Twisted gastrulation promotes BMP signaling in zebrafish dorsal-ventral axial patterning

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Summary

In vertebrates and invertebrates, the bone morphogenetic protein (BMP) signaling pathway patterns cell fates along the dorsoventral (DV) axis. In vertebrates, BMP signaling specifies ventral cell fates, whereas restriction of BMP signaling by extracellular antagonists allows specification of dorsal fates. In misexpression assays, the conserved extracellular factor Twisted gastrulation (Tsg) is reported to both promote and antagonize BMP signaling in DV patterning. To investigate the role of endogenous Tsg in early DV patterning, we performed morpholino (MO)-based knockdown studies of Tsg1 in zebrafish. We found that loss of *tsg1* results in a moderately strong dorsalization of the embryonic axis, suggesting that Tsg1 promotes ventral fates. Knockdown of *tsg1* combined with loss of function of the BMP agonist *tolloid* (*mini fin*) or heterozygosity for the ligand *bmp2b* (*swirl*) enhanced dorsalization, supporting a role for Tsg1 in specifying ventral cell fates as a BMP signaling agonist. Moreover, loss of *tsg1* partially suppressed the ventralized phenotypes of mutants of the BMP antagonists Chordin or Sizzled (Ogon). Our results support a model in which zebrafish Tsg1 promotes BMP signaling, and thus ventral cell fates, during DV axial patterning.

Key words: Twisted gastrulation, BMP signaling, Bone morphogenetic protein, Chordin, Zebrafish

Introduction

The bone morphogenetic protein (BMP) signaling pathway is essential for many developmental processes (Hogan, 1996), one of the earliest of which is to pattern cell fates along the dorsoventral (DV) axis in vertebrates and invertebrates (De Robertis and Sasai, 1996). In *Xenopus* and zebrafish, BMP signaling is required for the specification of ventral cell fates, whereas its restriction is necessary for proper specification of dorsal tissues (Holley and Ferguson, 1997). Genetic evidence implicating BMP signaling in DV patterning comes from a collection of zebrafish mutants that disrupt factors acting within a BMP signaling pathway (Hammerschmidt and Mullins, 2002). Mutations in genes encoding the ligands Bmp2b (Swirl) (Kishimoto et al., 1997; Nguyen et al., 1998) or Bmp7 (Snailhouse) (Dick et al., 2000; Schmid et al., 2000), the type I BMP receptor Alk8 (Lost-a-fin) (Bauer et al., 2001; Mintzer et al., 2001), or the intracellular effector Smad5 (Somitabun) (Kramer et al., 2002) result in the expansion of dorsal ectodermal and mesendodermal cell fates at the expense of nearly all ventral tissues.

Conversely, zebrafish mutants of the BMP antagonizing genes *chordin* (*chordino*) and *sizzled* (*ogon*) exhibit a reduction of dorsal and expansion of ventral cell types (Hammerschmidt et al., 1996). Extracellularly, Chordin, Follistatin and Noggin, which are expressed in dorsal domains, antagonize BMP signaling by directly binding and inhibiting BMP ligands, prohibiting activation of their receptors (De Robertis et al., 2000). Sizzled (Ogon) is unique as a BMP antagonist in its ventrally restricted expression pattern and its dependence on Chordin to antagonize BMP signaling (Yabe et al., 2003). Chordin is itself regulated by the Tolloid family of metalloproteases, which degrade Chordin and thus promote BMP signaling (Mullins, 1998). In zebrafish, mutations in *tolloid* [*mini fin*; *tolloid like 1* (*tll1*) – Zebrafish Information Network] result in the loss of the ventral fin fold, tissue derived from the ventral most region of the gastrula (Connors et al., 1999). This mildly dorsalized phenotype has led to a model in which zebrafish Tolloid (Mini fin) activity restricts Chordin, and thus promotes high levels of BMP signaling, at or near the end of gastrulation (Connors et al., 1999). The combined activity of these extracellular factors ensures proper patterning, and is consistent with a model in which dorsally produced BMP antagonists diffuse laterally to produce a gradient of BMP signaling (Holley and Ferguson, 1997; Thomsen, 1997), which imparts positional information and/or cell fate decisions along the DV axis.

