determine whether Tob could influence the ability of inhibitory Smad6 to inhibit BMP signaling. In *Xenopus* embryos, over-expression of inhibitory *Smad6* ventrally suppresses BMP signaling, thereby inducing an ectopic Spemann organizer resulting in the development of a secondary dorsal axis (Tsuneizumi et al., 1997; Hata et al., 1998; Nakayama et al., 1998a). Since Tob and Smad6 can physically interact in coimmunoprecipitation assays, we wondered whether Tob might enhance Smad6's ability to induce secondary axes in *Xenopus* embryos by cooperating with Smad6-mediated inhibition of BMP signaling.

We first determined the threshold concentration (i.e. minimum dose) of *Smad6* mRNA required to induce secondary axes in some embryos when ventrally injected at the four-cell-stage. We then compared the resulting percentage of secondary axes with the percentage of secondary axes after coinjection of *XTob2* mRNA together with Smad6. While injection of 1.75 ng of Smad6 mRNA induced secondary axes in some embryos (21%, $n = 24$), co-injection of 1.75 ng Smad6 mRNA together with 4 ng XTob2 mRNA induced secondary axes more frequently (48%, $n = 21$; Fig. 4A) than injection of *Smad6* mRNA alone. Similarly, at a higher dose of *Smad6* (3.5 ng), secondary axes were observed in 19% of the embryos ($n = 26$), while coinjection of 4 ng *XTob2* mRNA resulted in secondary axis formation in 64% of the embryos ($n = 25$). In contrast, injection of 3–5 ng of XTob2

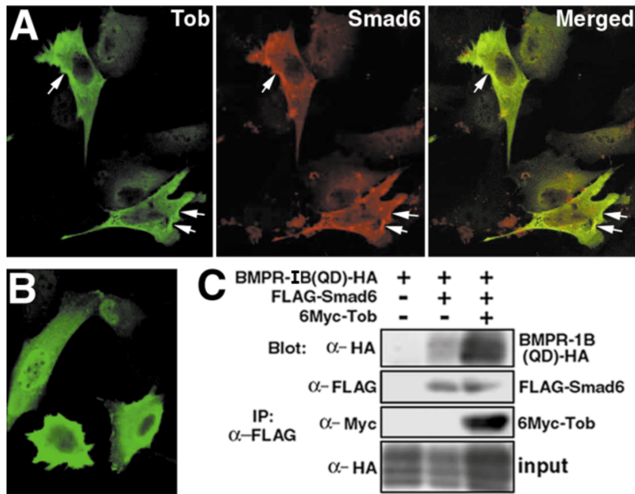

Fig. 5. Localization and physical interaction of Tob and Smad6. (A,B). Subcellular co-localization of Tob and Smad6. C2C12 cells were transfected with mouse Tob and mouse Smad6 constructs (A) or with mouse Tob alone (B). Cells were stained with anti-Tob monoclonal antibody and anti-Smad6 polyclonal antibodies (ZYMED Biotechnology). Images were obtained using a confocal microscope. The far right picture in Panel A shows a merged image. Arrows in (A) point to enrichment of Tob and Smad6 staining near the cell membrane. (C). Interactions between Smad6 and activated BMP receptor IB. COS7 cells were co-transfected with or without Myc-tagged Tob and the indicated Flag-tagged Smad and activated BMP receptor (BMPR-IB) constructs. Cell lysates were subjected to immunoprecipitation analysis using anti-Flag antibody, and coprecipitating BMPR-IB was detected by immunoblotting with anti-HA antibodies.

mRNA did not induce secondary axes (0%; $n = 46$; see Fig. 4B). From these results, we conclude that XTob2 is capable of enhancing the ability of Smad6 to inhibit BMP signaling in vivo.

