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Twisted gastrulation is required for forebrain specification and cooperates with Chordin to inhibit BMP signaling during *X. tropicalis* gastrulation

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Abstract

In the developing vertebrate embryo, proper dorsal–ventral patterning relies on BMP antagonists secreted by the organizer during gastrulation. The BMP antagonist chordin has a complex interaction with BMPs that is governed in part by its interaction with the secreted protein twisted gastrulation (tsg). In different contexts, tsg has activity as either a BMP agonist or as a BMP antagonist. Using morpholino oligonucleotides in *Xenopus tropicalis*, we show that reducing tsg gene product results in a ventralized embryo, and that tsg morphants specifically lack a forebrain. We provide new evidence that tsg acts as a BMP antagonist during *X. tropicalis* gastrulation since the tsg depletion phenotype can be rescued in two ways: by chordin overexpression and by BMP depletion. We conclude that tsg acts as a BMP antagonist in the context of the frog gastrula, and that it acts cooperatively with chordin to establish dorsal structures and particularly forebrain tissue during development.

Keywords: Twisted gastrulation; BMP antagonist; Chordin; Forebrain; *Xenopus tropicalis*; Morpholino oligonucleotides

Introduction

During early development in the *Xenopus* embryo, bone morphogenetic protein (BMP) signaling plays a major role in the specification of the dorsal–ventral axis (De Robertis et al., 2000; Harland, 2004; Harland and Gerhart, 1997; Niehrs, 2004). Transduction of BMP signals promotes ventralization and suppresses the formation of dorsal structures such as neural tissue, somites and notochord. Thus, dorsal development requires suppression of BMP signaling. In the gastrulating frog embryo, BMP antagonists are secreted by Spemann's Organizer and sequester BMPs from their target receptors. In the absence of secreted BMP antagonists, the embryo fails to form dorsal structures or neural tissue, demonstrating that BMP antagonism is required for these cell fates (Khokha et al., 2005).

Several BMP antagonists have been characterized, including noggin, chordin, follistatin, Xnr3 and the multifunctional antagonist cerberus (De Robertis et al., 2000; Harland and Gerhart, 1997; Niehrs, 2004). Ectopic ventral expression of these molecules results in the formation of secondary axes, where presumptive ventrolateral tissues develop with dorsal cell fates. Conversely, loss of BMP antagonists results in mispatterning of the embryo and a loss of dorso-anterior structures. For example, zebrafish embryos with null mutations in chordin are partially ventralized, while mice that are mutant for noggin and chordin have defects in anterior head formation (Bachiller et al., 2000; Schulte-Merker et al., 1997). Frog embryos depleted of chordin, noggin and follistatin completely fail to develop dorsal structures (Khokha et al., 2005).

While noggin, chordin, cerberus and perhaps follistatin all exert their antagonistic effects by binding directly to BMPs, the interaction between chordin and BMPs is particularly complex. Chordin binds BMP4 and BMP7 with high affinity, and prevents binding of BMP ligands to their cognate receptors, thus interfering with BMP signaling (Piccolo et al., 1996). Biochemical studies demonstrate that three cysteine-rich domains in the chordin protein bind BMPs (Garcia Abreu et al., 2002; Larrain et al., 2000). These individual domains will also bind BMPs following cleavage of chordin, albeit with lesser affinity than full-length chordin. Cleavage of chordin is mediated by the metalloproteinase xolloid (tolloid

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in other organisms), which targets both chordin and chordin–BMP complexes (Piccolo et al., 1997).

The interaction between chordin, xolloid and BMPs is modulated by another secreted protein, twisted gastrulation (tsg) (Chang et al., 2001; Oelgeschlager et al., 2000; Scott et al., 2001). In vertebrates, tsg binds both chordin and BMP with high affinity, forming ternary complexes. Based on the biochemical data, the interaction of chordin with BMPs can favor roles for tsg as either a BMP agonist or BMP antagonist. One study has proposed that tsg can act as both a BMP agonist and antagonist depending on the cleavage state of chordin (Larrain et al., 2001). According to this model, tsg promotes chordin function by enhancing the binding of chordin to BMP, forming stable ternary complexes and preventing BMP signaling (Scott et al., 2001). However, tsg/BMP/chordin complexes would also represent a superior substrate for cleavage of chordin by xolloid, lowering chordin's affinity for BMP and consequently promoting BMP signaling. Thus, a balance of BMP agonist/antagonist activity could be mediated by tsg according to the relative abundance of xolloid and chordin-bound BMP. In addition, the pro-BMP activity of tsg may be independent of BMP binding, again emphasizing its interactions with chordin (Oelgeschlager et al., 2003b).

In vivo evidence for the activity of twisted gastrulation in vertebrates has also been used to support a model of either BMP agonism or antagonism. Overexpression studies of *tsg* in zebrafish and in *Xenopus* have given seemingly contradictory results. In zebrafish, overexpression of tsg results in dorsalized embryos (Little and Mullins, 2004; Ross et al., 2001). Reported phenotypes of tailbud-stage *Xenopus* embryos injected with tsg mRNA vary from dorsalized to ventralized (Chang et al., 2001; Oelgeschlager et al., 2000; Scott et al., 2001). Microinjection of high concentrations of *tsg* mRNA inhibits the ability of injected chordin or its cleavage products to form secondary axes (Oelgeschlager et al., 2000). However, lower concentrations of *tsg* mRNA promote secondary axis formation by chordin (Scott et al., 2001) and induce ectodermal tissues to express dorsal rather than ventral markers (Chang et al., 2001).

Studies exploring the role of tsg by loss of function have led to similarly complex inferences. Genetic knockouts of *twisted gastrulation* (*twsg* in mammals) in the mouse lead to thymocyte defects that are consistent with a role in BMP antagonism, but also to bone defects that are more readily explained by a role as a BMP agonist (Nosaka et al., 2003). In some genetic backgrounds, loss of *twsg* is also accompanied by severe craniofacial and forebrain defects (Petryk et al., 2004). However, in most genetic backgrounds, *twsg* has no forebrain defects. Compound *twsg* mutant/BMP4 heterozygotes exhibit holoprosencephaly, while BMP7 homozygotes with one or more mutant copies of *twsg* demonstrate sirenomelia, suggesting that cooperative interactions may exist between tsg and BMPs in the mouse (Zakin and De Robertis, 2004; Zakin et al., 2005), though the diversity and the nature of the phenotypes makes a straightforward interpretation of twsg function difficult.

In *Xenopus*, loss-of-function analysis of chordin and tsg using morpholino oligonucleotides (MOs) and rescue experiments with noggin and chordin demonstrated a cooperative role between tsg and known BMP antagonists (Blitz et al., 2003). In this study, depletion of tsg resulted in embryos with reduced dorsal structures and expanded ventral structures, consistent with reduced BMP antagonism. Recently, however, another report in *Xenopus* indicated that depletion of tsg and BMP7 by MOs resulted in a cooperative effect (Zakin et al., 2005).

In zebrafish, loss-of-function studies have also led to complex conclusions. In an initial analysis, depletion of tsg resulted in a reduction of dorsal structures, with sub-inhibitory doses of chordin MO and tsg MO cooperating to expand ventral tissues (Ross et al., 2001). In more recent studies (Little and Mullins, 2004; Xie and Fisher, 2005), tsg depletion caused an expansion in dorsal tissues and a reduction of ventral tissues. In these experiments, sub-dorsalizing doses of tsg MO dorsalized *swirl* (bmp2b) heterozygotes and *mini-fin* (tolloid) mutants, indicating cooperation between tsg and BMPs. In addition, tsg knockdown also partially appeared to suppress the phenotypes seen in mutants of BMP antagonists.

Therefore in each of these model systems, articulating a general role for tsg is difficult. Tsg appears to have an activity that can either suppress or augment BMP signaling depending on the developmental context in which it is characterized. Therefore, in this study, we extended the previous analysis of tsg by characterizing the molecular and morphological effects of tsg loss of function in the diploid frog *Xenopus tropicalis*. We have used MOs to deplete tsg, chordin, BMP4 and BMP7, and have assayed the individual and combined effects of these gene products on embryonic patterning. We have identified a novel role for tsg in forebrain development in *X. tropicalis* and provide new evidence that *tsg* acts as a BMP antagonist during gastrulation.

