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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[1]. 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], enhances the antagonistic activity of Sog/chordin. In *Drosophila*, visualization of BMP signalling using anti-phospho-Smad staining[5] shows that the *tsg* and *sog* loss-of-function phenotypes are very similar. In S2 cells and imaginal discs, TSG and SOG together make 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[4]. 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[2,6–8]. 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)[9], whereas in *tld* mutants no *rho* expression is observed (Fig. 1A, d)[8]. 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 Zerknüllt (*zen*) (data not shown). To determine whether the response of these is indicative of different threshold levels of DPP signalling[10], we used an anti-phospho-Smad antibody[5] 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-

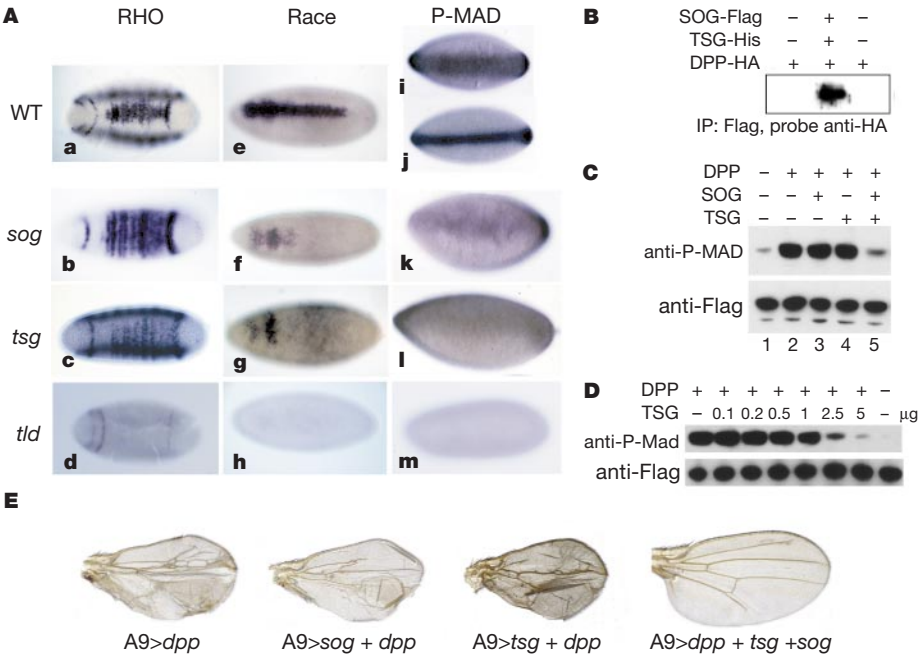

Figure 1 Tsg and SOG synergistically inhibit DPP signalling. **A**, The *tsg* loss-of-function phenotype is similar to that of *sog*. **a**–**h**, *In situ* hybridization of a Rhomboid RNA probe and a Race RNA probe to wild-type (WT), sog^{YS06}, tsg^{XB86} and tld^{B4} mutant embryos. Mutant embryos 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. **i**–**m**, Anti-phospho-MAD staining of WT (**i**, **j**), sog^{YS06} (**k**), tsg^{XB86} (**l**) and tld^{B4} (**m**) 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^{+2}, the supernatants were harvested and immunoprecipitated (IP) with anti-Flag antibody, transferred to a PVDF membrane and probed with anti-HA (12CA5 Roche) antibody as described[9]. **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[9,30] were crossed in the combinations indicated to the GAL4-A9 driver[30].