To determine whether this cooperation between XTob2 and Smad6 is due to inhibition of endogenous BMP signaling, we directly measured the level of endogenous BMP signaling in explanted animal caps using a BMP responsive reporter gene (Candia et al., 1997). This reporter gene, −385*Xvent2*/luc, contains a 385 bp promoter fragment of the *Xvent2* gene, a direct target of BMP signaling (Onichtchouk et al., 1996; Candia et al., 1997; Blitz et al., 2000). −385*Xvent2*/luc was microinjected into the animal pole of four-cell stage *Xenopus* embryos alone or together with various concentrations of *XTob2* and/or mouse *Smad6* mRNA. Animal caps were dissected at the blastula stage and luciferase activity was measured when control embryos reached the early gastrula stage. As shown in Fig. 4C, coinjection of *XTob2* mRNA together with *Smad6* mRNA led to stronger inhibition of reporter gene expression than injection of Smad6 or XTob2 alone. These reporter gene experiments were repeated and consistent results were obtained a minimum of three times. In addition, similar results were obtained when using mouse Tob mRNA (data not shown).

We also examined the effect of Tob on Smad6-mediated inhibition of BMP signaling in transfected cells. Overexpression of either mouse *Smad6* or mouse *Tob* in P19 cells weakly suppressed the activity of the −385*Xvent2*/luc reporter gene (Fig. 4D). However, when *Smad6* was coexpressed together with *Tob*, the suppression of *Xvent2* reporter activity became stronger than with *Smad6* or *Tob* alone. These data, together with our results in *Xenopus* embryos, strongly support the notion that Tob and Tob2 can cooperate with inhibitory Smad6 to inhibit BMP signaling in both *Xenopus* embryos and in cultured cells.

2.4. Tob co-localizes with Smad6 at the cell membrane and assists in complex formation between inhibitory Smads and BMP receptors

Tob and Smad1 co-localize in the nucleus in a BMP stimulation-dependent manner, indicating that Tob acts in the nucleus to modulate Smad1 function (Yoshida et al., 2000). However, substantial Tob staining is also observed in the cytoplasm and at the cell membrane in the presence of Smad1 and BMP signaling (Yoshida et al., 2000), suggesting that Tob may have other functions at these sites. Therefore we wished to determine the site of Tob and Smad6 co-localization in the cell to better understand how Tob and Smad6 act to inhibit BMP signaling. To this end, we transfected C2C12 cells with a mouse Tob construct alone or together with Smad6 and examined their subcellular localization. Interestingly, when cells were transfected with *Tob* and *Smad6*, Tob and Smad6 appeared to be co-localized and enriched at the plasma membrane when compared with the cytoplasm in most cells (Fig. 5A, arrows), while co-localization was occasionally observed in the cytoplasm. In contrast, in cells transfected with Tob alone (Fig. 5B), Tob was not enriched at the membrane when compared with the cytoplasm, and was predominantly localized in the cytoplasm and occasionally in the nucleus. These data suggest that Tob might influence the action of Smad6 at the plasma membrane. We also attempted to localize endogenous Tob and Smad6 proteins, but our current polyclonal antibodies did not recognize these proteins at their endogenous expression levels.

Next, we examined whether Tob can act as a cofactor to assist in the formation of a complex between Smad6 and BMP receptors. COS7 cells were transfected with Flag-tagged Smad6 and an HA-tagged constitutively active form of BMP receptor type IA or IB [BMPR-IA(QD) or IB(QD)] with or without Myc-tagged mouse Tob. Cell lysates were isolated and subjected to coimmunoprecipitation analysis using anti-Flag antibody, followed by immunoblotting with anti-HA antibody. A three-fold increase in complex formation was detected between BMPR-IB(QD) and Smad6 in lysates from cells cotransfected with *Tob* when compared to controls without *Tob* (Fig. 5C). When BMPR-IA(QD) was used for a similar coimmunoprecipitation assay, increased complex formation was detected, albeit weakly (data not shown). Taken together, we propose that Tob functions at the cell membrane to enhance the

inhibitory activity of Smad6 by assisting complex formation between Smad6 and activated BMP type I receptor. Attempts to coimmunoprecipitate Tob and BMP type I receptors (in the absence of Smad6) have failed to demonstrate a direct physical interaction between these proteins. Thus, at present, it is unclear whether Tob-BMP receptor interactions occur through direct contact or through indirect mechanisms such as interaction with Smad6 or other unidentified protein cofactors near the membrane.