Materials and methods

Ovulation, in vitro fertilization, and rearing of embryos

Ovulation of adult *X. tropicalis* (Nasco) and in vitro fertilization of eggs were conducted as described (http://tropicalis.berkeley.edu/home). Fertilized eggs were de-jellied in 3% cysteine in 1/9X modified frog ringer's solution (MR) for 10–15 min. Embryos were reared at room temperature (21–23°C) in 1/20X MR supplemented with 100 μg/ml gentamicin sulfate.

Morpholino oligonucleotide design and injection

Morpholino oligonucleotides (MOs) were designed to block translation of *X. tropicalis* tsg, chordin, BMP4 and BMP7 (Gene Tools, LLC). Sequences were as follows: tsg MO 5′TAGGAGAGAGGGCTTCATACTTGGC3′; chordin MO 5′CAAAGCATTTTTGTGGTAGCCCCGA3′; BMP4 MO 5′CCAGGAATCATGGTGTCTTGACAGA3′; BMP7 MO 5′TTACTATCAAAGCATTCATTTTGTC3′. MOs were resuspended in DEPC-treated 0.1 × MR to a concentration of either 1 mM or 2 mM. Fluorescent miniruby (Molecular Probes, (Lane and Sheets, 2002)) was co-injected as a tracer for injection. We injected both blastomeres at the two-cell stage (unless otherwise stated), with each cell receiving half the total MO dose. For tsg MO, a total dose of 5 ng/embryo is described as a "low dose", 10 ng/embryo is an "intermediate dose," and 20 ng/embryo is a "high dose". For chordin MO, the total dose was always 20 ng/embryo. For BMP 4 and BMP7, a total MO dose of 20 ng/embryo is described as a "low dose" and a total dose of 40 ng/embryo was described as a "high dose." Co-injection of chordin and tsg MOs was carried out at a total dose of 10 ng of tsg MO with 20 ng of chordin MO.

Targeting of the MO was confirmed by visualization of miniruby fluorescence. Because morphological criteria are used for staging and MO-injected embryos sometimes did not develop with normal or complete morphology, control embryos were used for staging, and then both control and injected embryos were fixed concurrently. When possible, morphological criteria such as the beginning of blastopore formation and the appearance of the cement gland, or molecular criteria such as the onset of specific gene expression (described in Results) were used to ensure that MO-injected embryos were not non-specifically delayed in development.

mRNA injection

Template for chicken chordin (a generous gift of Paul Wilson) was linearized with *Hin*dIII and transcribed using mMessage mMachine Sp6 RNA polymerase kits (Ambion). Resulting RNA was resuspended in DEPC-treated water. 50–100 pg were injected into the marginal zone of a single dorsal blastomere at the 4 cell stage. Rainbow trout tsg (*Oncorhynchus mykiss* EST tcay0033b.b.14, GenBank accession number BX317058) was subcloned into CS107 using *Eco*RI/*Not*I sites, then linearized for transcription with *Asc*I and transcribed as above. 200 pg of mRNA were injected into each dorsal blastomere at the 4 cell stage. In both cases, mRNAs were co-injected with GFP mRNA to allow tracing of the injection by fluorescence.

In situ hybridization

Clones were obtained from either the Sanger Center EST projects via the HGMP MRC Geneservice, from the IMAGE clone libraries, or obtained by PCR. Additional clone information as well as template preparation can be obtained at http://tropicalis.berkeley.edu/home. In order to avoid trapping of stain in the blastocoel cavity, blastula and some gastrula embryos were bisected with a razor blade prior to dehydration.

Results

Tsg Expression in X. tropicalis

We began our investigation of twisted gastrulation function in *X. tropicalis* by examining its expression in early embryonic stages. We find that tsg mRNA is detected in the animal portion of cleavage-stage embryos (Fig. 1A), suggesting the presence of maternal transcripts, as described for *X. laevis* (Oelgeschlager et al., 2000). Tsg is expressed broadly through the animal region at stage 8 (Fig. 1B), and is expressed asymmetrically at stage 9 (Fig. 1C). By mid-gastrulation (stages 10.5 and 11), we can no longer detect expression of tsg (Figs. 1D, E), suggesting that gastrula-stage expression of tsg in *X. tropicalis* may differ from *X. laevis*. However, we do find that zygotic tsg is expressed in the tailbud region of early tadpoles as is seen in *X. laevis* (stage 22, Figs. 1F, I) as well as in the cement gland.

Tsg knockdown

A major goal in pursuing the function of tsg was to determine the developmental effects of tsg knockdown using morpholino oligonucleotides (MOs). We characterized the tsg MO phenotype on the basis of morphological and molecular data at several stages throughout development. Embryos injected with a high dose of the tsg MO (10 ng per cell at the two-cell stage) appear normal through blastula stages, and initiate gastrulation normally, with formation of the dorsal lip

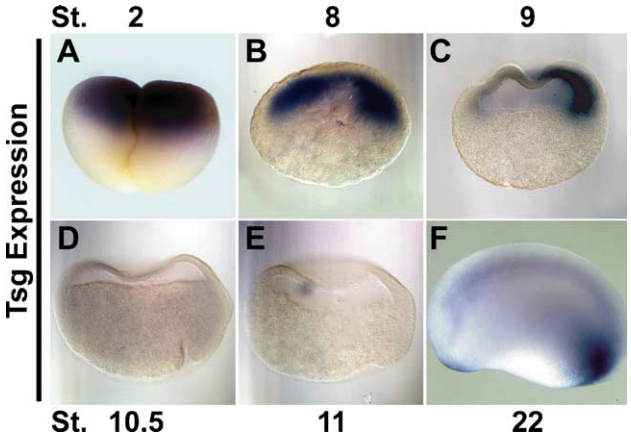

Fig. 1. Expression of tsg in *X. tropicalis* embryos. Embryos were collected at the 2-cell stage (A), stage 8 (B), stage 9 (C) stage 10.5 (D) stage 11 (E), and stage 22 (F), and assayed for expression of tsg. Panel A is a whole embryo shown in lateral view with animal to the top, panels B, C are bisected embryos shown with animal pole to the top. Panels D, E are bisected embryos shown with animal to the top and dorsal to the right, and panel F is a whole embryo shown laterally with anterior to the left.

of the blastopore at the same time as their uninjected siblings. By stage 11, however, morphological differences are apparent between injected and uninjected embryos, as tsg MO-injected embryos appear to close their blastopores at a slower rate than their uninjected siblings (Figs. 2A, a and g). To see if these morphological differences were reflected in differences at the molecular level, we examined the expression of several markers of dorsal–ventral patterning. At stage 11, the dorsal organizer markers *gsc* and *chordin* are expressed normally in tsg MO-injected embryos (Figs. 2A, b, h and c, i respectively). However, expression of the prospective muscle marker *myf-5* is greatly reduced (Figs. 2A, d, j), as has been noted previously in embryos depleted of BMP antagonists (Khokha et al., 2005). This suggested an early defect in graded dorsal–ventral identity in tsg-depleted embryos. Consistent with this hypothesis, expression of the ventral marker *sizzled* extends dorsally in tsg-depleted embryos (Figs. 2A, e, k), suggesting a broader ventrally-specified domain. We find that *vent-2* expression is excluded from the organizer in tsg-depleted embryos as it is in uninjected embryos (Figs. 2A, f, l), but that *vent-2* is consistently expressed through more of the dorsal animal portion of tsg MO-injected embryos than their uninjected siblings. Therefore, we find several lines of evidence to suggest that by mid-gastrulation, tsg-depleted embryos have a normal organizer, but have defects in dorsal–ventral patterning consistent with an expansion of ventral territory.