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[7,11,12] 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 amnioserosa 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*, *hnd* 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 concentration 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

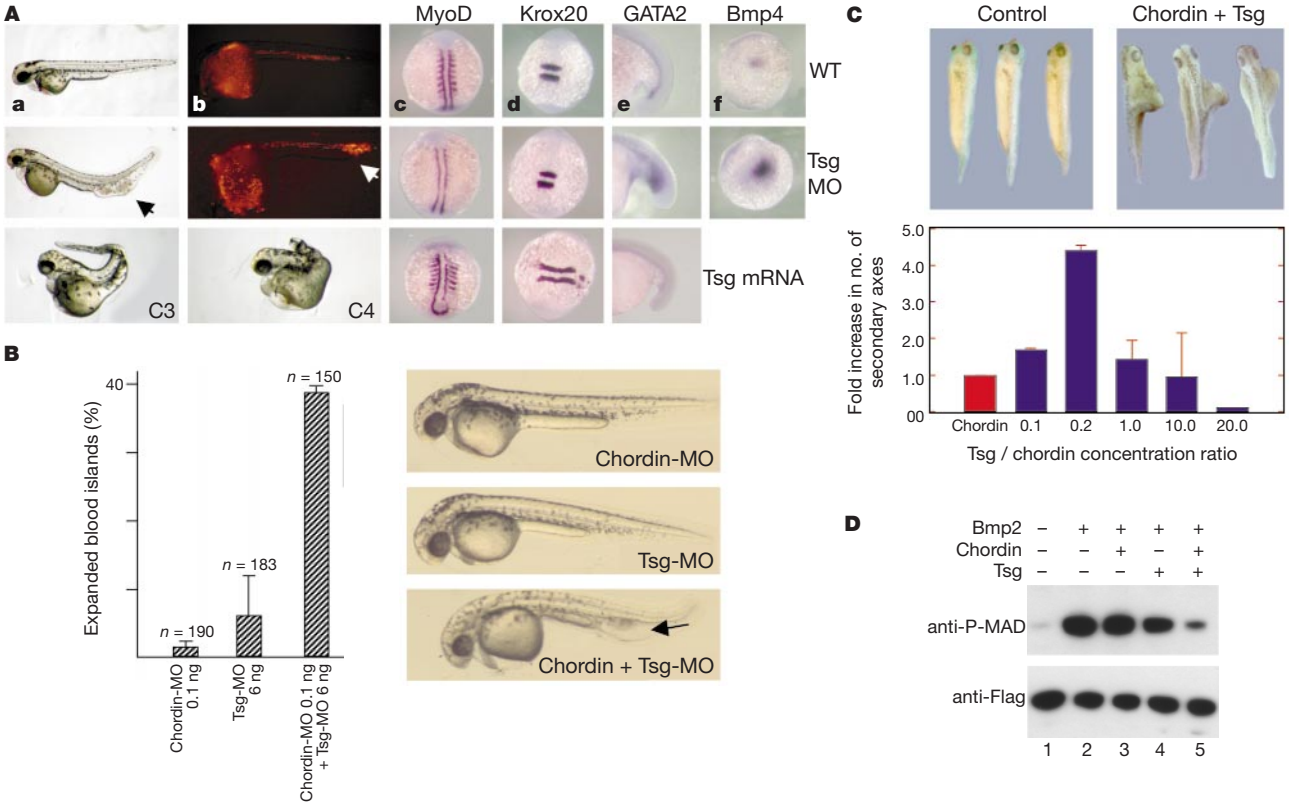

Figure 2 Vertebrate Tsg enhances chordin's antagonist function. **A**, Loss of zebrafish *tsg1* activity ventralizes zebrafish embryos whereas ectopic *tsg1* mRNA has dorsalizing activity. **a**, WT embryo (48 h). **b**, WT embryo injected with 9 ng UroD morpholino oligonucleotides. Blood cells are indicated by red staining. **c–f**, *In situ* hybridization. *myoD* (8 somite stage) (**c**); *krox20* (8 somite stage) (**d**); *GATA2* (22 somite stage) (**e**); and *bmp4* (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 of *bmp4* is expanded at the 3 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 mutants[17]. 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[13] to reduce the function of this gene in early zebrafish development. Injection of a *tsg1* morpholino oligonucleotide (*ztsg*-MO) produces a phenotype characteristic of expanded BMP signalling (Fig. 2A)[14,15]. Using morphological criteria and fluorescent red blood cells[13], 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 of *bmp4* 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[14,16]. Overall, this phenotype is very similar to that of *ogon*/*mercedes* mutants[14,15] and moderate *chordin* loss-of-function mutants, and represents a modest ventralized phenotype[13,14].