3. Discussion

BMP signaling functions in a vast and expanding number of diverse developmental processes. It has become increasingly clear that BMP signaling is regulated at a variety of levels including negative regulation by seemingly redundant secreted and intracellular inhibitory molecules (Cho and Blitz, 1998; von Bubnoff and Cho, 2001). Extracellular inhibitors include noggin, chordin, follistatin, cerberus, nodal-related 3, twisted gastrulation and related factors. Intracellular inhibitors include Smurfs, BAMBI, Ski, inhibitory Smads (I-Smads) 6 and 7 and Tob. Tob/BTG family proteins are generally thought to function in the nucleus. For example, BTG1 and BTG2 associate with the homeodomain transcription factor Hoxb9 and the estrogen receptor to regulate transcription (Prevot et al., 2000, 2001). Tob and Tob2 interact with nuclear factors like Caf1 and p90rsk1 to regulate cell proliferation (Ikematsu et al., 1999; Suzuki et al., 2001). Furthermore, Tob associates with Smads 1, 5, and 8 to inhibit BMP signaling in the nucleus (Yoshida et al., 2000). These pieces of evidence strongly suggest a nuclear function for Tob-related proteins in transcriptional regulation.

However, we have shown previously (Yoshida et al., 2000) and in this study (Fig. 5A) that Tob is also localized cytoplasmically and at the cell membrane suggesting that Tob proteins might also have role(s) outside the nucleus. We show here that Tob can directly interact with Smads 6 and 7 in vitro. In order to demonstrate the in vivo relevance of our finding, we have attempted to coimmunoprecipitate endogenous Tob and Smad6. However, due to the poor quality of the antibodies available to us, we were unable so far to detect coimmunoprecipitation of the endogenous proteins. Nevertheless, we show here that Tob and Tob2 can functionally cooperate with Smad6 in vivo to inhibit BMP signaling, in both *Xenopus* embryos and in cultured P19 cells.

A previous study has shown that Smad6 protein accumulates both at the membrane and in the nucleus (Nakayama et al., 1998a). Here we find that when *Tob* is over-expressed with *Smad6*, Tob co-localizes with Smad6 at the membrane. Immunoprecipitation analysis also reveals that Tob can assist in the formation of a stable complex between Smad6 and BMP type IB receptor. These new observations are consistent with the view that Tob inhibits BMP signaling by assisting in the formation or stabilization of a Smad6–BMP receptor complex at the plasma membrane. While our over-expression analyses favor the view that Tob and Smad6 act together at the membrane, perhaps along with an as yet unidentified factor, to inhibit BMP signaling, we have been unable to detect the co-localization of endogenous Smad6 and Tob proteins at the membrane. Thus, we cannot rule out the possibility that endogenous Tob acts in places other than the cell membrane, i.e. at nuclear or cytoplasmic levels.

Taking the latter observations together with the previously suggested nuclear role for Tob, we propose that Tob's major function in BMP inhibition in a given cell type may depend on the relative availability of R- and I-Smads. In some cells, Tob may act primarily in the nucleus to inhibit R-Smad action, whereas in other cells, Tob may function at the membrane in concert with Smad6 and/or 7 to inhibit receptor action. However, these two modes of signal regulation may not be mutually exclusive, as we cannot exclude the possibility that both processes may occur simultaneously within the same cell.