Although the activities of BMP ligands, the extracellular antagonist Chordin and the protease Tolloid are well agreed upon, the function of the secreted factor Twisted gastrulation (Tsg) in DV patterning in vertebrates has been less clear. Tsg displays both BMP promoting and antagonizing activities in gain-of-function and biochemical assays. Overexpression of Tsg at high levels in *Xenopus* embryos causes ventralization, indicative of high levels of BMP signaling (Oelgeschlager et al., 2000; Ross et al., 2001). When expressed with Tolloid or at high levels relative to Chordin, Tsg can act as an agonist of

BMP signaling (Larrain et al., 2001; Ross et al., 2001; Oelgeschlager et al., 2003). Consistent with this, Tsg can release BMP ligands from Chordin cleavage products (Larrain et al., 2001; Oelgeschlager et al., 2000), and Tsg enhances Tolloid-mediated proteolysis of Chordin (Larrain et al., 2001; Scott et al., 2001; Shimmi and O'Connor, 2003; Yu et al., 2000). Conversely, lower levels of overexpressed Tsg antagonize BMP signaling, causing dorsalization (Chang et al., 2001; Ross et al., 2001; Scott et al., 2001). Biochemically, Tsg binds with high affinity directly to BMP ligands and enhances the binding of Chordin to BMPs (Chang et al., 2001; Larrain et al., 2001; Oelgeschlager et al., 2000; Scott et al., 2001), suggesting that Tsg inhibits BMP signaling. One model taking into account these results proposes that Tsg activity depends on the cleavage status of Chordin, and thus on the activity of Tolloid: uncleaved Chordin would elicit strong binding between Tsg and BMPs to antagonize BMP signaling, whereas Tolloid-generated Chordin fragments, which possess residual anti-BMP activity, would be released from BMPs by Tsg to promote BMP signaling (Larrain et al., 2001). This model is based largely upon overexpression experiments, leaving unanswered the question of the endogenous role of Tsg as a pro- or anti-BMP factor in DV patterning of the embryonic axis.

We performed morpholino (MO)-based loss-of-function studies of *tsg1* (*tsgb* – Zebrafish Information Network) in the zebrafish and examined genetic interactions between *tsg1* and several BMP signaling component mutants. We found that knockdown of Tsg1 results in a moderately strong dorsalization, consistent with a loss of BMP signaling. We show a genetic interaction between *tsg1* and *bmp2b* (*swirl*), supporting a role for Tsg1 as a BMP signaling agonist. We demonstrate that *mini fin* (*tolloid*) and *tsg1* cooperate in the promotion of BMP signaling. We also show that Tsg1 knockdown can partially suppress the *chordino* and *sizzled* (*ogon*) ventralized phenotypes, indicating that Tsg1 can act as a pro-BMP ventralizing factor in the absence of Chordin, as well as in the absence of Sizzled (Ogon). We propose that the predominant role of Tsg1 in DV patterning in the zebrafish is to promote BMP signaling, and that this function involves mechanisms that do not rely exclusively on the presence of Chordin or Chordin fragments.

Materials and methods

Strains and genotyping

Mutant strains used were *mini fin*^{tm124a}, *swirl*^{tc300a}, *chordino*^{tt250} and *ogon*^{tm305}. Genotyping was performed as described [*mini fin*^{tm124a} by Connors et al. (Connors et al., 1999)]; *chordino*^{tt250} by Oelgeschlager et al. (Oelgeschlager et al., 2003); *swirl*^{tc300a} by Wagner and Mullins (Wagner and Mullins, 2002), except Taq$^{\alpha}$1 restriction enzyme was used to genotype *ogon*^{tm305} using primers rk1-5′ and rk1-3′ (Yabe et al., 2003)]. Genotyping was performed on all individual embryos pictured. The results are included in the tables, except Fig. 5C-H and Fig. 6C-F, where homozygous mutant parents were used.

RACE analysis of *tsg1* maternal transcript

RT-PCR and in situ hybridization indicated that *tsg1* transcripts are found in zebrafish one cell stage embryos (not shown). 5′ RACE was performed to obtain the 5′ untranslated sequence of maternal *tsg1* mRNA using GeneRacer kit (Invitrogen), according to the manufacturer's instructions. The sequence we obtained (up to the first codon of the open reading frame) was 5′ GCATTAGTTGAGTCTG-CTCCAGTTTAGTTCTGCTCAGCTCAGAGTTCGGCGGTAAAT-GCTCTGCAGCAAAACCTCGTGAGTTCAACATCTTCATTCTC-AAGGTCCGGTGGGCTCTGTTCGTGTCGTGATG 3′.