Increasing the level of zebrafish *tsg1* by injecting messenger RNA produced phenotypes characteristic of diminished BMP signalling[17–19] 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[17,19]. 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.

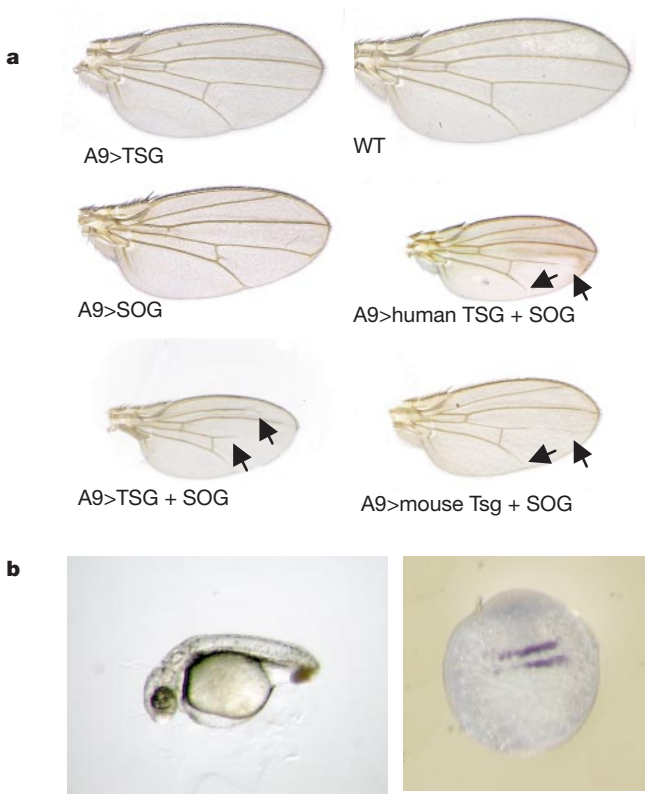

Figure 3 Functional equivalence of Tsg proteins. **a**, The GAL4-A9 driver was crossed to flies at 25 °C (first column) or 29 °C (second column) with the indicated transgenes. Arrows indicate loss of distal vein material. **b**, Injection of 30 pg *Drosophila* Tsg into zebrafish embryos results in a C3-like dorsalized phenotype (54%; n = 22) at 48 h after fertilization (left). Right, embryo injected with 30 pg *Drosophila tsg* exhibiting expansion of *krox20* at the 8 somite stage (compare with Fig. 2a).

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 modest 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[20]. 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[21] and is very similar to that produced by coexpression of the *Drosophila tsg* and *sog* 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[22–24], 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[24]. 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[25].

This is consistent with the finding that SOG diffusion is essential for activation of genes such as *race* that require high levels of DPP/SCW signalling (Fig. 1, see also ref. 26). In this model TLD would serve to modulate both the net movement of DPP and its release from the inhibitory complex by cleaving SOG[8,25]. The ability of TSG to enhance the rate of SOG cleavage may also be an important aspect of this model in that it helps ensure the proper timing of these rapid developmental events. It seems unlikely that TSG is needed to remove an inhibitory CR-containing fragment from DPP as the affinity of full-length SOG for DPP in the absence of TSG seems to be low. Likewise, in zebrafish the phenotype of reduced Tsg function is ventralized and not dorsalized as would be predicted if Tsg were primarily needed to release inhibitory CR fragments from ligand. In *Xenopus*, however, perhaps the endogenous levels of full-length chordin and CR fragments are higher than in zebrafish, thereby making the CR displacement activity of Tsg the more important biological function[24]. Determination of the *in vivo* levels of these proteins, along with a more careful analysis of the concentration optima for each type of reaction involving Tsg function, will be required before we can fully understand all of its *in vivo* activities. □