Distinguishing between these two possibilities (i.e. Smad1/5/8 sequestration vs. enhancement of Smad6/7-mediated inhibition) using loss-of-function analyses poses a significant challenge. One problem is that there are at least two *Tob* genes (*Tob* and *Tob2*), which may represent partially redundant proteins. Such a functional redundancy of Tob and Tob2 might explain the rather mild phenotype observed in mice lacking *Tob* (Yoshida et al., 2000): homozygous Tob knockout mice are viable and show no apparent phenotypic abnormalities for months after birth when an increase in bone mass is observed. Thus, inhibition of the function of one Tob may not reveal its mode of action due to redundancy. This may explain why we did not observe significant abnormalities in early *Xenopus* embryos when we reduced the expression of *XTob2* using morpholino anti-sense oligonucleotides, despite the fact that a similar dose of our morpholino was able to inhibit translation of XTob2 in vitro (results not shown). It remains to be seen whether this lack in phenotype is due to the presence of another *Tob* gene in *Xenopus* which has a partially redundant function to *XTob2*.

Another problem is that inhibition of Tob/Tob2 function would be predicted to give the same enhancement of BMP signaling by either mechanism (the nuclear function of Tob vs. the plasma membrane function), further complicating the analysis. In addition, it is often the case that increased BMP signaling, as might be observed the case when Tob/Tob2 functions are blocked, leads to feedback induction of other inhibitors of BMP signaling including I-Smads and extracellular BMP antagonists, possibly masking the effects of a loss of Tob function (Tsuneizumi et al., 1997; Afrakhte et al., 1998; Gazzerro et al., 1998; Takase et al., 1998; Pereira et al., 2000). In any case, further characterization of the protein–protein interactions between Tobs

and R- vs. I-Smads might provide a useful set of reagents for separately analyzing Tob's modes of action.

Finally, we show that Tob can associate with Smad7, an I-Smad, which inhibits both BMP and TGF-β receptor signaling (Nakao et al., 1997; Bhushan et al., 1998; Casellas and Brivanlou, 1998; Bai and Cao, 2002). Therefore, it is interesting to speculate that the functions of Tob/BTG family proteins as anti-proliferative factors may also involve I-Smad mediated inhibition of receptor signaling in response to TGF-β ligand. In conclusion, we anticipate that further in vivo characterization of Tob and Tob2 will lead to a better understanding of TGF-β signaling in general.

4. Experimental procedures

4.1. Yeast two-hybrid screen

An amino-terminal 236 amino acid fragment of human Tob was subcloned into pGBT9 (Clontech) to create a bait-expressing plasmid pGBT9-hTobN. PJ69-4A yeast carrying pGBT9-TobN were transfected with a human liver cDNA library fused with the GAL4 transcriptional activation domain. Three clones encoding human Smad6 were isolated after screening 3.5×10^4 clones.

4.2. Cell culture, immunoprecipitation and immunoblotting

COS7 and C2C12 cells were maintained in DMEM containing 10% FCS, and 15% FCS, respectively. P19 cells were maintained in α-MEM containing 10% FCS. COS7 cells were incubated with 50 mM MG-132 for 2 h, and solubilized in a buffer containing 10 mM Tris–HCl [pH 7.8], 150 mM NaCl, 1% Nonidet P-40, 1% aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, and 50 mM MG-132. Lysates were immunoprecipitated with an anti-Flag monoclonal antibody, followed by immunoblotting with specific antibodies as described previously (Yoshida et al., 2000). Antibodies used for blotting were anti-HA (Boehringer), anti-Myc (Santa Cruz Biotechnology), and anti-Flag (Sigma) antibodies. For all coimmunoprecipitation experiments, human *Smad1,4*, *8* and mouse *Smad5–7*, and mouse *Tob* were used. Activated BMP-RIA(QD) is human and HA-BMPR-IB(QD) receptor is mouse.

4.3. Cloning of Xenopus Tob2 and northern blot analysis

To identify a cDNA encoding *Xenopus Tob*, PCR was used to amplify *Xenopus* ovary cDNA using degenerated oligonucleotide primers. The degenerated primers derived from Tob homology domain of human Tob, Tob2, BTG1, BTG2, and ANA were 5′-GCGGATCCTGGT(T/T)(T/C)C-C(A/C/G/T)GA(A/G)CC-3′ and 5′-GCGAATTCTC(A/G)(T/A)A(A/C/G/T)GG(A/G)TC(A/C/G/T)A(T/C)CCA-3′. A 0.2 kb PCR product was subcloned and sequenced. Using this partial *Xenopus Tob2* cDNA as a probe, a full length *Xenopus Tob2* cDNA was isolated from an oocyte cDNA library (Clontech). Northern Blot assays were performed as described previously (Blitz and Cho, 1995). We used src as a loading control (Collett and Steele, 1993).

