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Nature, 410(6827)

0028-0836

Ross, JJ
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2001-03-01

10.1038/35068578

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Peer reviewed
Twisted gastrulation is a conserved extracellular BMP antagonist

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Bone morphogenetic protein (BMP) signalling regulates embryonic dorsal–ventral cell fate decisions in flies, frogs and fish. BMP activity is controlled by several secreted factors including the antagonists chordin and short gastrulation (SOG)2,3. Here we show that a second secreted protein, TwistEd gastrulation (Tsg)4, together makes a more effective inhibitor of BMP signalling than either of them alone. Blocking Tsg function in zebrafish with morpholino oligonucleotides causes ventralization similar to that produced by chordin mutants. Co-injection of sub-inhibitory levels of morpholines directed against both Tsg and chordin synergistically enhances the penetrance of the ventralized phenotype. We show that Tsgs from different species are functionally equivalent, and conclude that Tsg is a conserved protein that functions with SOG/chordin to antagonize BMP signalling.

TSG is required to specify the dorsal-most structures in the *Drosophila* embryo, for example amnioserosa. Mutations in the BMP-like ligands, Decapentaplegic (DPP) and Screw (SCW), the BMP inhibitory factor SOG, or the SOG-processing enzyme Tolloid (TLD), also cause loss of the amnioserosa, even though some of these products seem to have opposing biochemical functions. To place TSG activity relative to the biochemical function of these other factors, we examined its loss-of-function phenotype using molecular markers (Fig. 1A). The phenotype of tsg mutants (Fig. 1A, c, g, l) is most similar to that produced by loss of the BMP antagonist SOG (Fig. 1A, b, f, k) rather than that produced by loss of the ligands DPP or SCW (data not shown), or the SOG-processing protease TLD (Fig. 1A, d, h, m). In both tsg and sog mutants, the dorsal marker Rhomboid (rho) expands (Fig. 1A, b, c), whereas in tld mutants no rho expression is observed (Fig. 1A, d)4. In contrast, mutations in tsg, sog and tld eliminate expression of other presumptive amnioserosa markers including Race (Fig. 1A, g, f, h). Hindsight (data not shown) and Zerknöttl (zen) (data not shown).

To determine whether the response of these is indicative of different threshold levels of DPP signalling5, we used an anti-phospho-Smad antibody to directly visualize the levels of ligand signalling. Wild-type embryos accumulate phosphorylated mother against DPP (P-MAD) in an 18–20-cell-wide dorsal stripe at mid-cellulariza-

![Figure 1](image-url)

**Figure 1** Tsg and SOG synergistically inhibit DPP signalling. **A**. The tsg loss-of-function phenotype is similar to that of sog. a–h. In situ hybridization of a Rhomboid RNA probe and a Race RNA probe to wild-type (WT), sog and tsg mutant embryos. Mutant embryos were identified by the absence of lacZ expression supplied by a marked balancer chromosome. All embryos are at the mid cellular blastoderm or early gastrulation stage; anterior is to the left and the view is dorsal. a–m. Anti-phospho-MAD staining of WT (i), sog (j), sog (k) and tld (l) mutant embryos. The embryos in i and j are dorsal side up, and in k–m they are viewed laterally. **B**. TSG and SOG form a high-affinity complex with DPP. S2 cells were transfected with DPP-HA and either SOG alone or SOG and TSG. After induction with Cu+, the supernatants were harvested and immunoprecipitated (IP) with anti-Flag antibody, transferred to a PVDF membrane and probed with anti-HA (12CAS Roche) antibody as described. **C**. TSG and SOG synergistically inhibit DPP signalling in S2 cells. In lanes 2–5, 20 ng purified DPP was added. Lane 1 was mock treated with buffer. In lanes 3–5, 1.25 μg SOG, 1 μg TSG or 1 μg of each were premixed with DPP and added to cells. The top panel was probed with anti-P-MAD antibody; the bottom with anti-Flag antibody. **D**. TSG alone inhibits DPP signalling in S2 cells at high concentrations. Cells were transfected with MAD-Flag as above, and then treated with 20 ng DPP and the indicated levels of TSG. **E**. TSG and SOG together form an effective inhibitor of DPP in vivo. Transformant lines containing UAS dpp, sog and tsg constructs were crossed in the combinations indicated to the GAL4-A9 driver.
tion (Fig. 1A, i) that rapidly resolves into an 8–9-cell-wide stripe (Fig. 1A, j) of more intensely stained cells just as gastrulation starts. Although an underlying gradient of activity not detectable by this method may exist, these results instead suggest that DPP/SCW activity is distributed in a sharp on-off pattern that resolves into a narrow stripe of dorsal cells, which—posterior to the cephalic furrow—corresponds in width to those cells labelled by the amnio-serosa markers Race and Hindsight. In sog and tsg mutants (Fig. 1A, k, l), P-MAD fails to refine and intensify, whereas in tld mutants (Fig. 1A, m) P-MAD activity is below the level of detection in all dorsal cells. We suggest that the low, uniform levels of P-MAD seen in sog and tsg mutants are sufficient to activate rho, but not race, hndl or zen transcription.

