Tob1 Controls Dorsal Development of Zebrafish Embryos by Antagonizing Maternal β-Catenin Transcriptional Activity

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Summary

Maternal β-catenin and Nodal signals are essential for the formation of the dorsal organizer, which, in turn, induces neural and other dorsal tissue development in vertebrate embryos. Tob (Transducer of ErbB2) proteins possess antiproliferative properties and are known to influence BMP signaling, but their relationship to other signaling pathways and to embryonic patterning in general was unclear. In this study, we demonstrate that zebrafish tob1a is required for correct dorsoventral patterning. Mechanistically, Tob1a inhibits β-catenin transcriptional activity by physically associating with β-catenin and preventing the formation of β-catenin/LEF1 complexes. Although Tob1a can also inhibit the transcriptional activity of the Nodal effector Smad3, its role in limiting dorsal development is executed primarily by antagonizing the β-catenin signal. We further demonstrate that Tob family members across species share similar biochemical properties and biological activities.

Introduction

Vertebrate embryos are patterned along the dorsoventral axis during early development. The Wnt/β-catenin signaling pathway plays a pivotal role in dorsoventral patterning. By acting as a downstream transcription factor in the Wnt pathway, β-catenin is essential for the establishment of the dorsoventral axis (Bellipanni et al., 2006; Heasman et al., 1994; Kelly et al., 2000; Tao et al., 2005). During early embryogenesis in Xenopus and fish, maternal β-catenin in fertilized eggs is stabilized and translocated into the nuclei of blastomeres on the dorsal side (De Robertis and Kuroda, 2004; Schneider et al., 1996; Weaver and Kimelman, 2004), but it is degraded on the ventral side under the control of factors such as GSK3β, CK1α, APC, and Axin (Bienz, 1999; Polakis, 2002). This asymmetrical β-catenin activity later helps to establish the dorsal organizer, a signaling center in which other dorsal determinants and antagonists of ventralizing bone morphogenetic protein (Bmp) signals are zygotically activated (De Robertis and Kuroda, 2004). Therefore, the dorsalizing activity of β-catenin appears to be primarily controlled by differential stabilization at the protein level along the dorsoventral axis. It is not clear whether the dorsalizing activity of β-catenin in these species could also be controlled by modulation of its transcriptional activity, although its transcriptional activity has been shown to be regulated by Inhibitor of β-catenin and TCF (ICAT), and over-expression of ICAT inhibits Xenopus axis formation (Tago et al., 2000). In contrast to maternal Wnt/β-catenin activity, zygotic Wnt/β-catenin activity, which acts after the establishment of the dorsoventral axis, is required for the maintenance of nonaxial mesoderm identity and for the restriction of the size of the dorsal organizer (Hoppler et al., 1996; Lekven et al., 2001; Ramel et al., 2005; Ramel and Lekven, 2004).

Nodal proteins, members of the TGFβ superfamily, are essential for mesendoderm induction (Feldman et al., 1998; Gritsman et al., 1999; Zhou et al., 1993) and are also involved in dorsoventral patterning (Chen and Schier, 2001; Dougan et al., 2003; Gore et al., 2005; Gritsman et al., 2000). Nodal signals may act downstream of, or in parallel to, β-catenin in the organizer formation (Agius et al., 2000; Gore et al., 2005; Kelly et al., 2000; Rex et al., 2002; Shimizu et al., 2000; Wessely et al., 2004; Yang et al., 2002). Nodal signal transduction is mediated by the receptor-activated factors Smad2 and Smad3. In zebrafish, smad2, smad3a, and smad3b are maternally expressed (Dick et al., 2000; Pogoda and Meyer, 2002). However, it is not known which receptor-activated Smads are the major mediators of Nodal signals during early development of zebrafish.

Tob/Tob1 was identified as an ErbB2 binding protein that was able to inhibit the growth of NIH3T3 cells (Matsuda et al., 1996). Human TOB is highly expressed in quiescent T cells and is essential for suppressing T cell activation (Tzachanis et al., 2001). Decreased expression or inactivation of TOB in human cancer tissues (Ito et al., 2005; Iwanaga et al., 2003) and a higher tumor occurrence rate in Tob null mice (Yoshida et al., 2003) both point to an antitumor effect of Tob, but the underlying mechanisms are unclear. The developmental function of endogenous Tob has only been studied in mice. Tob-deficient mice have a greater bone mass, resulting from increased numbers of osteoblasts due to enhanced Bmp signals, indicating that Tob plays an inhibitory role in osteoblast proliferation (Yoshida et al., 2000). Nevertheless, Tob null embryos develop without apparent defects (Yoshida et al., 2000), although Tob is expressed maternally and ubiquitously in embryos (Yoshida et al., 1997). It is possible that other Tob family members may compensate for Tob function during embryogenesis. It remains unknown whether and how...
Tob members are involved in the early development of vertebrate embryos.