4.4. Embryo manipulations, whole-mount in situ hybridization and microinjection

Embryos were obtained by in vitro fertilization and cultured as previously published (Cho et al., 1991). Whole-mount in situ hybridizations were performed as described (Harland, 1991; Blitz and Cho, 1995) except for the use of BM purple (Boehringer–Mannheim Biochemicals) as colorimetric substrate and maleic acid buffer. The full length *Xenopus Tob2* cDNA was used for in situ hybridization. Synthetic mRNAs were prepared using the Message Machine Kit (Ambion). Mouse Smad6 mRNA for microinjection was prepared by transcribing an HA tagged pCS2 + -*Smad6* construct with SP6 RNA polymerase. The *XTob2* cDNA resulting from the library screening described above was subcloned into pBluescript RN3 (Lemaire et al., 1995). To synthesize *XTob2* mRNA for microinjection, this XTob2-pBS-RN3 construct was linearized with Not-I and transcribed with T3 RNA polymerase. Dorsoventral polarity was determined at the four-cell stage, and the indicated amounts of synthetic mRNAs were injected into the desired blastomeres of four-cell stage embryos at 4 nl each unless indicated otherwise. When assessing the percentage of secondary axes induced by ventrally injecting Smad6 and/or XTob2 mRNAs, we repeated all experiments at least three times to ensure the quality and consistency of the data.

4.5. Reporter gene assay and transient transfection

For luciferase reporter gene assays in *Xenopus* embryonic explants, 0.3 ng of −385 *Xvent2*/luc reporter DNA was microinjected into the animal pole region of four-cell stage embryos, alone or together with the indicated amounts of mouse Smad6 and/or XTob2 mRNA. At the blastula stage, animal caps were dissected manually, cultured for 3 h, homogenized, and subjected to luciferase assays. To account for inherent variability in luciferase counts between different batches of eggs, each experiment was carried out using eggs from a single female, and each luciferase count represents the average of usually ten animal caps. In addition, reporter gene experiments were repeated and consistent results were obtained a minimum of three times.

For luciferase reporter gene assays in P19 cells, the cells were transfected by the lipofection method with various combinations of the following plasmids: pME18S, pME18S-Tob-Flag (mouse *Tob*), pcDEF3-Smad1-Flag, pcDEF3-Smad6-Flag (mouse *Smad6*), pRL-TK, and *Xvent2*/luc. Cells were harvested and assayed for luciferase activity (a Dual-Luciferase Reporter System, Promega) 24 h after the addition of rhBMP2. Total amount of DNA per transfection

was equalized using empty vector DNA and a pRL-TK expression construct was co-transfected to normalize for transfection efficiency. Error bars were calculated from three independent experiments.

4.6. Immunofluorescence

C2C12 cells were transfected with mouse *Tob* expression vector together with the plasmid encoding mouse Smad6 protein. Cells were then treated and stained with anti-Tob monoclonal (IBM) and anti-Smad6 polyclonal antibodies (ZYMED), followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG and rhodamine-labeled anti-rabbit IgG, respectively. Image analysis was performed using a confocal laser-scanning microscope (Zeiss).

Acknowledgements

We thank Drs T. Imamura, M. Watanabe, Y. Yamanashi, J. Inoue, and T. Noda for critical discussions, and Yamanouchi Pharmaceutical for rhBMP2. We also thank Dr T. Koide, Mr S. Ogata and Mr S. Cilia for their help and assistance in *Xenopus Tob* isolation and whole mount in situ hybridization. This work was supported by grants from NIH and from the Ministry of Education, Science, Sports and Culture of Japan, from the Organization for Pharmaceutical Safety and Research of Japan, and from the Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists. The GenBank accession number for full-length *Xenopus Tob2* is AB056893.