Because gastrulation is a dynamic process with rapid and significant changes in gene expression, any slight delay in development caused by injection of MO might result in the appearance of substantial fate changes as an artifact of delay. However, in normal embryos *sizzled* expression does not begin until stage 11, and tsg MO-injected embryos not only express *sizzled* but show an expansion of this gene marker, suggesting that changes in other gene expression patterns are not due to delayed development. In addition, the ventralized phenotype of

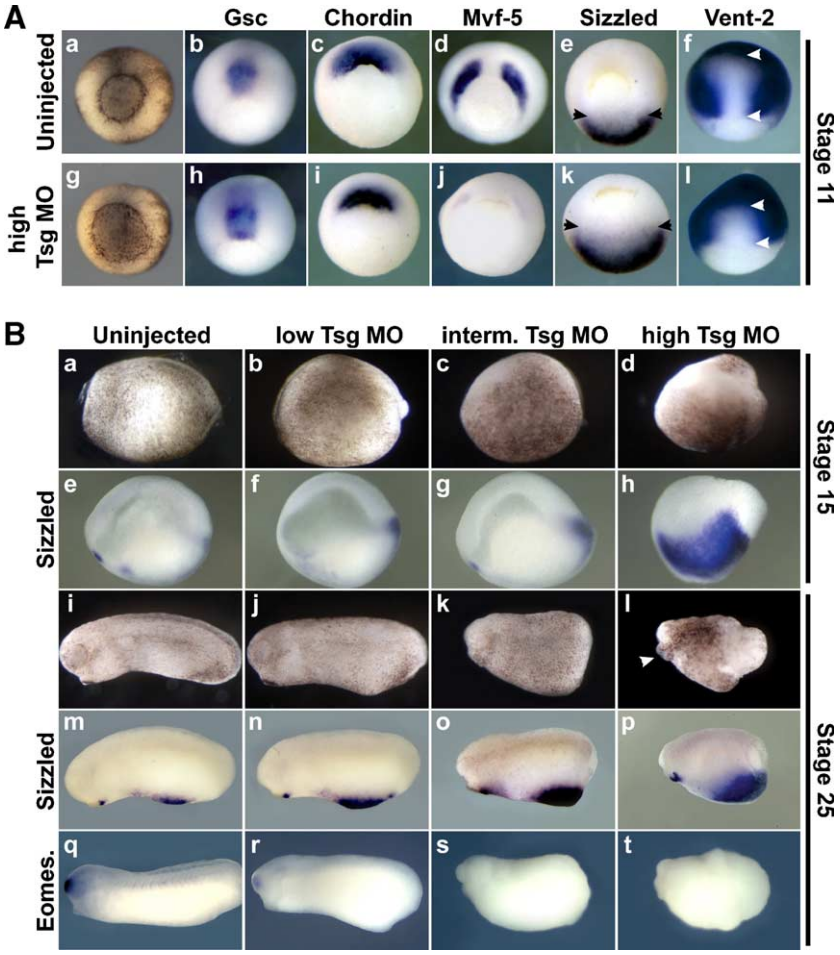

Fig. 2. Tsg morphants are ventralized. (A) Embryos were injected at the two-cell stage with a high dose of tsg MO (g–l) and compared to uninjected sibling control embryos (a–f) at stage 11 for changes in morphology and molecular marker expression as indicated. Arrows in panels e, k indicate the dorsal limit of *sizzled* expression, while arrows in panels f, l indicate the animal limit of *vent-2* expression. Embryos in panels f, l are shown from a dorsal view, all others are vegetal views with dorsal to the top. (B) Embryos injected with low (b, f, j, n, r), intermediate (c, g, k, o, s) or high (d, h, l, p, t) doses of tsg MO were compared to uninjected control siblings (a, e, i, m, q) for changes in morphology and expression of the ventral molecular marker *sizzled* (e–h, m–p) and the dorsal forebrain marker *eomesodermin* (q–t). All embryos are shown in lateral views with dorsal to the top, anterior to the left. Embryos were analyzed at stage 15 (a–h) or stage 25 (i–t). The arrowhead in panel l marks the cement gland.

tsg morphants persists later into neurula and tailbud-stage embryos when the dorsal–ventral axis is well-established (see below).

We then focused our analysis on later stages of development. One model for tsg function suggests that tsg can act as a BMP agonist or antagonist depending on its level of expression (Larrain et al., 2001). By injecting different doses of MO, we can deplete endogenous tsg levels to varying extents and test this hypothesis. We assessed expression of *sizzled* as a marker for ventral tissue, and expression of *eomesodermin* as a marker for the forebrain, the most dorso-anterior tissue. At the neurula stage (stage 15), low doses of the tsg MO have no noticeable effects on morphology compared to uninjected embryos, or on expression of *sizzled* (Figs. 2B, a, b, e, f). By tailbud stage (stage 25), minor morphological defects become apparent, with 25% of embryos showing a slight truncation of the anterior/posterior axis and a subtle expansion of ventral tissue marked by *sizzled* expression (Figs. 2B, j, n). Expression of *eomesodermin* is somewhat reduced at this dose (Figs. 2B, r), consistent with a reduction in dorso-anterior tissue. At an intermediate dose of tsg MO, morphological and molecular consequences are more severe. At stage 15 most embryos still appear wild-type, with a modest expansion of *sizzled* expression (Figs. 2B, c, g), but by stage 25 a clear truncation of the anterior/posterior axis is evident in all embryos (n = 88), with a markedly reduced head and an expansion of ventral tissue (Figs. 2B, k, o). Surprisingly, expression of *eomesodermin* was lost at this dose and at higher doses of tsg MO, suggesting a role for tsg in specification of the forebrain (Figs. 2B, s, t). Other anterior structures such as the cement gland were not lost at any MO dose (Figs. 2B, l, arrowhead). This loss of forebrain is discussed further below (Fig. 4).

The blastopore closure defects suggested by gastrula-stage embryos injected with a high dose of tsg MO are also reflected in tailbud-stage embryos injected with an intermediate dose of MO. 55% of embryos assayed at this stage have incompletely-closed blastopores. Embryos injected with a high dose of tsg MO are even more severely affected. 80% of embryos assayed at stage 15 in this experiment have open blastopores (n = 142)