As the phenotypes of tsg and sog mutants are similar, we sought to determine whether TSG can enhance the binding of SOG to ligand. Co-immunoprecipitation of DPP by SOG is greatly enhanced when these two factors are coexpressed in S2 cells along with TSG (Fig. 1B). To test whether the combination of SOG and TSG blocks DPP signalling better than SOG alone, we developed an S2 cell-culture assay for DPP signalling (Fig. 1C). At high concentrations TSG alone can block DPP signalling (Fig. 1D); however, at lower concentration, the combination of TSG and SOG together dramatically reduces the DPP-dependent accumulation of P-MAD much more efficiently than either could alone. In vivo overexpression of sog and tsg together can completely reverse the phenotype of ectopic dpp expression in the wing, whereas the expression of either alone has no effect. We conclude that a complex of TSG and SOG is an efficient antagonist of DPP signalling.

To determine whether Tsg is conserved among other species, we sought and found genes in the database related to Drosophila TSG in human, mouse, zebrafish and Xenopus. In addition, we found a second tsg-related sequence in Drosophila (tsg2) and obtained a second zebrafish tsg (tsg1) using degenerate polymerase chain reaction (PCR) methods. The protein products show extensive similarity with about 50% of 202 amino-acid residues matching in all four species (see http://darwin.bio.uci.edu/~marshlab/). The pairs of tsg genes in fly and fish are closer to each other than to tsg in any other species, suggesting independent gene-duplication events in these two species. We mapped the human, mouse and zebrafish (tsg1) genes by a combination of fluorescence in situ hybridization (FISH) or radiation hybrid mapping. The mouse gene maps to 17E1.3–E2, a region that is syntenic to 18p11.2–3 where the human homologue resides. In zebrafish, tsg1 is located at linkage group 24–74.5, which is syntenic to the human locus and indicates that all

Figure 2 Vertebrate Tsg enhances chordin’s antagonist function. A, Loss of zebrafish tsg1 activity ventralizes zebrafish embryos whereas ectopic tsg1 mRNA has dorsalizing activity. a, WT embryo (48 h). b, WT embryo injected with 9 ng Urd morpholino oligonucleotides. Blood cells are indicated by red staining. c–f, In situ hybridization. myoD (8 somite stage) (c); krox20 (8 somite stage) (d); GATA2 (22 somite stage) (e); andbmp4 (tail bud view, 3 somite stage) (f). Second row as above but injected with 12 ng tsg1 morpholino oligonucleotide. The filled and open arrows indicate ectopic blood islands (60%, n = 110, filled arrow; 60%, n = 100, open arrow). Caudal expression ofbmp4 is expanded at the 8 somite stage (48%; n = 25), and the blood marker GATA2 is expanded at the 22 somite stage of development (48%; n = 21). Paraxial expression of MyoD is significantly reduced at the 8 somite stage (38%; n = 33), and the anterior ectodermal marker Krox20 is moderately reduced at the 8 somite stage (49%; n = 39). The third row shows embryos after injection with zebrafish Tsg1 mRNA. At 48 h of development, Tsg1 mRNA-injected embryos display phenotypes indistinguishable from the C3–C4 class of dorsalized mutants17. Expression of myoD (50%; n = 8) and krox20 (70%; n = 10) is also significantly expanded at the 8 somite stage, while GATA2 and bmp4 are reduced. B, Enhancement of the zebrafish Tsg1 loss-of-function phenotype by sub-inhibitory loss of the chordin gene. Embryos were injected with a low dose of zebrafish Tsg1-MO, chordin-MO, or both, and assayed for blood island expansion (arrow). C, Effect of Xenopus Tsg on the ability of chordin to block BMP signalling in Xenopus. Chordin and Tsg were injected at the ratios indicated. The graph represents the combined results from five experiments. About 200 embryos were injected for each point. On the y axis 1.0 for chordin corresponds to 2–16% induction of secondary axes. The chordin mRNA concentration was 5 pg. D, Vertebrate Tsg and SOG synergistically inhibit BMP-2 signalling in Drosophila S2 cells. The experiment was carried out as in Fig. 1. The mouse protein concentrations were: bmp2, 0.2 ng; chordin, 1 μg; and Tsg, 0.5 μg.
three genes are probably functional orthologues.