References

Afrakhte, M., Moren, A., Jossan, S., Itoh, S., Sampath, K., Westermark, B., Heldin, C.H., Heldin, N.E., ten Dijke, P., 1998. Induction of inhibitory Smad6 and Smad7 mRNA by TGF-beta family members. Biochem. Biophys. Res. Commun. 249, 505–511.

Bai, S., Cao, X., 2002. A nuclear antagonistic mechanism of inhibitory Smads in transforming growth factor-beta signaling. J. Biol. Chem. 277, 4176–4182.

Bhushan, A., Chen, Y., Vale, W., 1998. Smad7 inhibits mesoderm formation and promotes neural cell fate in Xenopus embryos. Dev. Biol. 200, 260–268.

Blitz, I.L., Cho, K.W., 1995. Anterior neurectoderm is progressively induced during gastrulation: the role of the Xenopus homeobox gene orthodenticle. Development 121, 993–1004.

Blitz, I.L., Shimmi, O., Wunnenberg-Stapleton, K., O'Connor, M.B., Cho, K.W., 2000. Is chordin a long-range- or short-range-acting factor? Roles for BMP1-related metalloproteases in chordin and BMP4 autofeedback loop regulation. Dev. Biol. 223, 120–138.

Bradbury, A., Possenti, R., Shooter, E.M., Tirone, F., 1991. Molecular cloning of PC3, a putatively secreted protein whose mRNA is induced by nerve growth factor and depolarization. Proc. Natl Acad. Sci. USA 88, 3353–3357.

Candia, A.F., Watabe, T., Hawley, S.H., Onichtchouk, D., Zhang, Y., Derynck, R., Niehrs, C., Cho, K.W., 1997. Cellular interpretation of multiple TGF-beta signals: intracellular antagonism between activin/BVg1 and BMP-2/4 signaling mediated by Smads. Development 124, 4467–4480.

Casellas, R., Brivanlou, A.H., 1998. Xenopus Smad7 inhibits both the activin and BMP pathways and acts as a neural inducer. Dev. Biol. 198, 1–12.

Cho, K.W., Blitz, I.L., 1998. BMPs, Smads and metalloproteases: extracellular and intracellular modes of negative regulation. Curr. Opin. Genet. Dev. 8, 443–449.

Cho, K.W., Blumberg, B., Steinbeisser, H., De Robertis, E.M., 1991. Molecular nature of Spemann's organizer: the role of the Xenopus homeobox gene goosecoid. Cell 67, 1111–1120.

Collett, J.W., Steele, R.E., 1993. Alternative splicing of a neural-specific Src mRNA (Src+) is a rapid and protein synthesis-independent response to neural induction in *Xenopus laevis*. Dev. Biol. 158, 487–495.

Ebisawa, T., Fukuchi, M., Murakami, G., Chiba, T., Tanaka, K., Imamura, T., Miyazono, K., 2001. Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation. J. Biol. Chem. 276, 12477–12480.

Fletcher, B.S., Lim, R.W., Varnum, B.C., Kujubu, D.A., Koski, R.A., Herschman, H.R., 1991. Structure and expression of TIS21, a primary response gene induced by growth factors and tumor promoters. J. Biol. Chem. 266, 14511–14518.

Gazzerro, E., Gangji, V., Canalis, E., 1998. Bone morphogenetic proteins induce the expression of noggin, which limits their activity in cultured rat osteoblasts. J. Clin. Invest. 102, 2106–2114.

Guehenneux, F., Duret, L., Callanan, M.B., Bouhas, R., Hayette, S., Berthet, C., Samarut, C., Rimokh, R., Birot, A.M., Wang, Q., Magaud, J.P., Rouault, J.P., 1997. Cloning of the mouse BTG3 gene and definition of a new gene family (the BTG family) involved in the negative control of the cell cycle. Leukemia 11, 370–375.