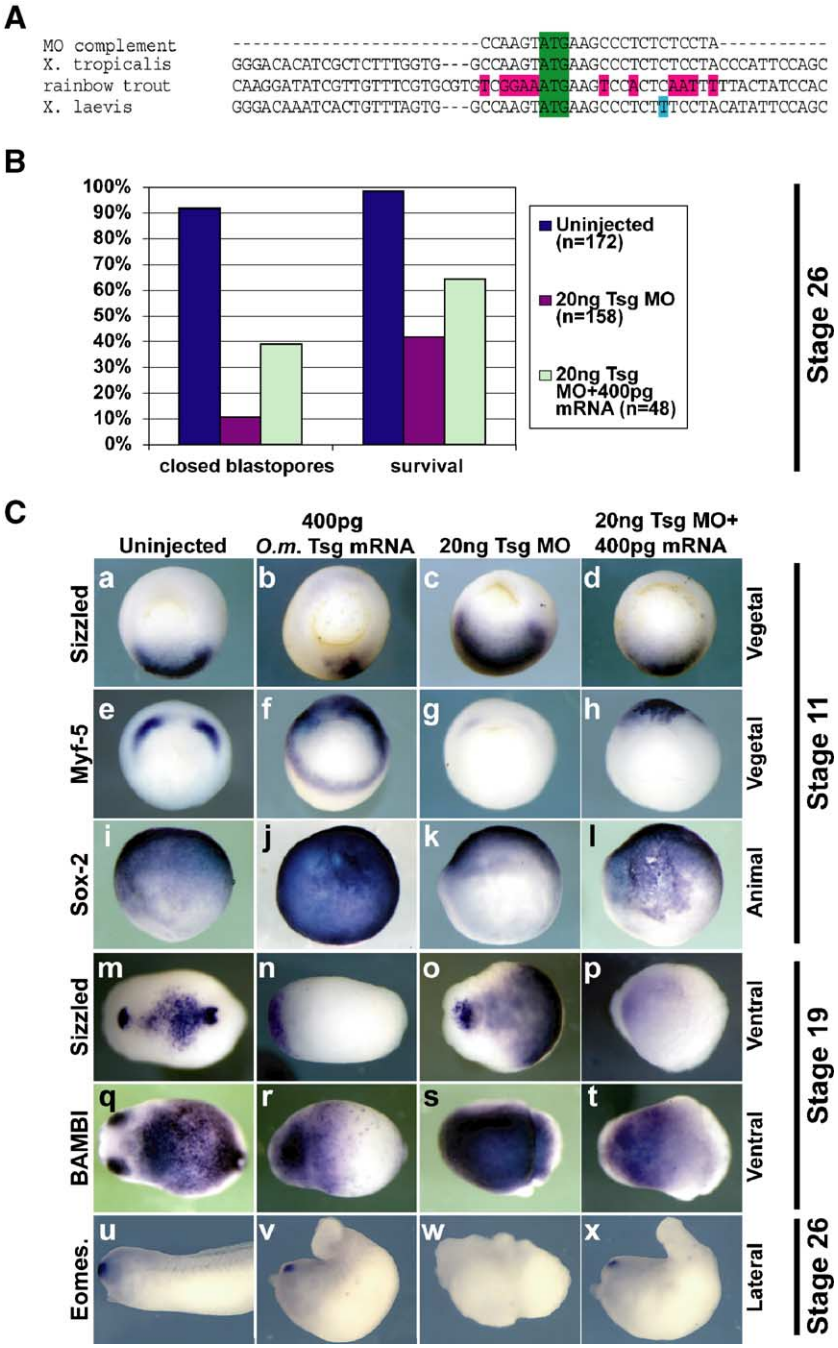

Fig. 3. Co-injection of tsg mRNA rescues the tsg morphant phenotype. (A) Alignment of the target sequence for the tsg MO used in this study with the 5′ region of mRNAs for *X. tropicalis*, *X. laevis* and *Oncorhynchus mykiss* (rainbow trout). The translational start site is shown in green, mismatches between *O. mykiss* and the tsg MO are shown in pink, and mismatches between *X. laevis* and the tsg MO are shown in blue. (B) Embryos were injected at the two-cell stage with 20 ng tsg MO or 20 ng tsg MO plus 400 pg of *O. mykiss* tsg mRNA, then assayed at stage 26 for survival and proper blastopore closure. The experiment was repeated with the same result. (C) Embryos were injected with 400 pg *O. mykiss* tsg mRNA (b, f, j, n, r, v), 20 ng tsg MO (c, g, k, o, s, w), or both (d, h, l, p, t, x) and compared to uninjected sibling control embryos (a, e, i, m, q, u) at stage 11 (a–l), stage 19 (m–t), or stage 26 (u–x) for expression of the molecular markers indicated at left. The view for each marker is indicated at right. For animal and vegetal views, dorsal is to the top while for ventral and lateral views, anterior is to the left.

(Figs. 2B, d) and many fail to neurulate properly. All embryos show dramatic upregulation and expansion of *sizzled* expression (Figs. 2B, h).

In addition to the expansion of ventral tissues and defects in blastopore closure, tsg morphants have a decreased rate of survival. While 100% of embryos survive through gastrulation, only 40% of embryos injected with this dose of MO survived to stage 25. Those that do survive show severe defects in blastopore closure, a dramatic reduction in the size of the head and dorsal structures, a severely truncated anterior/posterior axis and an expansion of ventral tissue, with a greatly extended domain of *sizzled* expression (Figs. 2B, l, p). As shown below, these defects are specifically due to depletion of tsg and can be rescued by overexpression of tsg mRNA.

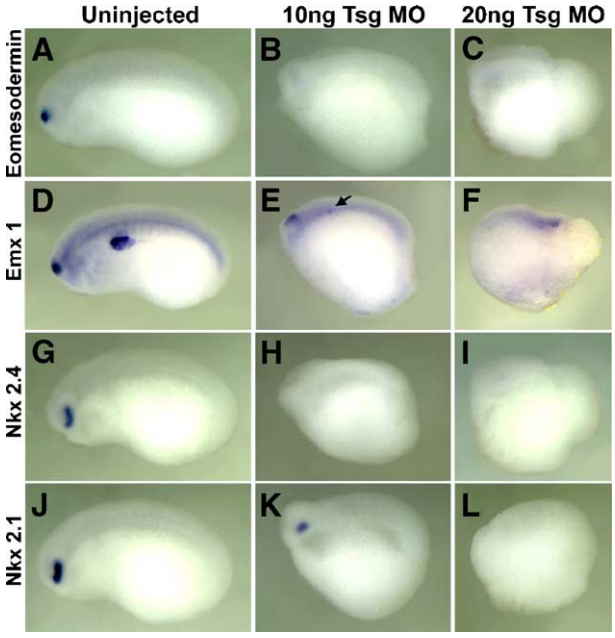

Fig. 4. Forebrain patterning is impaired in tsg morphants. Embryos were injected with intermediate (B, E, H, K) or high (C, F, I, L) doses of tsg MO and compared to uninjected sibling control embryos (A, D, G, J). Regional specific markers of the forebrain were assayed including the dorsal telencephalon markers *eomesodermin* (A–C) and *emx-1* (D–F), and the ventral telencephalon markers *Nkx2.1* (G–I) and *Nkx2.4* (J–L). All embryos are shown at stage 23 and are viewed laterally with anterior to the left.

Thus depletion of tsg results in the expansion of ventral tissues by morphological and molecular criteria in a dose-dependent manner. This loss-of-function phenotype is consistent with a reduction in BMP antagonist activity, and is similar to, but more dramatic than, tsg loss-of-function phenotypes that have been reported in *X. laevis* (Blitz et al., 2003; Zakin et al., 2005). In addition, we find that expression of the ventral marker *sizzled* is either unchanged or expanded in embryos injected with low to high doses of tsg, especially in embryos that are assayed at later stages of development. We do not find any evidence for a dorsalized morphology or loss of ventral marker expression by any dose of tsg MO, in contrast to similar studies in zebrafish or *Xenopus* (Little and Mullins, 2004; Zakin et al., 2005).

To ensure that the phenotypes we observed were specifically due to the loss of tsg and not to other non-specific consequences of the MO, we attempted to rescue tsg morphants by co-injecting embryos with tsg mRNA that was non-complementary to our MO. We chose rainbow trout (*Oncorhynchus mykiss*) tsg mRNA for this purpose, which encodes a protein 84% identical to *X. tropicalis* tsg but does not match our tsg MO at 10/24 base pair positions (Fig. 3A). We found that co-injection of 400 pg *O. mykiss* tsg mRNA with 20 ng (high dose) of tsg MO resulted in a dramatic improvement in blastopore closure and survival over embryos injected with tsg MO alone (Fig. 3B), suggesting that the morphological defects we observed were due specifically to the loss of tsg gene product.

We also analyzed expression of several molecular markers to see if the molecular consequences of the tsg MO were also rescued by co-injection with tsg mRNA. At mid-gastrula (stage 11), we noted that injection of 400 pg of *O. mykiss* tsg mRNA alone resulted in a reduction of *sizzled* expression, expansion of *myf-5* expression, and expansion of the pan-neural marker *sox-2* relative to uninjected sibling embryos (Figs. 3C, a, b, e, f, i, j). These changes in molecular marker expression are consistent with dorsalization, and are complementary to the effects we observed with tsg MO injection, in which embryos show expanded *sizzled* expression, reduced *myf-5* expression and reduced *sox-2* expression (Figs. 3C, c, g, k). Co-injection of tsg mRNA with tsg MO resulted in rescue of the expression levels of these markers relative to embryos injected with tsg MO or tsg mRNA alone.