Here, we report that tob1a, a maternally expressed Tob member, is required for proper dorsoventral patterning of zebrafish embryos. Overexpression of tob1a causes ventralized phenotypes, while tob1b knockdown leads to embryonic dorsalization. Tob1a binds to β-catenin and blocks the formation of the β-catenin/LEF1 transcriptional complexes, which provides another mechanism for limiting the dorsalizing activity of β-catenin. Additionally, Tob1a inhibits Smad3-induced embryonic dorsalization by physically interacting with and preventing Smad3 from binding to one of its cofactors.

Results

Spatiotemporal Expression of tob1a

We identified zebrafish tob1a by screening a zebrafish cDNA library and using tob1b as a probe. The zebrafish Tob1a protein shares a sequence identity of 79.6%, 81.4%, and 79.3% to zebrafish Tob1b, human TOB1, and mouse Tob/Tob1, respectively.

We examined the spatiotemporal expression pattern of tob1a during zebrafish embryogenesis by using whole-mount in situ hybridization. Similar to tob1b, the tob1a transcript is present in the fertilized eggs and in all blastodermal cells at the 1000-cell and the 30% epiboly stages (Figures 1A–1C). At around the 40% epiboly stage, tob1a expression becomes restricted to the germ ring, where mesoderm precursors reside (Figure 1D). During gastrulation and segmentation, tob1a is expressed in the prechordal mesoderm/hatching gland, the notochord, and the tail bud (Figures 1E–1J), and it exhibits a different expression pattern from that of tob1b during the same periods (Shi et al., 2004). At 24 hr postfertilization (hpf), tob1a expression was detected primarily in the hatching gland, the lens, somites, and the notochord (Figure 1K).

We next tested how tob1a expression was regulated by examining the probable regulatory roles of the major signaling pathways involved in early embryonic development. The expression of tob1a during gastrulation was induced by overexpression of the Nodal ligand squint (sqt) (Figures 1L and 1L'), but it was inhibited by overexpression of the Nodal antagonist lefty1 (Figures 1M and 1M') and in Nodal-deficient MZoep mutant embryos (Figures 1N and 1N'), suggesting that Nodal signaling is required for maintaining zygotic tob1a expression. However, the level of maternal tob1a mRNA was not reduced in MZoep mutants (data not shown), suggesting that maternal tob1a expression is Nodal independent. Likewise, overexpression of β-cateninJN mRNA, which encodes a constitutively active β-catenin lacking the first 45 N-terminal residues, induced ectopic expression of tob1a (Figures 1O and 1O'). Conversely, when Wnt/β-catenin signaling was blocked by injecting a β-catenin-2 morpholino, tob1a expression was inhibited (Figures 1P and 1P'). Taken together, our data suggest that both Nodal and canonical Wnt signals are required for sustaining zygotic tob1a expression. We found that tob1a expression in embryos injected with bmp2b mRNA or in swirl mutants that are homozygous for a bmp2b mutation (Kishimoto et al., 1997) was not altered (data not shown), implying that zygotic expression of tob1a may be independent of Bmp signals.

Tob1a Has Ventralizing Activity in Embryonic Patterning

To investigate the role of tob1a in embryonic development, we first injected zebrafish embryos with synthetic tob1a mRNA. Injection with 400 ng tob1a mRNA caused 53% of the embryos to lose their head and notochord at 24 hpf (Figure 2B; Figure S1, see the Supplemental Data Available with this article online); these phenotypes are characteristic of embryonic ventralization and resemble those seen in ichabod mutant embryos that lack maternal β-catenin-2 expression (Bellipanni et al., 2006; Kelly
et al., 2000). The expression of the shield-specific genes *chordin* and *goosecoid* was decreased at the shield stage (Figures 2I and 2J), and the expression of the anterior neuroectoderm marker *otx2* was also decreased at the bud stage (Figure 2K). In contrast, the ventral markers *bmp2b*, *eve1*, and *gata2* expanded dorsally during gastrulation, and the hematopoietic marker *gata1* had a larger expression domain in the blood island at 24 hpf (Figures 2L–2O). The ratios of embryos with altered marker gene expression are summarized in Figure S1. These results indicate that *tob1a* overexpression is able to ventralize embryos.

To investigate the role of endogenous *tob1a*, a morpholino antisense oligonucleotide (tob1a-MO), which was able to block production of the Tob1a-GFP fusion protein from a tob1a-GFP fusion expression plasmid (data not shown), was injected into one-cell embryos. Embryos injected with 12 ng tob1a-MO showed normal morphology. Data were averaged from three independent experiments and are expressed as means plus standard deviations. (D) An embryo coinjected with 100 pg tob1a mRNA and 12 ng tob1a-MO showed normal morphology. Data were averaged from three independent experiments and are expressed as means plus standard deviations. (E) Wild-type embryo. A caudal ventral region was enlarged in the insert so that the notochord (n) and the caudal ventral fin (cvf) can be seen (same for other pictures). (B) Injection with 400 pg tob1a mRNA resulted in loss of the head and notochord. (C) Injection with 12 ng tob1a-MO led to loss of the caudal ventral fin. Data were averaged from three independent experiments and are expressed as means plus standard deviations. (D) An embryo coinjected with 100 pg tob1a mRNA and 12 ng tob1a-MO showed normal morphology. Data were averaged from three independent experiments and are expressed as means plus standard deviations. (E) Wild-type embryos. (F) Embryos injected with 12 ng tob1a-MO showed early tail protrusion. (G) Embryos coinjected with 100 pg tob1a mRNA and 12 ng tob1a-MO showed normal tail morphology. (H) The ratios of dorsalized embryos, as shown in (F), in three independent experiments after injections. Data were averaged from three independent experiments and are expressed as means plus standard deviations. The number of calculated embryos is indicated below each bar.