Harland, R.M., 1991. In situ hybridization: an improved whole-mount method for Xenopus embryos. Methods Cell Biol. 36, 685–695.

Hata, A., Lagna, G., Massague, J., Hemmati-Brivanlou, A., 1998. Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. Genes Dev. 12, 186–197.

Hogan, B.L., 1996. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes Dev. 10, 1580–1594.

Ikematsu, N., Yoshida, Y., Kawamura-Tsuzuku, J., Ohsugi, M., Onda, M., Hirai, M., Fujimoto, J., Yamamoto, T., 1999. Tob2, a novel anti-proliferative Tob/BTG1 family member, associates with a component of the CCR4 transcriptional regulatory complex capable of binding cyclin-dependent kinases. Oncogene 18, 7432–7441.

Imamura, T., Takase, M., Nishihara, A., Oeda, E., Hanai, J., Kawabata, M., Miyazono, K., 1997. Smad6 inhibits signalling by the TGF-beta superfamily. Nature 389, 622–626.

Kavsak, P., Rasmussen, R.K., Causing, C.G., Bonni, S., Zhu, H., Thomsen, G.H., Wrana, J.L., 2000. Smad7 binds to smurf2 to form an E3 utiquitin legase that targets the TGFβ receptor for degradation. Mol. Cell 6, 1365–1375.

Kimura, N., Matsuo, R., Shibuya, H., Nakashima, K., Taga, T., 2000. BMP2-induced apoptosis is mediated by activation of the TAK1-p38 kinase pathway that is negatively regulated by Smad6. J. Biol. Chem. 275, 17647–17652.

Lemaire, P., Garrett, N., Gurdon, J.B., 1995. Expression cloning of Siamois, a Xenopus homeobox gene expressed in dorsal–vegetal cells of blastulae and able to induce a complete secondary axis. Cell 81, 85–94.

Massagué, J., 2000. How cells read TGF-beta signals. Nat. Rev. Mol. Cell. Biol. 1, 169–178.

Matsuda, S., Kawamura-Tsuzuku, J., Ohsugi, M., Yoshida, M., Emi, M., Nakamura, Y., Onda, M., Yoshida, Y., Nishiyama, A., Yamamoto, T., 1996. Tob, a novel protein that interacts with p185erbB2, is associated with anti-proliferative activity. Oncogene 12, 705–713.

Matsuda, S., Rouault, J., Magaud, J., Berthet, C., 2001. In search of a function for the TIS21/PC3/BTG1/TOB family. FEBS Lett. 497, 67–72.

Miyazono, K., Kusanagi, K., Inoue, H., 2001. Divergence and convergence of TGF-beta/BMP signaling. J. Cell Physiol. 187, 265–276.

Montagnoli, A., Guardavaccaro, D., Starace, G., Tirone, F., 1996. Overexpression of the nerve growth factor-inducible PC3 immediate early gene is associated with growth inhibition. Cell Growth Differ. 7, 1327–1336.

Nakao, A., Afrakhte, M., Moren, A., Nakayama, T., Christian, J.L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N.E., Heldin, C.H., ten Dijke, P., 1997. Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. Nature 389, 631–635.

Nakayama, T., Gardner, H., Berg, L.K., Christian, J.L., 1998a. Smad6 functions as an intracellular antagonist of some TGF-beta family members during Xenopus embryogenesis. Genes Cells 3, 387–394.

Nakayama, T., Snyder, M.A., Grewal, S.S., Tsuneizumi, K., Tabata, T., Christian, J.L., 1998b. Xenopus Smad8 acts downstream of BMP-4 to modulate its activity during vertebrate embryonic patterning. Development 125, 857–867.

Onichtchouk, D., Gawantka, V., Dosch, R., Delius, H., Hirschfeld, K., Blumenstock, C., Niehrs, C., 1996. The Xvent-2 homeobox gene is part of the BMP-4 signalling pathway controlling [correction of controlling] dorsoventral patterning of Xenopus mesoderm. Development 122, 3045–3053.