Later molecular consequences of tsg MO injection are also rescued by mRNA overexpression. At late neurula (stage 19), expression of the BMP signaling target *BAMBI* (Onichtchouk et al., 1999) and of the ventral marker *sizzled* (Collavin and Kirschner, 2003) is greatly expanded in embryos injected with tsg MO compared to uninjected sibling embryos (Figs. 3C, m, o, q, s). This expansion is rescued by co-injection with tsg mRNA (Figs. 3C, p, t). The loss of *eomesodermin* expression caused by tsg MO injection is also restored by co-injection with tsg mRNA (Figs. 3C, w, x). Together, these data demonstrate that embryos in which tsg is overexpressed are dorsalized, while embryos depleted of tsg are ventralized in a manner that can be rescued by overexpression of tsg.

Twisted gastrulation is required in forebrain development

The absence of *eomesodermin* expression in embryos depleted of tsg suggested a requirement for tsg in the specification of the forebrain. To characterize this forebrain defect, we analyzed regional specific markers.

The dorsal telencephalon expresses *eomesodermin* and *emx1* while the ventral forebrain expresses *nkx2.1* and *nkx2.4* (Lupo et al., 2002). Injection of an intermediate dose of tsg MO results in a severe reduction of both dorsal and ventral telencephalic markers in stage 25 embryos (Fig. 4). Expression of the dorsal telencephalon marker *eomesodermin* is absent at this dose (Fig. 4B), as is expression of the ventral telencephalon marker *nkx2.4* (Fig. 4H), while expression of the dorsal marker *emx1* and of the ventral marker *nkx2.1* is markedly reduced (Figs. 4E, K respectively). *Emx1* is also expressed in the developing kidney, and this expression is retained in small bilateral domains in embryos injected with an intermediate dose of tsg MO (Fig. 4E, arrowhead). The reduction in expression is consistent with the finding that the kidney is induced by anterior somites (Seufert et al., 1999). At a high dose of tsg MO, expression of all forebrain markers is absent in stage 25 embryos (Figs. 4C, F, I, L). This loss of forebrain markers cannot be attributed to a loss of all anterior-most structures, because ventral–anterior structures such as the cement gland are retained (Fig. 2). Similarly, the preserved expression of en-2, *krox-20,* and *otx-2* (Fig. 5, below) indicates that other markers of brain regionalization are maintained. Thus, these data demonstrate a requirement for twisted gastrulation in dorsal and ventral forebrain development.

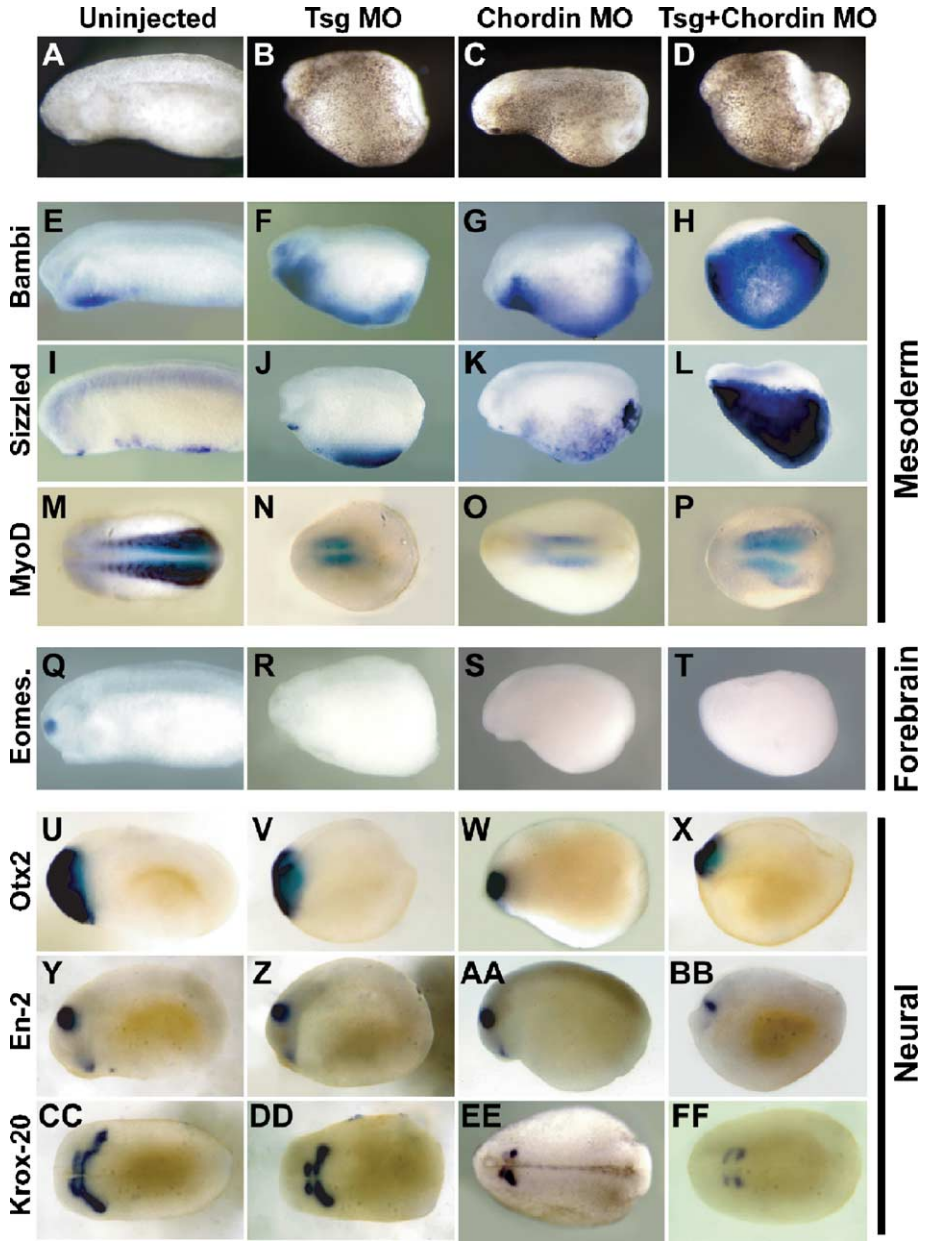

Fig. 5. Depletion of chordin and tsg reduces dorsal tissues and results in a cooperative expansion of ventral tissues. Embryos were injected at the two-cell stage with tsg MO (B, F, J, N, R, V, Z, DD), chordin MO (C, G, K, O, S, W, AA, EE), or both MOs (D, H, L, P, T, X, BB, FF) and compared to uninjected sibling control embryos (A, E, I, M, Q, U, Y, CC). Embryos were assayed for expression of the ventral markers *BAMBI* (E–H) and *sizzled* (I–L) and the somite marker *myoD* (M–P). Embryos were also assayed for expression of several regional specific markers of the neural plate: *eomesodermin* (Q–T), *otx2* (U–X), *en-2* (Y–BB) and *krox-20* (CC–FF). All embryos are between stages 22–25. Embryos stained for expression of *myoD* (M–P) and *krox-20* (CC–FF) are viewed dorsally with anterior to the left, all others are viewed laterally with anterior to the left.

Cooperative effects of Tsg and chordin

A central goal of this study was to establish whether tsg behaves as a BMP agonist or antagonist in the context of normal *Xenopus tropicalis* development. If tsg is a BMP antagonist that cooperates with the known BMP antagonist chordin, one would predict that loss of tsg function would augment the chordin loss of function phenotype. We therefore co-injected MOs directed against tsg and chordin and assayed their combined effects on development. The chordin MO causes a ventralized phenotype characterized by a reduction in head size and expansion of ventral tissue similar to the phenotype seen in *X. laevis* (Fig. 5C) (Oelgeschlager et al., 2003a). At the tailbud stage, embryos co-injected with these two morpholinos showed severe ventralization and loss of dorsal structures (Fig. 5D). The head is reduced and the anterior/posterior axis is dramatically shortened. Co-injection of both MOs results in severe defects in blastopore closure (95% open blastopores at stage 25, $n = 183$) and a pronounced expansion of ventral tissue as compared to dorsal tissue. These phenotypes are more frequent and severe in co-injected embryos than in embryos injected with either MO alone.