(I–O) Expression patterns of marker genes in (I–O) wild-type or embryos injected with either (I–O) 400 pg tob1a mRNA or (I–O) 15 ng tob1a-MO. Embryos for *chordin*, *otx2*, and *eve1* are shown in animal pole views with dorsal oriented toward the right; the embryo for *goosecoid* is shown in a dorsal view with the animal pole oriented toward the top; and embryos for *bmp2b* and *gata2* are shown in lateral views with dorsal oriented toward the right. Developmental stages are indicated at the bottom. Relevant statistical data are presented in Figure S1.

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is required for normal dorsoventral patterning of zebrafish embryos.

**Tob1a Binds to and Attenuates the Transcriptional Activity of β-Catenin**

To find clues for the mechanisms underlying tob1a function, we went on to identify Tob1a-interacting proteins by using yeast two-hybrid screening and Tob1a as bait. One of the identified Tob1a binding proteins is β-catenin. To confirm their physical interaction, we performed reciprocal immunoprecipitation of overexpressed Myc-Tob1a and Flag-β-catenin in mammalian cells. The result indicated that Myc-Tob1a was coimmunoprecipitated with Flag-β-catenin (Figure 3A). Immunostaining in HeLa cells revealed that Tob1a was located in both the cytoplasm and nuclei, and that Tob1a overlapped well with β-catenin in the nucleus (Figure 3B). Thus, β-catenin is a binding partner of Tob1a.

We then investigated the effect of Tob1a on β-catenin-mediated Wnt signaling by using the Wnt signal-responsive reporter LEF1-luciferase. In transfected mammalian cells, luciferase expression was induced by overexpression of β-catenin and LEF1, but this induction was inhibited by coexpression of tob1a in a dose-dependent manner (Figure 3C). When the LEF1-luciferase DNA was injected into single-cell embryos, luciferase expression was induced by endogenous Wnt signaling at the bud stage (Figure 3D). The reporter expression in embryos was inhibited by overexpression of tob1a mRNA, but it was enhanced by knockdown of tob1a. Apparently, Tob1a negatively regulates canonical Wnt signaling both in vitro and in vivo.

The next question is how Tob1a inhibits β-catenin-mediated canonical Wnt signaling. It has been known that cytoplasmic β-catenin degrades in the absence of Wnt signals through the formation of β-catenin/Axin/GSK/APC complexes, and that activation of Wnt signaling allows for the release of β-catenin from the degradation complexes, followed by translocation into the nucleus (Logan and Nusse, 2004). The nuclear β-catenin interacts with the transcription factor LEF1 to activate target gene expression (Behrens et al., 1996; Hsu et al., 1998). Our domain-based mutagenesis analyses revealed that the armadillo repeats 4–6 of β-catenin, a region that is required for binding LEF1/TCF factors, were obligatory for Tob1a binding (Figure S2)(Graham et al., 2000; von Kries et al., 2000). Thus, Tob1a may compete with LEF1/TCF for β-catenin. Immunoprecipitation experiments in mammalian cells revealed that the amount of HA-LEF1 coimmunoprecipitated with Flag-β-catenin decreased in the presence of the increasing amount of Myc-Tob1a (Figure 3E), although neither the Flag-β-catenin level nor the HA-LEF1 level in total cell lysates was altered. Of note, we did not detect any direct binding of Tob1a to LEF1 (data not shown). These results suggest that Tob1a inhibits the interaction between β-catenin and LEF1 solely by competing with LEF1 for β-catenin. The possibility that Tob1a has a role in β-catenin degradation was tested by coimmunoprecipitation of β-catenin and its degradation complex components Axin and APC in mammalian cells. It was found that overexpression of Myc-Tob1a had no effect on β-catenin binding to APC or Axin (Figure S3). Taken together, these data suggest that Tob1a inhibits the transcriptional activity of β-catenin by preventing β-catenin from binding to a LEF1/TCF factor. The same mechanism is also used by another β-catenin antagonist, ICAT (Tago et al., 2000).