Methods

Isolation of *tsg* clones and gene mapping

Human *TSG* complementary DNA clones (accession numbers AW160804, AA905905, AI222228, AA486291, AI018381, AI379897 and AA758784) 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 zebrafish *tsg1* was isolated from an epiboly cDNA library (S. Ekker) using two degenerate primers (5′ primer: 5′-TG(CT)TG(CT)AA(AG)GA(CTAG)TG(CT)(CTA)T-3′ and 3′ primer: 5′-CC(CTG)A(CT)(AG)GA(CT)TC(AG)CA(AG)CA-3′). These primers amplified a 0.5-kilobase (kb) fragement that was used as a probe to identify a 1.2 kb cDNA from a zebrafish epiboly 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 16-kb genomic mouse *Tsg* fragment as a probe. The chromosomal assignment and band designation were determined by sequential G-banding to FISH. The zebrafish (*Danio rerio*) *tsg1* gene was mapped to linkage group 24 at 74.5 cM using a mouse–fish radiation hybrid panel[27].

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 zebrafish 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[9]. Conditioned medium containing SOG-Myc was applied to a $1 \times 10\text{-cm}^2$ S-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 1×10-cm Q-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 1×4-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 100 mM imidazole. Fractions containing Tsg-His were pooled 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 2×10^7 cells per dish. After 3 days, the cells were collected and split into 20 samples. One microgram Tsg-His and/or 1.25 μg SOG-Myc (Fig. 1C), or 0.5 μg mouse Tsg-protein C and/or 1 μg chordin-His (R&D Systems) (Fig. 2D) were premixed for 3 h at room temperature (RT) with 10^{-9} M Dpp or 10^{-11} 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 $1 \times$ 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[5] and anti-Flag M2 antibody (Kodak) at 1/2,000 dilution, followed by incubation in secondory antibody (horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse; Jackson Laboratory) and developed using ECL substrate (Pierce).

Morpholino oligonucleotides

We obtained Morpholino oligonucleotides from Gene Tools, LLC (Corvallis). We selected sequences on the basis of the design parameters recommended by the company. The zebrafish chordin morpholino oligonucleotide was as described[13]. *tsg1*-MO 5′-CTGATG ATGATGATGAAGACCCCAT-3′.

Embryo manipulations and microinjections

Morpholino oligonucleotides were injected as described[13]. For *Xenopus* injections, embryos were obtained by *in vitro* fertilization and cultured as described[28]. Microinjections of mRNA were performed at the 4-cell stage in $0.3 \times$ MMR, 3.5% Ficoll. We determined dorsal–ventral polarity of early cleavage stage embryos using pigmentation differences[28].

In situ hybridization and antibody staining

Hybridization to *Drosophila* and zebrafish embryos was as described[4,29]. The rabbit anti-phospho Mad antibody was a gift from P. ten Dijke and used at 1/2,000 dilution. Staining was visualized using an alkaline phosphatase-coupled secondary antibody (Promega Laboratories).

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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[1–6]. 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 patterning[13–15], 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 secreted factors, and mouse and *Xenopus* Tsg by low-stringency hybridization using human TSG as the probe. These vertebrate Tsgs have a high sequence homology to each other (more than 80% identical) and are about 30% identical to *Drosophila* TSG at the amino-acid level (data not shown). Tsg is expressed maternally and in all developmental stages in *Xenopus*, and at least from gastrula stages onward in mouse (data not shown). Expression of Tsg is also detected in a variety of adult tissues in both mouse and human (data not shown).