The zebrafish tsg1 gene is expressed uniformly in early embryos, whereas zebrafish tsg2 is only expressed at later stages (data not shown). Hence, we focused our analysis on zebrafish tsg1 and used morpholino oligonucleotides to reduce the function of this gene in early zebrafish development. Injection of a tsg1 morpholino oligonucleotide (tsg-MO) produces a phenotype characteristic of expanded BMP signalling (Fig. 2A). Using morphological criteria and fluorescent red blood cells, we found that embryos develop expansions of the ventral fin region that correspond to ectopic blood islands (Fig. 2A, arrowheads), a tissue derived from ventral mesoderm. Injected embryos also show an expansion of GATA2, loss of paraxial mesoderm (visualized with the marker myoD), and a mild reduction of anterior ectodermal tissues (detected by staining for krox20). Caudal expression ofbmp4 is also expanded in these embryos (Fig. 2A), while the anterior ectodermal marker otx2 is reduced (data not shown). Treated embryos also exhibit an expansion in apoptotic cells ventral to the yolk extension (data not shown), similar to dino and mercedes mutants. Overall, this phenotype is very similar to that of gata2a and mercedes mutants and moderate gata2a loss-of-function mutants, and represents a modest ventralized phenotype.

Increasing the level of zebrafish tsg1 by injecting messenger RNA produced phenotypes characteristic of diminished BMP signalling including reduced axial length with loss of ventral fin (Fig. 2A), an expansion of myoD and krox20, and a reduction in GATA2. This is a phenocopy of the C3–C4 class of dorsalized mutant embryos, similar to that of the Snailhouse (BMP7 homologue) and Piggytail mutations. Furthermore, the dorsalizing effect of zebrafish Tsg1 mRNA partially reverses the ventralizing effect of tsg1 (9 ng tsg1-MO caused 47 ± 2%, n = 376, ventralized embryos; 9 ng tsg1-MO plus 30 pg Tsg1 mRNA resulted in 19 ± 8%, n = 270, ventralized embryos) suggesting that loss of tsg1 is responsible for the phenotype. We conclude that loss of tsg1 leads to embryos with a ventralized phenotype, whereas ectopic expression of tsg1 leads to a dorsalized embryonic phenotype.

As our Drosophila data suggested that one function of TSG is to co-operate with SOG to inhibit BMP signalling, we asked whether the same relationship is true in vertebrates by determining whether a moderate reduction of zebrafish chordin activity could enhance the effect of a moderate reduction in tsg1 activity. Sub-inhibitory levels of a zebrafish chordin morpholino oligonucleotide and tsg1-MO were injected into wild-type embryos, and the effect on ectopic blood island development was scored. These two morpholino oligonucleotides synergistically enhanced blood island expansion (Fig. 2B), supporting the view that both of these gene products co-operatively inhibit BMP signalling. As with the Drosophila components, we found that the combination of purified mouse chordin and Tsg was better able to inhibit mouse BMP-stimulated phosphorylation of Mad in S2 cells than either could alone (Fig. 2D).

We also tested for synergy between Tsg and chordin mRNA in Xenopus embryos by co-injecting their mRNAs and scoring for enhancement of secondary axis formation. Co-injection of Xenopus Tsg and chordin reveals a dose–response optimum. When a sub-inhibitory dose of chordin mRNA is supplemented with increasing levels of Tsg mRNA, the fraction of embryos exhibiting a secondary axis increases up to 4.5-fold over chordin alone at a 1/5 ratio of Tsg/chordin mRNA. However, if the Tsg/chordin ratio is increased to 1:1 or higher, the number of secondary axes is reduced to basal levels and the resulting tadpoles have normal morphology. Injection of 150 pg Tsg alone (the highest concentration of Tsg mRNA used in these experiments) had no effect on embryonic development. Notably, if we increase the level of Tsg relative to chordin in the S2 experiments, we do not see a reversal of the inhibition phenotype (data not shown), suggesting that additional factors probably modulate the in vivo response. Taken together, we conclude that, like Drosophila TSG, vertebrate Tsg can co-operate with chordin to inhibit BMP signalling.