To further consolidate a competitive role of Tob1a against β-catenin/LEF complex formation, we performed a series of experiments to identify the interacting interface and functional domains of Tob1a that are required for antagonizing the transcriptional activity of β-catenin (Figures 3F–3H). Tob1a mutants ND5, MD2, and MD4, all of which lack the Box B domain that is conserved among Tob family members, failed to bind to β-catenin, while other deletion mutants retained the ability to bind β-catenin (Figure 3G). Furthermore, overexpression of ND5, MD2, or MD4 did not inhibit β-catenin/LEF1-induced Wnt reporter expression in mammalian cells. Therefore, the Box B domain of Tob1a is responsible for β-catenin interaction and is essential for conferring its anti-β-catenin activity.

**Tob1a Antagonizes the Dorsalizing Activity of Maternal β-Catenin in Zebrafish Embryos**

We next tested the genetic interaction between tob1a and β-catenin-mediated Wnt signaling that is essential for the formation of the dorsal organizer. Injection with 5 pg zebrafish β-catenin ΔN mRNA led to dorsalized phenotypes at the 5-somite stage, including abnormal anterodorsal accumulation of cells, epiboly arrest, and even two body axes (Figures 4A, 4C, and 4E). Overexpression of β-catenin ΔN also enhanced expression of the dorsal marker chordin, the axial mesoderm marker no tail (ntl), and the anterior neuroectodermal marker otx2, but it inhibited expression of the ventral markers bmp2, eve1, and gata2 (Figures 4A–4D). When the same amount of β-catenin ΔN mRNA and 100 pg tob1a mRNA were coinjected, the majority of embryos had normal morphology and marker gene expression (Figures 4A–4D). In contrast, coinjection with tob1a-MO and β-catenin ΔN caused more severe dorsalized phenotypes and greater changes in marker gene expression (Figures 4A–4D). In addition, tob1a-MO and β-catenin ΔN coinjection led to an increased percentage of embryos with two axes (Figure 4E). These results suggest that tob1a can antagonize the dorsalizing activity of β-catenin during the early development of zebrafish embryos.

In zebrafish, the maternal effect mutation ichabod results in severely ventralized phenotypes with loss or a reduction of dorsaotier derivatives due to decreased maternal expression of β-catenin-2 (Bellipanni et al., 2006; Kelly et al., 2000). Morpholino knockdown of β-catenin-2 causes the same ventralized phenotypes seen in ichabod (Bellipanni et al., 2006). We used a β-catenin-2 morpholino (IFICAT2MO) to further investigate the interaction between the maternal β-catenin signal and tob1a (Figure 5A). Embryos injected with tob1a-MO showed not only expansion of chordin and otx2 expression, but increased expression of bozozok (boz), which is a direct target of the maternal β-catenin signal (Leung et al., 2003; Ryu et al., 2001). In addition, tob1a-MO injection caused the expression domain of the posterior neuroectoderm marker hoxb1b to expand ventrally with vegetal shift of its anterior border. On the other hand, injection with 20 ng IFICAT2MO abolished
expression of boz, chordin, otx2, and hoxb1b. The expression of these markers was not recovered by simultaneous knockdown of tob1a and β-catenin-2, suggesting that tob1a genetically acts upstream of β-catenin-2, consistent with a direct inhibitory effect of Tob1a on the transcriptional activity of β-catenin.

We tested for any effect of overexpression of several tob1a deletion mutants in zebrafish embryos. Like
full-length tob1a, overexpression of the C-terminal-truncated mutant CD2, which retains the first 112 residues and inhibitory activity in vitro (Figures 4F and 4G), caused a decrease of the dorsal markers boz, chordin, and gsc in 71% (n = 34), 66% (n = 29), and 46% (n = 26) of the embryos (Figure 5B), respectively. However, overexpression of either the mutant ND5, which lacks the first 105 residues at the N terminus, or the mutant MD4, which lacks the Box B domain required for binding β-catenin, resulted in a slight decrease in expression of these markers in only 7%–15% of the embryos (n = 27–33) (Figure 5B). These results imply that direct binding of Tob1a to β-catenin is required for inhibiting dorsal development in vivo.

We also examined genetic interactions between tob1a and other mediators or targets of the Wnt/β-catenin signaling pathway. It was noted that left overexpression alleviated the ventralizing effect of tob1a overexpression (Figure S4A). Overexpression of a dominant negative LEF/TCF protein (tcf3ΔN) inhibited expression of chordin, and this effect was not rescued by tob1a knockdown (Figure S4B and legend). This result is as expected since Tcf3ΔN can bind to the target promoters but is unable to bind β-catenin. Together, these observations support the notion that tob1a inhibits dorsal development of the zebrafish embryo by antagonizing Wnt/β-catenin signals.