Morpholino and mRNA injections

Morpholinos were obtained from Gene Tools, LLC. Lyophilized powder was resuspended in water, then diluted into 1×Danieau buffer (Nasevicius and Ekker, 2000) for injection of 1 nl into one-cell stage embryos. The sequence of MO1 was as described by Ross et al. (Ross et al., 2001); MO4, 5′ TAAACTGGAGCAGACTCAACTAATG 3′; MO5, 5′ CGCCGAACTCTGAGCTGAGCAGAAC 3′; a four nucleotide mismatch MO to MO1 (mismatch MO1), 5′ CTCAT-GTTGATGATGAACACCGCAT 3′; a 5 nucleotide mismatch MO to MO5 (mismatch MO5), 5′ CCCCCAACTCTCAGCTCAGCACAAC 3′.

mRNA was in vitro transcribed as described (Nguyen et al., 1998) using SP6 mMessage mMachine kits (Ambion) and injected into embryos at the 1- to 2-cell stage. *ztsg1* cDNA without its endogenous secretion signal was subcloned by PCR from zTsg1-pT3TS (a gift from M. O'Connor) into a derivative of pCS2 containing the *Xenopus* Chordin signal peptide and a FLAG epitope downstream of the signal peptide (Oelgeschläger et al., 2000). To create FLAG-tagged *ztsg1* with its endogenous secretion signal, the Chordin secretion signal was replaced with *ztsg1* sequence using standard molecular biology procedures.

tsg1 RNA was transcribed from these *Not*I linearized plasmids. *tsg2* RNA was transcribed from pCS2-ztsg (a gift from M. Oelgeschläger and E. M. De Robertis) linearized with *Not*I. All injections were performed on at least three separate occasions. For rescue experiments, MO was injected first, followed by a second injection of mRNA into a random subset of the MO-injected embryos. Embryos injected with MO alone were then compared with those that were co-injected with mRNA.

Western blot analysis

Wild-type embryos were injected with mRNA encoding FLAG-tagged *tsg1*. A subset of these embryos were subsequently injected with 32 ng MO1 or mismatch MO1. Batches of five embryos were lysed in 20 μl SDS-PAGE loading buffer (Sambrook et al., 1989), boiled for five minutes, centrifuged for five minutes and the supernatants subject to SDS-PAGE analysis on 12% gels. After transferring to PVDF, membranes were probed with 1:1600 dilution of anti-FLAG antibody (Sigma) followed by 1:3000 dilution of HRP-conjugated sheep anti-mouse antibody (Amersham Biosciences) and detection with ECL plus western blotting detection kit (Amersham Biosciences) according to the manufacturer's instructions.

Cell death assay and in situ hybridization

Labeling with digoxigenin-conjugated nucleotides by terminal deoxynucleotidyl transferase was performed on embryos fixed in 4% paraformaldehyde using ApopTag Detection Kit (Intergen) according to the manufacturer's instructions. After incubation with alkaline phosphatase-conjugated anti-digoxigenin antibody, staining was performed with 330 mg/ml NBT and 170 mg/ml BCIP (Sigma). Whole mount in situ hybridization was performed as described (Nguyen et al., 1998) with probes against *bmp4* (Chin et al., 1997), *pax2.1* (Krauss et al., 1991), *krox20* (Oxtoby and Jowett, 1993), *myod* (Weinberg et al., 1996), *eve1* (Joly et al., 1993), *gsc* (Stachel et al., 1993), *foxb1.2* (formerly *fkd3*) (Odenthal and Nüsslein-Volhard, 1998), *dlx3* (Akimenko et al., 1994), *gata2* (Detrich et al., 1995) and *chd* (Miller-Bertoglio et al., 1997). All images were taken from an MZ12.5 stereomicroscope (Leica) with a ColorSNAP-cf digital camera (Photometrics) and processed using Adobe Photoshop.