Pereira, R.C., Economides, A.N., Canalis, E., 2000. Bone morphogenetic proteins induce gremlin, a protein that limits their activity in osteoblasts. Endocrinology 141, 4558–4563.

Prevot, D., Morel, A.P., Voeltzel, T., Rostan, M.C., Rimokh, R., Magaud, J.P., Corbo, L., 2001. Relationships of the antiproliferative proteins BTG1 and BTG2 with CAF1, the human homolog of a component of the yeast CCR4 transcriptional complex: involvement in estrogen receptor alpha signaling pathway. J. Biol. Chem. 276, 9640–9648.

Prevot, D., Voeltzel, T., Birot, A.M., Morel, A.P., Rostan, M.C., Magaud, J.P., Corbo, L., 2000. The leukemia-associated protein Btg1 and the p53-regulated protein Btg2 interact with the homeoprotein Hoxb9 and enhance its transcriptional activation. J. Biol. Chem. 275, 147–153.

Rouault, J.P., Falette, N., Guehenneux, F., Guillot, C., Rimokh, R., Wang, Q., Berthet, C., Moyret-Lalle, C., Savatier, P., Pain, B., Shaw, P., Berger, R., Samarut, J., Magaud, J.P., Ozturk, M., Samarut, C., Puisieux, A., 1996. Identification of BTG2, an antiproliferative p53-dependent component of the DNA damage cellular response pathway. Nat. Genet. 14, 482–486.

Rouault, J.P., Rimokh, R., Tessa, C., Paranhos, G., Ffrench, M., Duret, L., Garoccio, M., Germain, D., Samarut, J., Magaud, J.P., 1992. BTG1, a member of a new family of antiproliferative genes. EMBO J. 11, 1663–1670.

Suzuki, T., Matsuda, S., Tsuzuku, J.K., Yoshida, Y., Yamamoto, T., 2001. A serine/threonine kinase p90rsk1 phosphorylates the anti-proliferative protein Tob. Genes Cells 6, 131–138.

Takase, M., Imamura, T., Sampath, T.K., Takeda, K., Ichijo, H., Miyazono, K., Kawabata, M., 1998. Induction of Smad6 mRNA by bone morphogenetic proteins. Biochem. Biophys. Res. Commun. 244, 26–29.

Tsuneizumi, K., Nakayama, T., Kamoshida, Y., Kornberg, T.B., Christian, J.L., Tabata, T., 1997. Daughters against dpp modulates dpp organizing activity in Drosophila wing development. Nature 389, 627–631.

Tzachanis, D., Freeman, G.J., Hirano, N., van Puijenbroek, A.A., Delfs, M.W., Berezovskaya, A., Nadler, L.M., Boussiotis, V.A., 2001. Tob is a negative regulator of activation that is expressed in anergic and quiescent T cells. Nat. Immunol. 2, 1174–1182.

von Bubnoff, A., Cho, K.W., 2001. Intracellular BMP signaling regulation in vertebrates: pathway or network? Dev. Biol. 239, 1–14.

Weinstein, D.C., Hemmati-Brivanlou, A., 1999. Neural induction. Annu. Rev. Cell Dev. Biol. 15, 411–433.

Yoshida, Y., Matsuda, S., Ikematsu, N., Kawamura-Tsuzuku, J., Inazawa, J., Umemori, H., Yamamoto, T., 1998. ANA, a novel member of Tob/BTG1 family, is expressed in the ventricular zone of the developing central nervous system. Oncogene 16, 2687–2693.

Yoshida, Y., Tanaka, S., Umemori, H., Minowa, O., Usui, M., Ikematsu, N., Hosoda, E., Imamura, T., Kuno, J., Yamashita, T., Miyazono, K., Noda, M., Noda, T., Yamamoto, T., 2000. Negative regulation of BMP/Smad signaling by Tob in Osteoblasts. Cell 103, 1085–1097.