**tob1a May Control Zygotic Wnt/β-Catenin Signals for Ventrolateral Mesodermal Development during Gastrulation**

To investigate the involvement of tob1a in regulating Wnt8-stimulated zygotic Wnt/β-catenin activity, tob1a
knockdown was performed along with and in combination with wnt8 knockdown by using wnt8MO2 (Lekven et al., 2001), which was followed by examination of a set of markers (Figure 5C). The expression of boz was increased in late blastulas by tob1a knockdown (Figure 5A), but it was not affected by wnt8 knockdown (data not shown). This could be explained by the fact that tob1a only affects maternal Wnt/β-catenin activity when the zygotic Wnt/β-catenin signal is not available at such early stages. The expression domains of chordin at the shield stage and ofx2 at the bud stage were expanded by either tob1a or wnt8 knockdown, and they were further expanded by simultaneous knockdown of tob1a and wnt8, which excludes the possibility that tob1a primarily inhibits endogenous Wnt8 activity in dorsal and anterior neuroectodermal development. The expression of the ventrolateral mesoderm marker tbx6 was markedly reduced by wnt8 knockdown. Knockdown of tob1a appeared to slightly enhance tbx6 expression and partially rescued the effects of wnt8 knockdown on tbx6, indicating that tob1a may inhibit zygotic Wnt/β-catenin activity in ventrolateral mesodermal development. Ectopic expression of tob1a suppressed a wnt8 knockdown-induced increase of chordin and ofx2 expression, but it strengthened the inhibitory effect of wnt8 knockdown on tbx6. This suggests that, in the absence of Wnt8 activity, ectopic tob1a is still able to inhibit maternal Wnt/β-catenin in the organizer formation as well as zygotic Wnt/β-catenin in ventrolateral mesodermal development. Alternatively, these results may point to additional, Wnt-independent roles for tob1a in the mesoderm, and we examine this possibility below.

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**Figure 5. Genetic Interactions between tob1a and Maternal or Zygotic Wnt/β-Catenin Signals**

(A) Interaction between tob1a and maternal Wnt/β-catenin activity. After injection with 20 ng βcat2MO, 20 ng tob1a-MO, or both (indicated on the top) at the one-cell stage, embryos were examined for boz and chordin (chd) expression at the 30% epiboly stage and for ofx2 and hoxb1b expression at the 90% epiboly stage (indicated on the right). Embryos are shown in lateral views with dorsal oriented toward the right for boz and hoxb1b, in animal pole views with dorsal oriented toward right for chd, and in anterodorsal views with anterior oriented toward the top for ofx2.

(B) Effect of overexpression of tob1a mutants on early dorsoventral patterning. Embryos were injected with 400 pg of each mRNA species at the one-cell stage and were examined for boz expression at the oblong stage and for chd and goosecoid (gsc) expression at the shield stage. Embryos are shown in lateral views with dorsal oriented toward the right for boz, in animal pole views with dorsal oriented toward the right for chd, and in dorsal views with animal pole oriented toward the top for gsc. For details for tob1a mutants, see Figure 3F.

(C) Interaction between tob1a and zygotic Wnt/β-catenin activity. Injections were done at the one-cell stage at the dose of 20 ng for tob1a-MO, 8 ng for wnt8MO2, or 400 pg for tob1a mRNA. Embryos are shown in dorsal views with dorsal oriented toward the right for chd at the shield stage, in lateral views with dorsal oriented toward the right for tbx6 at the 75% epiboly stage, and in anterodorsal views with anterior oriented toward the top for ofx2 at the 90% epiboly stage.

**Tob1 Inhibits Smad3-Mediated TGFβ Signaling**

Yoshida et al. (2000) have previously demonstrated that mouse Tob1 binds various Smads, and that Tob1 inhibits Bmp signaling in vitro. We found that in
HEK293T cells overexpressed Tob1a strongly bound to Smad3, Smad4, Smad6, and Smad7 and weakly bound to Smad1 and Smad2; however, binding to Smad5 was almost undetectable (Figure 6A). Furthermore, Tob1a interacted with both zebrafish Smad3a and Smad3b (Figure 6B).

We then determined the effect of Tob1a on Bmp signaling by using the reporter BRE-luciferase that contains Bmp-specific responsive elements (BRE). In cultured HepG2 cells, tob1a overexpression inhibited the luciferase expression induced by a constitutively active Bmp receptor, BMPRIA-QD (Figure S5), which was
consistent with the effect of mouse Tob1 on Bmp signaling (Yoshida et al., 2000). In zebrafish embryos, however, BRE-luciferase expression was stimulated by tob1a overexpression, but it was inhibited by tob1a knockdown.

The effect of tob1a on TGFβ signaling was tested by using the reporter CAGA12-luciferase, which includes 12 tandem repeats of the Smad binding site (CAGA) in the promoter (Dennler et al., 1998). The expression of this reporter was stimulated by overexpression of the constitutively active ALK4 or Smad3 in Mv1lu cells. The ALK4-induced reporter expression was slightly attenuated by coexpression of tob1a (Figure 6C). However, the Smad3-induced reporter expression was more drastically inhibited by coexpression of tob1a (Figure 6D), implying that Tob1a suppresses Smad3 activity more effectively. In zebrafish embryos, the expression of the same reporter was inhibited by tob1a overexpression, but it was enhanced by tob1a knockdown (Figure 6E). These data suggest that Tob1 acts as a negative regulator of TGFβ signals in vitro as well as in vivo.