Results

No alterations in DV patterning by low level Tsg1 knockdown

To study the function of Tsg1 in early development, we injected an antisense MO oligonucleotide to inhibit Tsg1 translation. This MO, designated MO1, is complementary to the first 25 nucleotides of the *tsg1* open reading frame and of identical sequence to a MO used previously (Ross et al., 2001). Injection of 8 ng of MO1 into wild-type embryos resulted in the formation of an edema in the ventral tail vein in 61% of embryos (Fig. 1A,B; Table 1). In these embryos, the tail vein is dilated and blood pools within it. This phenotype is first visible at about 28 hours post fertilization (hpf) as a mild edema, which becomes prominent by 48 hpf (Fig. 1A,B; Table 1). A less severe phenotype was evident in 24% of the embryos, as decreased blood flow through the ventral tail vein (not shown). In all MO-injected embryos, we observed a reduction in anterior tissues at 48 hpf (compare bars in Fig. 1A,B). Surprisingly, the morphants did not display duplications of ventral tail fin tissue, which are typical of ventralized zebrafish mutants (see Figs 5, 6). These defects are similar to those seen in a previous study (Ross et al., 2001), where it was concluded that *tsg1* morphants resemble weakly ventralized zebrafish mutants.

To determine if patterning was affected in MO1-injected embryos, we examined gene expression markers of dorsally and ventrally derived tissues. During early somitogenesis stages, we did not observe changes in the expression of *pax2.1* or *krox20*, which mark dorsal ectodermal gastrula derivatives of the midbrain-hindbrain boundary and rhombomeres 3 and 5, respectively (Fig. 1G,K), or the extent of *myoD* expression to indicate dorsal mesoderm (Fig. 1H,L). Neither did we observe alterations in ventrally derived tissue, as revealed by *pax2.1* expression in the pronephric system (Fig. 1H,L) or *bmp4* ventral tail bud expression (Fig. 1J,N). Additionally, the most anterior expression domains of *bmp4* and *pax2.1* were not altered (asterisks in Fig. 1I,J,M,N). Based on these results, we conclude that 8 ng of MO1 does not induce significant alterations in DV patterning. Thus, our results are incongruent with those of Ross et al. (Ross et al., 2001), who reported a reduction in dorsal markers and an expansion in ventral markers during somitogenesis stages. We subsequently injected doses of 12, 15, and 16 ng of MO1. Although we saw increased penetrance and severity of the vein phenotype, we did not observe alterations in the expression domains of *pax2.1*, *krox20*, *myoD*, *bmp4* or *gata2* with these amounts of MO (not shown).

As a change in early patterning could not account for the reduction of anterior structures at 48 hpf, we investigated if the defects were specific or non-specific effects of MO1. To test this, we co-injected in vitro transcribed *tsg1* or *tsg2* mRNA, which lack sequence complementary to MO1, and assayed for rescue of the defects. We found that either mRNA could rescue the vein edema in a large fraction of embryos (Fig. 1C, Table 1). However, we did not detect rescue of the anterior structures by co-injection of *tsg* mRNA (Fig. 1C). Examination of live embryos revealed a high degree of cell death that became prominent at 24 hpf, a common nonspecific effect of MOs (Heasman, 2002; Nasevicius and Ekker, 2000). To determine if the decrease in anterior structures could be attributed to cell death, we used a TUNEL-based assay to label the nuclei of dying cells. We observed a substantial increase in cell death in anterior CNS tissues of morphants (Fig. 1D,E), which could not be rescued by RNA co-injection (Fig. 1F). We attribute the reduction in anterior tissues at 48 hpf to nonspecific, MO-induced cell death.