These phenotypes are similar to those described in *X. laevis* (Blitz et al., 2003), and are consistent with the hypothesis that tsg acts as a BMP antagonist and acts cooperatively with chordin.

Since dorsal–ventral patterning is dependent on a proper balance of BMP signaling, reduction of BMP antagonists should lead to a reduction in dorsal cell fates and to an expansion of ventral cell fates. To ascertain if tsg cooperates with chordin to expand ventral tissues, we assayed *BAMBI* and *sizzled*. These markers were notably expanded in embryos injected with tsg or chordin MOs (Figs. 5E–L). Expression of both *BAMBI* and *sizzled* is dramatically increased in embryos co-injected with both tsg and chordin MOs (Figs. 5H, L). In these co-injected embryos, the vast majority of the embryo is converted to ventral tissue, with the exception of a narrow domain at the extreme dorsal edge that retains a dorsal fate. These data strongly suggest that tsg and chordin act cooperatively to generate normal patterning along the dorsal–ventral axis, since reduction of both proteins leads to more dramatic ventralization than the loss of either protein individually.

Embryos injected with tsg and chordin MOs also show reduced expression of dorsal markers. The somite marker *myoD* is reduced in intensity and the volume of *myoD*-positive tissue in embryos injected with each MO and both MOs (Figs. 5M–P), consistent with the reduction in *myf-5* expression seen in younger embryos. To characterize anteroposterior patterning of the neural plate, we assayed *otx2* (anterior), *engrailed-2* (midbrain–hindbrain border), and *krox-20* (hindbrain) expression, which were all slightly reduced in late neurula-stage embryos injected with intermediate amounts of tsg MO or chordin MO, and were also reduced but not lost in embryos co-injected with both MOs (Figs. 5U–FF). The boundaries of *en-2* and *krox-20* retain their relative anterior/posterior positions. At tailbud stages, expression of the forebrain marker *eomesodermin* was usually lost in embryos injected with either tsg MO (83%, $n = 24$) or chordin MO (89%, $n = 18$) alone, and was always lost in embryos injected with both MOs (Figs. 5Q–T), suggesting that chordin is also required for specification of the forebrain, and that high levels of BMP antagonism are generally required to establish forebrain tissue. We conclude that depletion of tsg and chordin results in reduction of regional markers of the neural plate and in a loss of forebrain. We find that loss of chordin and tsg, whether individually or together, always results in an overall reduction of dorsal structures and expansion of ventral structures, with a cooperative ventralizing effect when both gene products are knocked down. We find no evidence for a rescue of the chordin loss-of-function phenotype by tsg depletion, as would be expected if tsg inhibited chordin activity, but conclude rather that tsg and chordin act cooperatively as BMP antagonists in the context of embryonic development.

Rescue of tsg with knockdown of BMPs

Knockdown of tsg and chordin results in a more severe expansion of ventral tissues than knockdown of either gene product alone, strongly suggesting that tsg acts as a BMP antagonist during development. BMP antagonists maintain a precise regulation of BMP signaling across the dorsal–ventral axis. According to this model, if BMP antagonists are depleted, the balance of BMP signaling should be regained by a compensating depletion of BMPs. A phenotype generated by knockdown of a BMP antagonist should thus be rescued by depletion of BMPs (Khokha et al., 2005). We confirmed the effectiveness of this strategy in this context by reducing BMP4 and BMP7 gene products to rescue the effects of chordin MO injection in tailbud-stage embryos (Supplementary Fig. 1). Co-injection of the BMP MOs in this manner rescues the reduction in head size and loss of *eomesodermin* expression seen in chordin MO-injected animals (78%, $n = 45$). Expression of *sizzled* is also reduced to wild-type levels.

We then investigated whether the tsg knockdown phenotype could be similarly rescued by co-injection with BMP MOs. Tsg has been shown to interact biochemically with both BMP4 and BMP7 (Oelgeschlager et al., 2000; Zakin and De Robertis, 2004; Zakin et al., 2005). We injected an intermediate dose of tsg MO, which causes a severely ventralized phenotype (Fig. 2), with varying doses of BMP4 and BMP7 MOs. Fig. 6A shows that co-injection of tsg MO with BMP MOs results in a significant improvement in blastopore closure. A combination of tsg MO injected with 40 ng of either BMP4 or BMP7 MO or with 20 ng of both BMP4 and BMP7 MOs results in 30–40% more closed blastopores in stage 25 embryos than in embryos injected with tsg MO alone. Injection of BMP MOs alone had no effect on blastopore closure.

Embryo morphology was also rescued by co-injection of BMP MOs with tsg MO, as shown in Fig. 6B. Co-injecting 40 ng of BMP4 MO with an intermediate dose of tsg MO resulted in 85% of embryos showing at least some rescue of head size and length of the anterior/posterior axis, with 37% of embryos showing extensive rescue of these features and a nearly-wildtype morphology, including reduced ventral tissue and wild-type tailbud morphology. Similar results were seen for co-injection of 40 ng BMP7 MO with tsg MO. An even more dramatic rescue effect was seen in embryos co-injected with tsg MO and 20 ng of both BMP4 and BMP7 MOs. 96% of these embryos showed some morphological rescue, and 74% have extensive rescue of head morphology, length of the A/P axis, and amount of ventral tissue. At these doses, injection of BMP MOs alone had modest effects on embryo morphology (Supplementary Fig. 2), although at higher doses, we do observe phenotypes similar to those previously reported (Reversade et al., 2005).

Our finding that BMP knockdown effectively rescues the tsg MO phenotype again supports the hypothesis that tsg acts as a BMP antagonist in the context of *X. tropicalis* gastrulation.

BMP knockdown rescues forebrain and ventral mesoderm in embryos depleted of tsg

To characterize the extent of rescue caused by co-injection of BMP MOs with tsg MO in more detail, we examined the

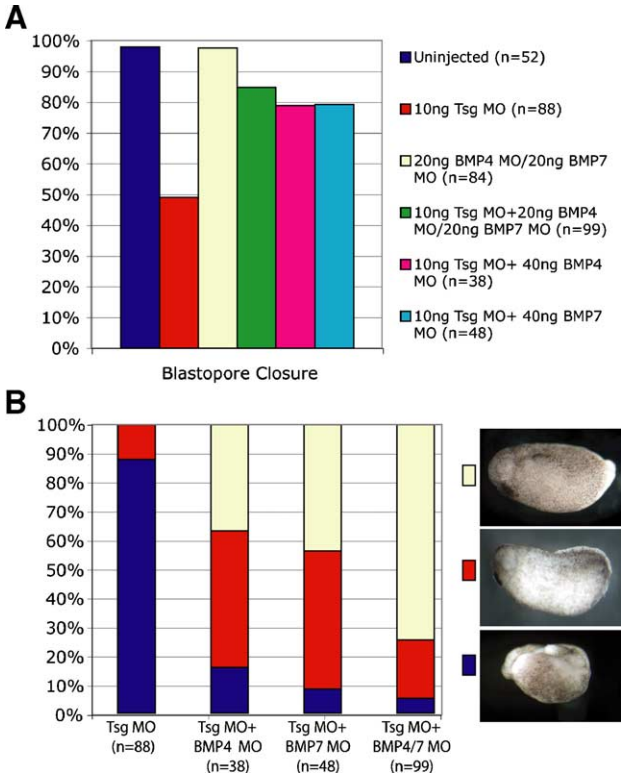

Fig. 6. BMP depletion rescues tsg morphants morphologically. (A) The incidence of blastopore closure was quantified in embryos injected at the two-cell stage with tsg MO, BMP MOs, or varying combinations of tsg and BMP MOs as indicated. The experiment was repeated with similar results. (B) Co-injection of tsg MO with BMP MOs rescues embryos morphology to varying extents. Embryos were injected at the 2-cell stage with 10 ng of tsg MO and either 40 ng of BMP4 MO, 40 ng of BMP7 MO, or 20 ng of both BMP4 and BMP7 MOs. For each combination, the percentage of embryos observed with each degree of rescue was quantified. Representative embryos for each category of rescue are included at right. The category represented in blue indicates embryos with minimal or no rescue of head morphology or embryo length. Embryos represented by the red category had mild to moderate rescue of head morphology and/or embryo length. Embryos represented by the yellow category showed significant rescue of head morphology, embryo length and overall morphology. For panels A and B, all embryos were injected at the 2-cell stage with the combination of MOs indicated, and were assayed at stage 25 for blastopore closure and morphology.