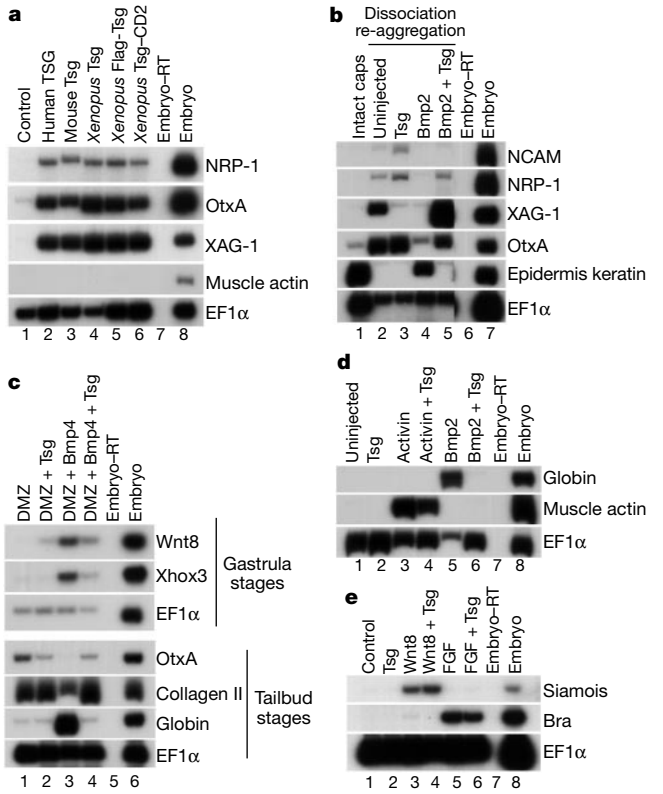

Figure 1 Vertebrate Tsgs inhibit BMP signalling in embryonic explants. **a**, Vertebrate Tsgs induce expression of cement gland and neural markers in animal caps. **b**, *Xenopus* Tsg blocks epidermal induction and neural inhibition by Bmp2 in dissociated animal-cap cells. **c**, *Xenopus* Tsg blocks ventralization of dorsal marginal-zone explants by Bmp4. **d**, *Xenopus* Tsg blocks mesodermal induction by Bmp2, but not activin. **e**, *Xenopus* Tsg does not interfere with the Wnt or FGF signalling. In animal-cap assays, RNAs were injected into animal poles of both cells of 2-cell-stage embryos. Animal caps were dissected at blastula stages (stage 9) and incubated to gastrula (stage 11, **e**) or neurula stages (stage 20, **a**, **b**, **d**). In **b**, the caps were dissociated at blastula stages for 4 h before re-aggregation and incubation to neurula stages, as described[16]. In the marginal-zone assay, RNAs were injected into the two dorsal blastomeres of 4-cell-stage embryos. Dorsal marginal-zone explants were dissected at early gastrula stage (stage 10) and incubated to mid-gastrula (stage 11) or tailbud (stage 28) stages. Xhox3, Wnt8 and globin are ventral markers, whereas OtxA and collagen II are dorsal markers. The weak induction of Wnt8 is not always observed. In **e**, the basic FGF protein (Sigma) was added at 100 ng ml^{-1} to the animal caps at the blastula stages. The amount of RNA injected into the embryos was: 2 ng, all Tsg; 0.5 ng, Bmp2; 0.5 ng, Bmp4; 5 pg, activin; and 50 pg, Wnt8.

To study the function of Tsgs, we first analysed their activities in *Xenopus* ectodermal explants (animal caps). As shown in Fig. 1a, human, mouse and *Xenopus* Tsg induce the cement gland and the neural markers XAG-1, OtxA and NRP-1 with comparable efficiency, suggesting that these vertebrate Tsgs function similarly in *Xenopus*. The induction of cement gland and neural markers in animal caps in the absence of mesoderm is normally associated with inhibition of the BMP signalling[16–18], so we therefore addressed whether Tsg could directly block the activity of BMP. We first examined the effect of Tsg on ventralization of the ectodermal cells by BMPs. As described previously[16], intact animal caps express high levels of epidermal keratin. This expression is suppressed when caps from blastula stages are dissociated for 4 h (Fig. 1b, lanes 1 and

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