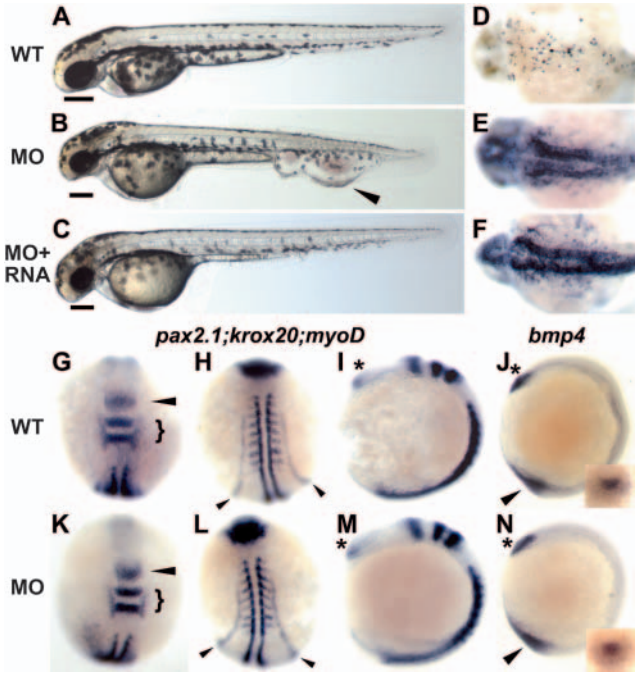

Fig. 1. Low-level *tsg1* knockdown causes a ventral tail vein edema and cell death in anterior structures, but does not alter patterning during early somitogenesis. Uninjected embryo (A); a wild-type embryo injected with 8 ng MO1 (B) displays a slightly reduced head and a large edema in, or dilation of, the ventral tail vein (arrowhead), in which the blood accumulates and circulation slows. (C) The vein edema can be rescued by injection of *tsg1* mRNA (10% with edema phenotype, *n*=133, not shown) or *tsg2* mRNA (8% with edema phenotype, *n*=106), whereas the reduction in head size is unaltered. Scale bars in A-C show the diameter of the eye as a measure of the extent of anterior tissues. TUNEL analysis as a measure of cell death in 24 hours post fertilization (hpf) uninjected wild-type (D) and 8 ng MO1-injected sibling (E) embryos. Cell death is not rescued by co-injection of *tsg* mRNA (F). (D-F) Anterior towards the left, dorsal view posterior to the MHB boundary. (G-N) Whole-mount in situ hybridization of uninjected embryos (G-J) or embryos injected with 8 ng MO1 (K-N). (G,K) Expression of *pax2.1* (arrowhead) and *krox20* (bracket) in the mid/hindbrain (MHB) boundary and rhombomeres 3 and 5, respectively. (H,L) More posterior views of the embryos in G and K. *pax2.1* expression in the pronephric system (arrowheads) and *myoD* in anterior somitic mesoderm is unchanged. (I,M) Lateral views of untreated or injected embryos showing no alterations in anterior expression of *pax2.1* in the eye field (asterisk). (J,N) Expression of *bmp4* anteriorly (asterisk) and in the tail bud (arrowhead) at the three-somite stage is normal in injected embryos. Insets show tail bud views. (G-I,K-M) Eight somites. (J,N) Three somites. (G,H,K,L) Dorsal views with anterior upwards. (I,J,M,N) Lateral views, anterior is upwards.

Strong knockdown of Tsg1 results in dorsalization

We investigated whether the 8-16 ng *tsg1* knockdown may be an incomplete loss of *tsg1* function by examining the effects of injecting increasing amounts of MO1. We found that 32 ng

Table 1. Vein edema phenotype and RNA rescue

	Edema	Vein disruption*	Wild type
MO† only (*n*=1603)	61%	24%	15%
MO+*tsg1* RNA‡ (*n*=133)	10%	58%	34%
MO+*tsg2* RNA§ (*n*=106)	8%	23%	67%

*This class of embryos displayed decreased or aberrant circulation in the ventral tail vein, but not the edema pictured in Fig. 1B.
†8 ng of MO1 injected per embryo.
‡1 pg *tsg1* mRNA.
§10 pg *tsg2* mRNA.