expression of anteroposterior neural markers and ventral mesodermal markers. Given the loss of forebrain tissue in embryos depleted of tsg, we analyzed two forebrain markers, *eomesodermin* and *xbf-1*. Forebrain expression of both markers is rescued by co-injection with BMP 4 and 7 MO (Fig. 7, compare T, X to R, V). The expression *of krox-20* is also restored to wild-type levels by co-injection with BMP MOs (Figs. 7J, L). Expression of the midbrain–hindbrain boundary marker *en-2* and of the posterior spinal chord marker HoxB9 is unaffected by either tsg or BMP MOs, and these markers retain their relative anterior/posterior boundaries (Figs. 7F–H). The expression patterns of these dorsal markers in embryos injected with only BMP4 and 7 MOs are similar to uninjected embryos (Figs. 7G, K, O, S, W).

Ventral markers are also restored to wild-type levels by co-injection of tsg MO with BMP 4 and 7 MOs. The ventral marker *sizzled*, which is substantially expanded by tsg MO injection (Fig. 7Z), is rescued to wild-type levels by co-injection with BMP 4 and 7 MOs (Fig. 7BB). The expression of *sizzled* in embryos injected with a low dose of either BMP 4 or BMP 7 MOs alone is similar to wild type (Fig. 7AA). However, expression of *sizzled* and of *BAMBI* is reduced when high doses of BMP4/7 MOs are injected (data not shown).

Rescue of tsg with chordin mRNA

To test our hypothesis that tsg acts as a BMP antagonist further, we attempted to rescue the intermediate tsg phenotype with a dose of chordin mRNA that is sufficient to induce secondary axes (Larrain et al., 2000). Co-injection of chordin mRNA in tsg MO-injected embryos results in a dorsalized morphology characterized by a large head and truncated anterior/posterior axis (Fig. 8C). The frequency of blastopore closure defects (18.8%, n = 32), is reduced in embryos co-injected with chordin mRNA as compared with embryos injected with tsg MO alone (59%, n = 88). This rescue of blastopore closure is comparable to that achieved by co-injecting tsg MO with BMP4 and BMP7 MOs (Fig. 6A: 85% closed blastopores, n = 99). Secondary axes are occasionally observed in late tailbud stage embryos injected with tsg MO and chordin mRNA (15%, n = 32), but at a reduced frequency from embryos injected with chordin mRNA alone (28%, n = 38). Expression of *sizzled* is restored to wild-type levels in embryos co-injected with tsg MO and chordin mRNA (Figs. 8A–C). Expression of *eomesodermin* is also recovered in these embryos (Figs. 8D–F). This recovery of marker expression supports our prediction that loss of tsg function can be rescued by chordin mRNA, consistent with the hypothesis that tsg and chordin work collaboratively as BMP antagonists.

Discussion

Tsg is required for forebrain development in X. tropicalis

We have found that reduction in tsg function results in a complete loss of dorsal and ventral forebrain marker expression at tailbud stages. This loss is specific to the forebrain and does not reflect a general loss of anterior structures, since the cement gland, another anterior structure, is preserved. Neither can this loss of forebrain be attributed to a failure of brain differentiation or specification in general, since other markers for midbrain and hindbrain such as *engrailed* and *krox-20* are preserved with only a slight reduction in the domain of expression. Otx-2, which marks both the forebrain and the midbrain, is reduced but not lost, and based on the loss of expression of all markers specific to the forebrain, we suggest that the remaining Otx-2 expressing tissue has midbrain identity. The absence of forebrain marker expression seen in tsg morphants is rescued by reducing BMP signaling through knockdown of BMP4 or BMP7, and by overexpression of the BMP antagonist chordin, indicating that forebrain specification requires adequate BMP antagonism. A

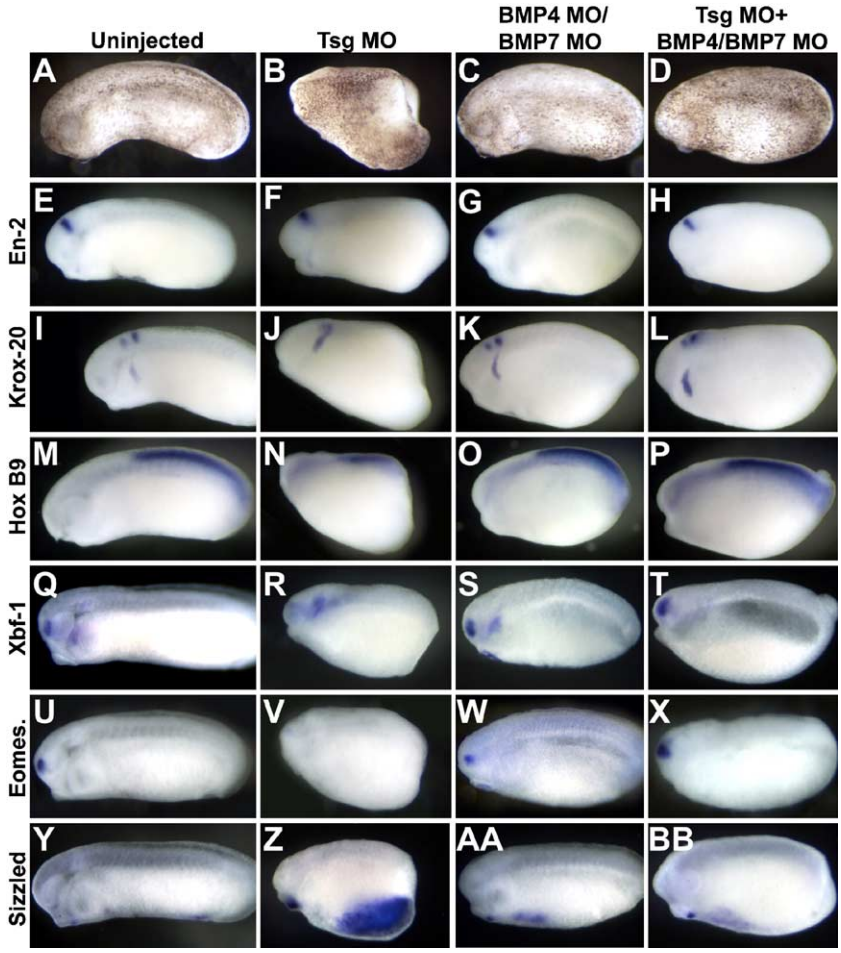

Fig. 7. BMP depletion rescues patterning defects in tsg morphants. Expression of region-specific markers were analyzed at stage 25 in uninjected embryos (A, E, I, M, Q, U, Y), and in embryos injected with 10 ng of tsg MO (B, F, J, N, R, V, Z), 20 ng each of BMP4 and BMP7 MOs (C, G, K, O, S, W, AA) or a combination of 10 ng tsg MO and 20 ng of BMP4 and BMP7 MOs (D, H, L, P, T, X, BB). Markers assayed included the midbrain–hindbrain boundary marker *en-2* (E–H), the hindbrain marker *krox-20* (I–L), the spinal cord marker *hoxb 9* (M–P), the forebrain markers *Xbf-1* (Q–T) and *eomesodermin* (U–X) and the ventral marker *sizzled* (Y–BB). All views are lateral with anterior to the left and dorsal to the top.

requirement for BMP antagonists in forebrain specification has been described in the mouse (Bachiller et al., 2000), and we also find that loss of chordin function results in loss of forebrain marker expression, which is recovered by simultaneous loss of BMPs. The requirement for tsg in specifying the forebrain can be compensated by overexpression of chordin. Thus, our findings support a model in which the biochemical interaction between tsg and chordin results in a potentiation of chordin function, and that in the absence of tsg, more chordin is required to achieve proper patterning of the