As a final test of the functional equivalence of the vertebrate and invertebrate tsg genes, we expressed the human and mouse genes under the control of the UAS promoter in flies, and injected Drosophila TSG mRNA into zebrafish embryos. The phenotype of animals expressing human TSG and Drosophila sog in wing discs (Fig. 3a) resembles that of dpp shortvein alleles and is very similar to that produced by coexpression of the Drosophila sog and tsg genes (Fig. 3a; see also ref. 9). When injected into zebrafish, Drosophila tsg produces a dorsalized phenotype equivalent to that produced by zebrafish tsg1, which includes reduced axial length and expansion of krox20 (Fig. 2A; compare with Fig. 3b) and myoD (data not shown).

Our experiments, and those of others, suggest that Tsg has three molecular functions. First, it can synergistically inhibit Dpp/BMP action in both Drosophila and vertebrates by forming a tripartite complex between itself, SOG/chordin and a BMP ligand (Fig. 1B, see also refs 9, 24). Second, Tsg seems to enhance the Tld/BMP-1-mediated cleavage rate of SOG/chordin and may change the preference of site utilization (O.S. and M.B.O., unpublished observations; see also refs 9, 23). Third, Tsg can promote the dissociation of chordin cysteine-rich (CR)-containing fragments from the ligand. Different organisms may exploit each of these properties to different degrees during development depending on the relative in vivo concentrations of each molecule. We propose that in Drosophila and zebrafish the primary function of Tsg is to form a tripartite complex between itself, SOG/chordin and a BMP ligand. In Drosophila, this complex acts to redistribute a limiting amount of DPP, such that activity is elevated dorsally at the expense of being lowered laterally. The net driving force for this redistribution is likely to be diffusion of SOG from its ventral source of synthesis.
with buffer C containing 1 M NaCl, the column was eluted with buffer C containing 100 mM Tris-HCl, pH 8.0 (buffer C). After washing with buffer A containing 300 mM NaCl, the column was eluted with buffer A, Sepharose column (Pharmacia) equilibrated with 100 mM MOPS-Na, pH 6.0 (buffer A).

...His (R&D Systems) (Fig. 2D) were premixed for 3 h at room temperature (RT) with and/or 1.25 g antibody at 1/5,000 dilution 5 and anti-Flag M2 antibody (Kodak) at 1/2,000 dilution, Flag-Mad for 3 h at RT. The cells were spun down and lysed by 1X SDS-PAGE buffer. The preparation was denatured and separated by SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The membrane was probed with anti-Phospho Mad PS1 antibody at 1/5,000 dilution 1 and anti-Flag M2 antibody (Kodak) at 1/2,000 dilution, followed by incubation in secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse; Jackson Laboratory) and developed using ECL substrate (Pierce).

**Methods**

**Isolation of tsg clones and gene mapping**

Human TSG complementary DNA clones (accession numbers AW166808, AA905905, AI222228, AA468291, A100381, A1379897 and AA757878) were obtained from Research Genetics. One clone (AI018381) was sequenced in its entirety, additional 5’ sequence was obtained from published EST sequences. Mouse Tsg cDNA clone (accession number AW258143) was also obtained from Research Genetics. The zebrin tsg1 was isolated from an ephod CDNA library (E. Ekker) using two degenerate primers (5’ primer: 5'-TG/CT/GG/CA/GG/TA/GG/CT/CA/GA/TG-3’ and 3’ primer: 5’-CC/CT/CT/A/GT/CA/GA/AA/GA/GA/CA-3’). These primers amplified a 0.4-kb fragment that was used as a probe to identify a 1.2-kb cDNA from a zebrin cDNA library. We sequenced this clone using standard methods. The human TSG locus was mapped against the Stanford G3 hamster–human radiation hybrid panel using primer pairs at the beginning, middle and end of the TSG mRNA. This placed human TSG between STS markers D18 and D18 within cytogenetic band 18p11.2. The mouse Tsg was mapped by FISH using a mouse–fish hybridization panel 17.

**Altering signal peptides**

While conducting these studies, we found that the secretion signals of the mammalian and Drosophila genes are incompatible with the other species. To circumvent these secretion-related problems, we used PCR to replace the human and mouse signal sequences with the Drosophila sequence and also the Drosophila signal peptide with the zebrin sequence (details are available on request).