Table 2. Dorsalization phenotype of *tsg1* knockdown and RNA rescue

	Class 4*	Class 3†	Wild type
MO1‡ only (*n*=1110)	20%	47%	33%
MO4§ only (*n*=414)	47%	23%	30%
MO5¶ only (*n*=1150)	38%	48%	13%
MO5¶+*tsg1* RNA** (*n*=243)	11%	29%	60%
MO5¶+*bmp2b* RNA†† (*n*=68)	10%	11%	76%
MO5¶+*smad5* RNA‡‡ (*n*=57)	2%	3%	86%

*Similar to embryos in Fig. 2O,Q.
†Similar to embryos in Fig. 2N,P.
‡32 ng MO1 per embryo.
§32 ng MO4 per embryo.
¶25 ng MO5 per embryo.
**2pg *tsg1* RNA per embryo.
††12 pg *bmp2b* RNA per embryo.
‡‡20 pg *smad5* RNA per embryo.

of MO1 appeared to dorsalize the embryo moderately to moderately strong, similar to class 3 (C3) and class 4 (C4) dorsalizations (Table 2; Fig. 2K,O, not shown) (Mullins et al., 1996). At no dose did we observe defects consistent with a ventralization. To verify the specificity of MO1, we designed two additional MOs, MO4 and MO5, targeted against non-overlapping sequences in the 5′ untranslated region. We found that injection of 32 ng of MO4 or 25 ng of MO5 induced a moderately strong dorsalization (Fig. 2; Table 2), similar to that of MO1.

We assayed patterning during gastrulation and early somitogenesis to determine if the presumptive dorsalization reflects a change in pattern formation. During mid-gastrula stages, we found that morphants displayed more restricted expression domains of the ventral markers *eve1, dlx3,* and *gata2* (Fig. 2A-C,F-H). Concomitantly, the expression of *foxb1.2* and *chordin,* which are normally dorsally restricted, were expanded into lateral domains (Fig. 2D,E,I,J), consistent with an alteration in DV patterning during the gastrula period when BMP signaling is thought to act. During early somitogenesis, we examined injected embryos for expression of *pax2.1*, *krox20* and *myoD*, and found that all expression domains of dorsally derived tissues were expanded (Fig. 2L,M,P,Q), indicating a dorsalized phenotype. As controls for the specificity of the dorsalized appearance, we injected up to 60 ng of two different mismatch MOs, which did not cause dorsalization (not shown). To test for the ability of the MOs to block translation, we injected 32 ng of MO1 or its four base mismatch control into embryos that were co-injected with a FLAG-tagged *tsg1* mRNA containing the MO1 binding site. We found that MO1 blocked translation of *tsg1*, whereas the