To understand the mechanism underlying negative regulation of TGFβ signaling by Tob1a, the effect of Tob1a on Smad3 activity was investigated. We did not test the effect of Smad2 because it interacted with Tob1a weakly and its roles in the development of zebrafish embryos are uncertain. The Smad3 binding domain of Tob1a was mapped to the Box B domain (Figure S6A), which is also essential for associating with β-catenin (Figures 3F and 3G). The amount of Flag-Smad3 bound to HA-Tob1a was progressively reduced when levels of coexpressed Myc-β-catenin increased (Figure S6B), suggesting that a competition between Smad3 and β-catenin for Tob1a binding may exist. The Tob1a binding domain of Smad3 was mapped to the MH2 domain (Figure 6F), which is responsible for interaction with and activation of the type I receptor, formation of homomeric and heteromeric Smad complexes, nucleocytoplasmic shuttling, and interaction with transcription cofactors or corepressors (Shi and Massague, 2003). We asked if the interaction between Tob1a and the MH2 domain of Smad3 affected the association of Smad3 with other binding partners or Smad3 activation. Our data demonstrated that tob1a overexpression in mammalian cells had no effect on Smad3 association with caALK4 (Figure S7), the level of phosphorylated Smad3 (Figure S7B), or Smad3/Smad4 complex formation (Figure S7C). In contrast, tob1a overexpression inhibited the interaction of Smad3 with the C-terminal half of transcriptional coactivator p300 in a dose-dependent manner (Figure 6G). It has been previously demonstrated that the C-terminal half of p300/ CBP binds to the MH2 domain of Smad3 and augments the transcriptional activity of Smad3 (Feng et al., 1998; Janknecht et al., 1998; Nishihara et al., 1998; Shen et al., 1998). Thus, we propose that Tob1a inhibits Smad3-activated transcription by preventing Smad3 from binding to coactivator p300.

Tob1a Inhibits Smad3-Induced Dorsalization in Zebrafish Embryos

We investigated genetic interactions between tob1a and smad3a/smadi3b in zebrafish embryos. When injected with smad3b mRNA, embryos were dorsIALIZED with an ovoid shape and laterally expanded somites at the 5-somite stage (Figure 6H). Coinjection with tob1a mRNA lessened smad3b-induced dorsalization, and coinjection with tob1a-MO intensified the dorsIALIZED phenotypes. These effects were confirmed by examination of the expression of the dorsal markers chordin and gsc as well as the ventral markers eve1 and gata2 at the shield stage (Figure 6I; Figure S8). Similar results were obtained with smad3a (Figure S9). Thus, tob1a has activity opposite to that of smad3a and smad3b during the early development of zebrafish embryos. However, as we discuss below, tob1a may primarily function to antagonize maternal β-catenin-mediated signaling rather than Smad3-mediated signaling in dorsal development.

Tob Proteins Are Functionally Conserved

We have previously identified the tob1b gene in zebrafish, but we failed to reveal its biological functions (Shi et al., 2004). In mammals, there are two members of the Tob family, Tob1 and Tob2 (Ajima et al., 2000; Yoshida et al., 1997). We asked if different members of the Tob family from various species share similar biochemical properties and biological functions.

We first tested if Tob1 and β-catenin interact with each other at their endogenous levels. β-catenin was found to be present in the immunoprecipitate obtained from human HEK293T cells with anti-TOB1 antibody (Figure 7A), indicating that endogenous TOB1 and β-catenin interact with each other in human cells. When cooverexpressed in HEK293T cells, Flag-β-catenin was coimmunoprecipitated with Myc-tob1b, Myc-Tob1, or Myc-Tob2 (Figure 7B). Like zebrafish tob1a, zebrafish tob1b, mouse Tob1, or mouse Tob2 attenuated β-catenin/LEF1-stimulated LEF1-luciferase expression (Figure 7C). Likewise, endogenous SMAD3 was associated with Tob1 in human cells (Figure 7D); overexpressed zebrafish Tob1b, mouse Tob1, or mouse Tob2 was able to bind to overexpressed Smad3 (Figure 7E) and inhibit Smad3-induced CAGA12-luciferase expression (Figure 7F). Taken together, these data suggest that interaction with β-catenin and Smad3 and inhibition of their transcriptional activities are common intrinsic properties of Tob proteins (Figure 7H).

Biological functions of zebrafish tob1b and mouse Tob1 and Tob2 were assessed by overexpression in zebrafish embryos. Injection with zebrafish tob1b and mouse Tob1 or Tob2 mRNA resulted in ventralized embryos with loss of the head and the notochord at 24 hpf. The dorsal markers chordin and gsc and the anterior neuroectodermal marker otx2 all displayed a reduction in expression, while the ventral markers bmp2b and gata1 had expanded domains of expression (Figure 7G; Figure S10). It appears that these tob genes all are capable of modulating dorsal tissue development upon overexpression regardless of their origins.