**Production and purification of recombinant proteins**

Recombinant proteins SOG-Myc, Tsg-His and Dpp-haemagglutinin (HA) were produced as described 18. Conditioned medium containing SOG-Myc was applied to a 1 X 10-cm 5’-Sepharose column (Pharmacia) equilibrated with 100 mM MOPS-Na, pH 6.0 (buffer A). After washing with buffer A containing 300 mM NaCl, the column was eluted with buffer A containing 750 mM NaCl, and the fractions were combined and stored for further use. Conditioned medium containing Tsg-His was applied to a 1 X 10-cm 5’-Sepharose column (Pharmacia) equilibrated with 50 mM Tris-HCl, pH 7.5 (buffer B). After washing with buffer B containing 200 mM NaCl, the column was eluted with buffer B containing 500 mM NaCl and the fractions were combined and applied to a 1 X 4-cm Ni-NTA agarose column (QIAGEN) equilibrated with 100 mM Tris-HCl, pH 8.0 (buffer C). After washing with buffer C containing 1 M NaCl, the column was eluted with buffer C containing 1 M NaCl and dialysed against 50 mM Tris-HCl, 150 mM NaCl, pH 7.4.

**Signalling assays**

Ten micrograms of Flag-tagged MAD were transfected in S2 cells at 2 X 10^5 cells per dish. After 3 days, the cells were collected and split into 20 samples. One microgram Tsg-His and/or 1.25 mg SOG-Myc (Fig. 1), or 0.5 mg mouse Tsg-protein C and/or 1 mg chordin antibody (R&D Systems) (Fig. 2D) were premixed for 3 h at room temperature (RT) with 10^7 M Dpp or 10^7 M BMP2 (R&D Systems) and then incubated with S2 cells expressing Flag-Mad for 3 h at RT. The cells were spun down and lysed by 1 X SDS–PAGE buffer. The supernatants were separated by SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The membrane was probed with anti-Phospho Mad PS1 antibody at 1/5,000 dilution 1 and anti-Flag M2 antibody (Kodak) at 1/2,000 dilution, followed by incubation in secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse; Jackson Laboratory) and developed using ECL substrate (Pierce).

**Embryo manipulations and microinjections**

Embryo manipulations were injected as described 13. For Xenopus injections, embryos were obtained by in vitro fertilization and cultured as described 14. Microinjections of mRNA were performed at the 4-cell stage in 0.3 X MMR, 3.5% Ficoll. We determined dorsal–ventral polarity of early cleavage stage embryos using pigmentation differences 15.

**in situ hybridization and antibody staining**

Hybridization to Drosophila and zebrin embryos was as described 16. The rabbit anti-Phospho Mad antibody was a gift from P. ten Dijke and used at 1/1,000 dilution. Staining was visualized using an alkaline phosphatase-coupled secondary antibody (Promega Laboratories).

Received 28 August 2000; accepted 8 January 2001.
Twisted gastrulation can function as a BMP antagonist

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Bone morphogenetic proteins (BMPs), including the fly homologue Decapentaplegic (DPP), are important regulators of early vertebrate and invertebrate dorsal-ventral development. An evolutionarily conserved BMP regulatory mechanism operates from fly to fish, frog and mouse to control the dorsal-ventral axis determination. Several secreted factors, including the BMP antagonist chordin/Short gastrulation (SOG)7–12, modulate the activity of BMPs. In Drosophila, Twisted gastrulation (TSG) is also involved in dorsal-ventral patterning13–15, yet the mechanism of its function is unclear. Here we report the characterization of the vertebrate Tsg homologues. We show that Tsg can block BMP function in Xenopus embryonic explants and inhibits several ventral markers in whole-frog embryos. Tsg binds directly to BMPs and forms a ternary complex with chordin and BMPs. Coexpression of Tsg with chordin leads to a more efficient inhibition of the BMP activity in ectodermal explants. Unlike other known BMP antagonists, however, Tsg also reduces several anterior markers at late developmental stages. Our data suggest that Tsg can function as a BMP inhibitor in Xenopus; furthermore, Tsg may have additional functions during frog embryogenesis.

We isolated human Twisted gastrulation (TSG) in a screen for Xenopus homeobox gene function. Cell 67, 1111–1120 (1991).

NATURE | VOL 410 | 22 MARCH 2001 | www.nature.com
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