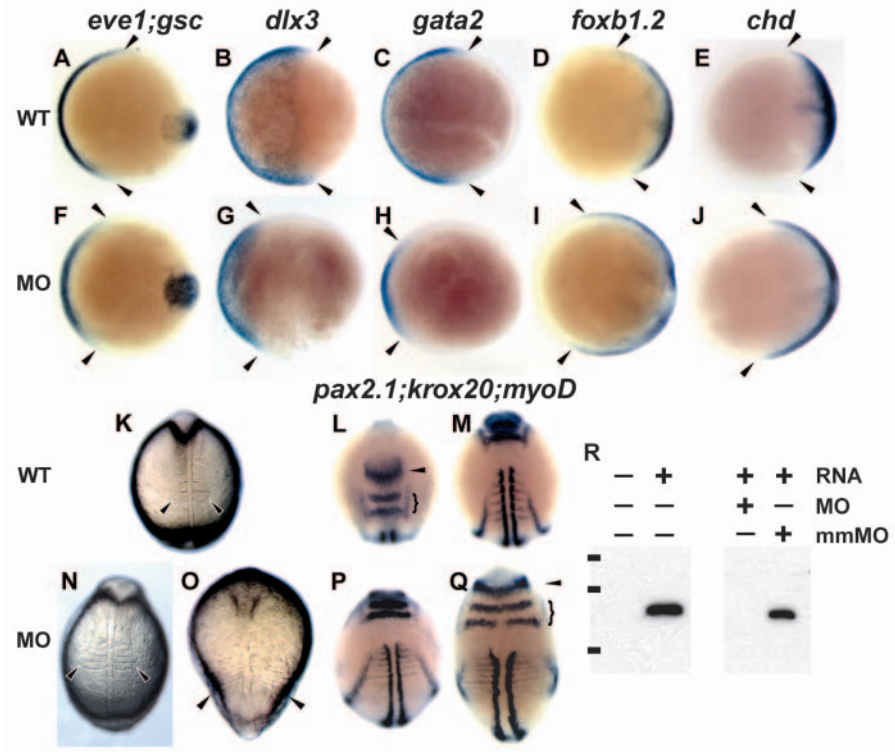

Fig. 2. High level *tsg1* knockdown dorsalizes the embryo. Uninjected embryos (A-E,K-M) compared with siblings injected with 25 ng MO5 (F-J,N,P,Q) or 32 ng MO1 (O). (A-J) Whole-mount in situ hybridization at 80% epiboly (mid-gastrulation); animal pole views, dorsal towards the right. Reduced ventral domain of *eve1* (*n*=32/54), *dlx3* (*n*=25/41) and *gata2* (*n*=28/50) expression (A-C,F-H) and expanded *foxb1.2* (*n*=34/46) and *chordin* (*chd*) (*n*=16/30) expression in dorsal regions (D,E,I,J) in injected compared with uninjected embryos (delineated by arrowheads) was observed. *gsc* expression in the dorsal midline prechordal plate mesoderm is not affected (A,F), similar to results in other dorsalized BMP signaling pathway mutants in zebrafish (Mullins et al., 1996; Nguyen et al., 1998). (K-Q) During somitogenesis, injected embryos display dorsalized characteristics similar to class 3 (N) and class 4 (O) dorsalized mutants, as assessed by the lateral extent of somites in live embryos (arrowheads in K,N,O), by whole-mount in situ hybridization with *myod* in anterior somitic mesoderm (M,P,Q), and by expansion of *pax2.1* (arrowhead) and *krox20* (bracket) expression in the MHB and rhombomeres 3 and 5, respectively (L,Q). The embryos in N,P display a class 3 or moderate dorsalization, whereas those in O,Q exhibit a greater expansion of the somitic mesoderm and neural tissue, similar to a class 4 dorsalization. (L) Dorsoanterior view; (M) a more posterior view of the same uninjected embryo. (R) Anti-FLAG western blot on lysates of embryos injected with FLAG-tagged *tsg1* alone or with 32 ng MO1 or mismatch MO1. Bars to the left of the blot represent the positions of protein standards with molecular weights of 50, 37 and 25 kDa.

Table 3. Dorsalization of *bmp2b* (*swirl*) heterozygotes by sub-dorsalizing *tsg1* knockdown

	C4	C3	C2	C1	Not dorsalized*
Uninjected siblings (*swirl*/+ and +/+; *n*=175)	0	0	0	0	100%
swirl/+ (*n*=143)†	21%	28%	36%	13%	2%
+/+ (*n*=152)†	1%	1%	2%	6%	90%

*Uninjected embryos were wild type. Injected embryos were not dorsalized, but most exhibited the tail vein edema phenotype.

†Embryos from *swirl* (*bmp2b*) heterozygotes crossed to wild-type fish were injected with 16 ng MO1, classified morphologically at 1 dpf and then genotyped.

mismatch control did not (Fig. 2R). Furthermore, we could partially rescue the MO-induced dorsalization by co-injection of 2 pg of *tsg1* mRNA (Table 2). Taken together, these results demonstrate that a strong knockdown of Tsg1 function by high levels of *tsg1* MO induces specific dorsalization phenotypes, strongly suggesting that the in vivo role of Tsg1 in zebrafish is to promote the specification of ventral cell fates.

Tsg genetically interacts with Bmp2b

To examine if Tsg1 promotes ventral cell fates by promoting BMP signaling, we tested whether *tsg1* interacts genetically with known BMP pathway component mutants. We injected a sub-dorsalizing amount of *tsg1* MO1 (16 ng) into embryos from a cross between a *swirl* (*bmp2b*) heterozygote and a wild type fish. In contrast to the uninjected, phenotypically wild type *swirl* (*bmp2b*) heterozygotes, greater than 95% of the *tsg1* MO-injected heterozygotes displayed dorsalized phenotypes at 24 hpf, ranging from weak (class 1, 13%; class 2, 36%), moderate (class 3, 28%) to strong (class 4, 21%) (Fig. 3B-D; Table 3). Importantly, 90% of injected wild-type sibling embryos were not dorsalized (Fig. 3A and Table 3). Dorsalization was also evident during somitogenesis by an expansion of dorsally expressed markers in the MO1-injected *swirl* heterozygotes (Fig. 3E-J). Furthermore, we found that 12 pg of *bmp2b* mRNA or 20 pg of mRNA encoding the intracellular BMP effector Smad5 could rescue the *tsg1* MO5 dorsalization (Table 2). Our ability to rescue Tsg loss-of-function with multiple components of a BMP signaling pathway, as well as the genetic interaction between loss of *swirl* (*bmp2b*) and *tsg1* function indicates that Tsg1 functions endogenously to promote BMP signaling in dorsoventral patterning.