Discussion

In this study, we have demonstrated that tob1a exerts a ventralizing role in dorsoventral patterning of zebrafish embryos. Mechanistically, Tob1a inhibits transcriptional activity of β-catenin by preventing it from binding to Tcf/coactivators, and it represses the transcriptional activity of Smad3 by competing for cofactor p300.
Generally, \textit{tob}1\textit{a} overexpression is associated with a reduction of dorsal marker gene expression and an increase of ventral marker gene expression during late blastulation and early gastrulation, while its knockdown produces opposite effects, such as smaller, thinner tails. Since \textit{tob}1\textit{a} knockdown expands the expression of the maternal \textit{\beta}-\textit{catenin} target gene \textit{boz}, but is unable to rescue the knockdown effect of maternal \textit{\beta}-\textit{catenin}-2 (Figure 5A), we conclude that at least some of \textit{tob}1\textit{a}’s ventralizing effects are attributable to its inhibition of maternal \textit{\beta}-\textit{catenin} activity. However, we could not formally exclude the possibility that \textit{tob}1\textit{a} also inhibits

![Figure 7. Tob Proteins from Different Species Have Conserved Activity](image-url)
zygotic Wnt/β-catenin activity in ventrolateral mesodermal development after the establishment of the dorsal organizer, because tob1a knockdown slightly enhances the expression of the ventrolateral mesodermal marker tbx6 (Figure 5C). The effect of tob1a on maternal Wnt/β-catenin activity in dorsal development might obscure its effect on zygotic Wnt/β-catenin activity in ventrolateral mesodermal development, and vice versa.

Smad2/Smad3-mediated Nodal signals are involved in organizer formation as well as dorsal mesodermal development (Tian and Meng, 2006). Despite the fact that tob1a possesses anti-Smad3 activity, there are a number of reasons that lead us to believe that tob1a controls the dorsal development of zebrafish embryos mainly by antagonizing the dorsalizing activity of maternal Wnt/β-catenin activity. First, unlike the maternal-effect ichabod mutants that lack the head and the notochord at 24 hpf, embryos with defective Nodal signaling, such as sqt;cyc double mutants (Fieldman et al., 1998) and MZoep mutants (Gritsman et al., 1999), are able to develop a head with correct anteroposterior patterning, though the notochord is missing. Overexpression of tob1a results in ventralized phenotypes similar to those of ichabod mutants, but not to sqt;cyc or MZoep mutants (Figure S1). Similar to wild-type embryos, MZoep mutants can be ventralized by tob1a overexpression (Figure S11), indicating that the ventralizing activity of tob1a may be independent of Oep-mediated Nodal signals. Second, tob1a negatively regulates the expression of the early dorsal marker boz (Figure 5), which is a direct binding target of Lef1 (Leung et al., 2003) and is activated by maternal Wnt/β-catenin activity (Fekany et al., 1999). When maternal Wnt/β-catenin activity is blocked by knocking down β-catenin-2, boz expression is not rescued by tob1a knockdown. On the other hand, β-catenin-induced dorsalized phenotypes are rescued by tob1a overexpression but are enhanced by tob1a knockdown (Figure 4).

We note that knockdown of tob1a causes a slight increase of the mesodermal marker ntl in the germ ring at the shield stage and an increase of the endoderm markers sox32 and sox17, while tob1a overexpression exerts an opposite effect on these markers (Figure S11). Thus, we would not exclude the possibility that tob1a inhibits the activities of Nodal/Smads in mesodermal and endodermal development. Because zygotic expression of the Nodal genes sqt and cyc requires maternal Wnt/β-catenin activity (Bellipanni et al., 2006; Kelly et al., 2000; Shimizu et al., 2000), it is not clear whether tob1a controls the Nodal signaling pathway in these developmental processes directly or indirectly.

A previous study in mice demonstrates that Tob1 inhibits Bmp signals in a poorly understood mechanism in osteoblast proliferation (Yoshida et al., 2000). We found that in zebrafish embryos tob1a overexpression stimulates expression of the BRE-luciferase reporter, while tob1a knockdown reduced its expression. A possible explanation is that tob1a can, in fact, activate endogenous Bmp signaling via an unknown mechanism. A more plausible explanation is that tob1a’s inhibition of β-catenin-dependent dorsal development indirectly results in a reduction of the dorsal expression of BMP antagonists and thus disinhibits bmp2b.

Although tob1b, another tob1 gene in zebrafish, is also maternally expressed (Shi et al., 2004) and its overexpression slightly ventralizes embryos, its knockdown does not cause detectable dorsalized phenotypes (data not shown). It is possible that these two tob1 genes in the zebrafish genome have undergone functional partitioning during evolution. The mouse and human genomes contain Tob1 and Tob2 genes, and they are maternally expressed (Ajima et al., 2000; Ikematsu et al., 1999; Yoshida et al., 1997). We have shown that, like zebrafish tob1a, both mouse Tob1 and Tob2 inhibit β-catenin-mediated Wnt signaling in vitro and ventralize zebrafish embryos upon overexpression, suggesting a role in early embryonic patterning. In mice, Tob1 null embryos develop to term without detectable defects (Yoshida et al., 2000). This may be attributed to the redundant function of Tob1 and Tob2 during embryogenesis. It is therefore reasonable to suggest that double knockout in the Tob1 and Tob2 loci would reveal their involvement in the early patterning of mammalian embryos.