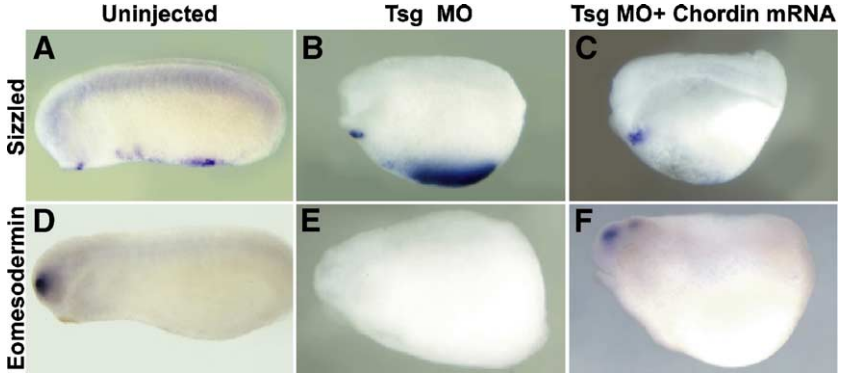

Fig. 8. Chordin overexpression rescues tsg morphants. Expression of the ventral mesodermal marker *sizzled* was examined in stage 25 uninjected embryos (A), and in sibling embryos injected with intermediate doses of tsg MO (B), or an intermediate dose of tsg MO plus chicken chordin mRNA (C). Expression of the dorsal telencephalon marker *eomesodermin* was also compared among uninjected embryos (D), sibling embryos injected with an intermediate dose of tsg MO (E), and siblings injected with an intermediate dose of tsg MO and chicken chordin mRNA. All views are lateral with anterior to the left and dorsal to the top.

forebrain. It is also possible that the BMP binding activity of tsg allows it to act as a BMP antagonist independently of chordin, and that tsg and chordin act additively and independently to specify the forebrain. A specific requirement for tsg in specifying the forebrain has not been previously demonstrated. In one mouse study, genetic knockouts of tsg cause craniofacial defects and loss of head structures (Petryk et al., 2004), but these effects are restricted to particular genetic backgrounds and were not described in other mouse loss-of-function studies (Nosaka et al., 2003) except in the compound tsg mutant and BMP4 heterozygote (Zakin and De Robertis, 2004). Thus we show that the BMP antagonist activity of tsg is required for the development of the forebrain in *Xenopus*.

Twisted gastrulation is a BMP antagonist in the Xenopus gastrula

The activity of twisted gastrulation has been controversial. While there is ample biochemical evidence for a direct interaction between tsg, BMPs and chordin, the in vivo role of tsg has been more difficult to establish with certainty. Because of the apparent contradiction among overexpression studies (Oelgeschlager et al., 2000; Scott et al., 2001), it has been suggested that low doses of twisted gastrulation protein might result in BMP antagonist-like activity while high doses promote the release of BMPs from chordin, thus potentiating BMP signaling. Here, we report that the activity of twisted gastrulation with respect to BMP signaling is exclusively antagonistic in the context of *X. tropicalis* gastrulation. Titration of the tsg MO reveals a dose-dependent increase in ventral cell fates by morphological and molecular criteria, with reduction of dorso-anterior structures and extensive expansion of ventral mesoderm. At no dose of MO do we observe any evidence for dorsalization. This argues against a model where tsg can have both BMP agonist and BMP antagonist roles depending on concentration, at least at physiological levels within the gastrulating embryo. Instead we favor a role for strict BMP antagonism in the context of early *X. tropicalis* development. Our analysis of mid-gastrula stage tsg morphants reveals that ventral territory is already expanded while prospective neural tissue is reduced, indicating that tsg acts as a BMP antagonist during gastrulation when these tissues are first being specified. The high levels of zygotic expression of tsg in the ventral tissues of tailbud-stage embryos suggest that it may also have a role in maintaining repression of BMP signaling activity later in development. Overexpression of tsg leads to opposite phenotypes, namely an increase in neural tissue and decrease in ventral tissue, and can also rescue the patterning defects seen in tsg depleted embryos. We note that the morphology of embryos co-injected with tsg MOs and BMP MOs is more nearly wild-type than that of embryos co-injected with tsg MO and tsg mRNA. We take this as evidence that the depletion approach offered by concurrent MO injection results in more nearly physiological levels of BMP signaling than can be achieved by combined knockdown and overexpression of tsg, which may be complicated by non-physiological timing or location of tsg overexpression.

Our loss of function evidence demonstrates that tsg is a crucial molecule in *X. tropicalis* development. Depletion of tsg, results in multiple phenotypes in gastrula, neurula, and tailbud-stage embryos: loss of the forebrain, reduction in dorsal tissues, expansion of ventral tissues, and gastrulation defects as evidenced by open blastopores and resultant loss of viability. While not a focus of the present study, blastopore closure defects represent another specific phenotype in tsg-depleted embryos. Many of these defects are exacerbated by a simultaneous loss of chordin. Importantly, the morphological and molecular phenotypic characteristics of tsg knockdown can all be rescued by a controlled loss of BMPs or increase of chordin, collectively supporting a cooperative interaction between chordin and tsg and opposing BMP signaling. The tsg loss of function phenotype described here for *X. tropicalis* and elsewhere for *X. laevis* is more severe than that caused by genetic knockouts in mice or MO-based knockdown in zebrafish (Blitz et al., 2003; Little and Mullins, 2004; Nosaka et al., 2003; Petryk et al., 2004; Scott et al., 2001; Zakin and De Robertis, 2004), and suggests that tsg is particularly crucial for regulating BMP signaling in *Xenopus* species.

Our evidence for tsg function in *X. tropicalis* supports other MO-based loss of function experiments conducted in *X. laevis* (Blitz et al., 2003). However, another recent report in *X. laevis* describes a contrasting finding: that injection of a tsg MO results in reduced expression of the ventral marker *sizzled* in late gastrula stage embryos, which is further reduced by co-injection of a BMP7 MO (Zakin et al., 2005). This is in sharp contrast to the findings in this study. The reason for this discrepancy is uncertain. One possibility is the difficulty of achieving consistent gene product knockdown in the allotetraploid *X. laevis.* Polymorphisms and the effects of gene copy number can affect morpholino binding and consequent phenotypic effects, which has been reported for *X. laevis* (Khokha et al., 2002; Piepenburg et al., 2004). Because *X. tropicalis* is inbred and has a diploid genome, designing effective MOs is simplified. Our data demonstrate a dramatically ventralized phenotype in tsg knockdown embryos with consistent expansion of ventral markers and loss of dorso-anterior markers at several doses of tsg MO, and we find no evidence for dorsalization by loss of tsg. These morphological and molecular consequences of tsg knockdown can be rescued both by loss of BMP gene product and by overexpression of chordin, demonstrating the specificity of these phenotypic effects, which are clearly consistent with a role for tsg as a BMP antagonist rather than an enhancer of BMP function.

Our data also contrast with conclusions drawn from recent loss-of-function experiments in zebrafish that argue in favor of a role for tsg in promoting BMP signaling (Little and Mullins, 2004; Xie and Fisher, 2005). However, in early patterning of the zebrafish, the role of tsg has also been controversial, with an earlier report suggesting that depletion of tsg results in a phenotype indicative of a BMP antagonist (Ross et al., 2001). A remaining possibility to reconcile the differences in loss-of-

function analyses of tsg in frogs and zebrafish is that at the biochemical level, tsg can act both as a BMP agonist (competing with chordin for BMP binding) and antagonist (strengthening the association between chordin and BMPs), and that this duplicity of roles is exploited differently in different animals. Our data demonstrate an exclusive function for tsg in cooperating with chordin to antagonize BMP signaling in the *X. tropicalis* gastrula, and suggest that though tsg may be biochemically plastic, its in vivo role in this system is restricted to BMP antagonism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.10.022.

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