Knockdown of tob1a only causes weak dorsalized phenotypes in zebrafish embryos. One possibility is that maternal Tob1a protein compensates for reduction caused by tob1a knockdown. The existence of tob2 genes in the zebrafish genome, which may have redundant functions with tob1a, or other β-catenin antagonists, e.g., ICAT, could also compromise the net effect of tob1a knockdown. Furthermore, if tob1a inhibits both maternal and zygotic Wnt/β-catenin activities, which have opposite effects on dorsal development, the severity of the overall phenotypes caused by tob1a knockdown may be reduced.

Tob proteins and Btg proteins share sequence homology at their N termini as well as antiproliferative properties (Matsuda et al., 2001). Biological functions for most Btg proteins are unknown. Wessely et al. (2005) recently found that overexpression of xBtg-x, a Btg family member in Xenopus, induced axis duplication and effectively rescued the ventralizing effects caused by UV irradiation in Xenopus embryos, and that knockdown of xBtg-x did not cause developmental defects. They further demonstrated that xBtg-x overexpression activated the maternal β-catenin pathway in the early frog embryo and stimulated β-catenin-dependent transcription in mammalian cells, although interaction of xBtg-x with β-catenin was not detected. We found that Tob1a was able to antagonize xBtg-x-enhanced expression of the LECF1-luciferase reporter in mammalian cells (Figure S12), but overexpression of xBtg-x in zebrafish embryos failed to cause detectable changes. Nevertheless, the opposite effects of zebrafish Tob1a and Xenopus xBtg-x on β-catenin signaling pathway may represent general differentiation between Tob and Btg proteins or species-specific divergence.

Increased Wnt signaling has been linked to tumorigenesis (Moon et al., 2004). Mutations that result in up-regulation of the nuclear β-catenin level and tumor development are usually associated with malfunction or loss-of-function of the components required for β-catenin degradation. Similarly, down-regulation of TOB expression as well as inactivation of the TOB protein have been detected in human tumor tissues (Ito et al., 2005; Iwanaga et al., 2003). Our current finding that Tob proteins are direct antagonists of β-catenin...
suggests that decreased Tob activity may coincide with an increase in the β-catenin signaling level. Therefore, it will be of great interest to investigate the β-catenin signaling level in those tumor tissues with reduced TOB activity.

**Experimental Procedures**

**Fish Strains**

Wild-type embryos of the Tuebingen strain and the mutant strain oeg−/+ were used. MZoep mutant embryos were generated as reported by Gritsman et al. (1999).

**Gene Identification and Construction Generation**

A plasmid containing tob1a cDNA was identified from a zebrafish cDNA library. To screen for proteins interacting with Tob1a, we used the BD Matchmaker Two-Hybrid System (Clonetech) to make pGBK7-tob1a bait and an AD fusion zebrafish cDNA library. A total of 32 interacting proteins, one of which was β-catenin, were identified. Mouse Tob1 and Tob2 were isolated by RT-PCR from a mouse brain cDNA library. For overexpression in mammalian cells, the coding sequence of a gene of interest was subcloned into vector pCMV5 targeting nucleotides are underlined) were used as controls. The morpholino (5'-TTCGCGTCAATCCTTTAG-3') was designed to have six mispaired nucleotides (5'-TCAAGCTGTTATTATGCTGCGCTGC-3'), mispaired nucleotides are underlined) were used as controls. The morpholino targeting β-catenin-2 (5'-CAAGCTGTTATTATGCTGCGCTGC-3') was subcloned into vector pxT7. Mutational constructs were generated by PCR and verified by sequencing.

**Luciferase Reporter Assays**

Luciferase reporter assays were performed in cell culture and in zebrafish embryos essentially as described previously (Sun et al., 2006; Zhang et al., 2004).

**Whole-Mount In Situ Hybridization and Microinjection**

Digoxigenin-UTP-labeled antisense RNA probes were generated by in vitro transcription and were used for whole-mount in situ hybridization. In vitro synthesis and purification of mRNA were performed by sequencing. Luciferase reporter assays were performed in cell culture and in zebrafish embryos essentially as described previously (Sun et al., 2006; Zhang et al., 2004).

**Immunoblotting and Coimmunoprecitation**

Cell culture, immunoblotting, and coimmunoprecipitation were performed as described previously (Rui et al., 2004; Zhang et al., 2004). Mouse monoclonal antibody against human Tob1 was purchased from Sigma-Aldrich. Rabbit polyclonal antibody against human Smad3 was the product of ZYMED Laboratories. Anti-phospho-Smad3 antibody was purchased from Cell Signaling Technology.

**Supplemental Data**

Supplemental Data include 12 figures and are available at http://www.developmentalcell.com/cgi/content/full/11/2/225/DC1/.

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**References**


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