Tsg cooperates with *tolloid* (*mini fin*) to promote BMP signaling

As discussed above, previous work in *Xenopus* suggests that the pro-BMP activity of Tsg depends on the ability of Tolloid to cleave Chordin into fragments (Larrain et al., 2001). These fragments contain residual anti-BMP activity, which Tsg can inactivate. To address whether Mini Fin (Mfn) (Tolloid) is required for Tsg1 function, we injected 12.5 ng of MO5 into the progeny of a cross of *mfn*^{tm124a} heterozygous adults, which carry a presumptive null mutation in *tolloid* (Connors et al., 1999). The *mfn* mutation produces a weakly dorsalized (class 1) phenotype, which is characterized by loss of ventral fin fold tissue (Fig. 4A,B) (Connors et al., 1999). Whereas most (94%, Table 4) *tsg1* MO5-injected wild-type or heterozygous siblings were not dorsalized, a large majority of *mfn* homozygous mutants (76%, Table 4) exhibited a significantly stronger dorsalized phenotype (class 3, Fig. 4C,E) than did uninjected

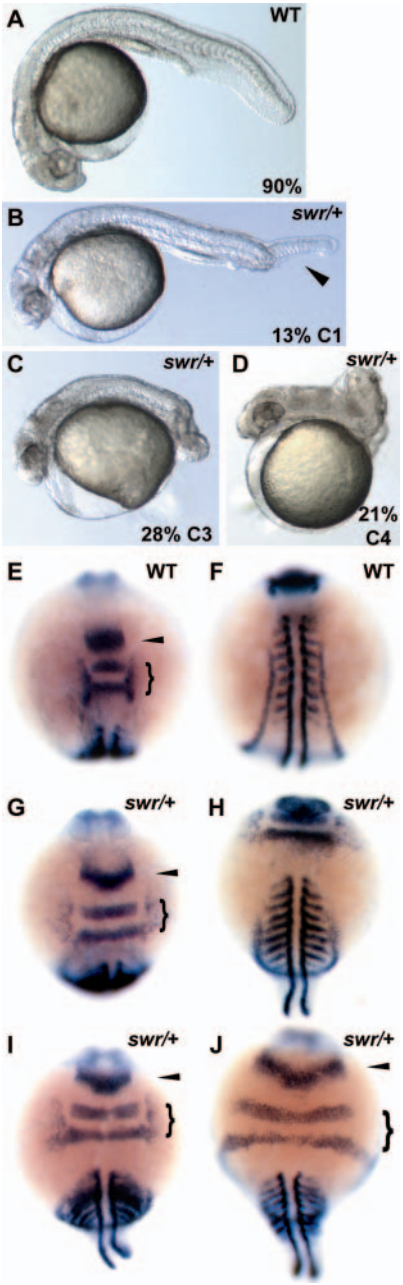

Fig. 3. Interaction between sub-dorsalizing *tsg1* knockdown and *swirl* (*bmp2b*) heterozygotes. All embryos were injected with 16 ng *tsg1* MO. At 24 hpf, an injected wild-type embryo was not dorsalized (A), whereas injected *swirl* (*bmp2b*) heterozygous siblings were dorsalized. The extent of dorsalization was categorized as follows: (B) weak, class 1 (arrowhead indicates partial loss of the ventral tail fin); (C) moderate, class 3; and (D) strong, class 4. (E-J) Whole-mount in situ hybridization of *myod*, *pax2a* and *krox20* in 16 ng *tsg1* MO1 injected embryos at the eight-somite stage. Injected wild-type embryos were not affected (E,F), whereas heterozygotes displayed a range of dorsalizations (G,H, moderate; I, strong; J, stronger). (F,H) More posterior views of the same embryos shown in E,G, respectively. (J) The somites extend around the circumference, which is typical of a strong, class 4 dorsalization.

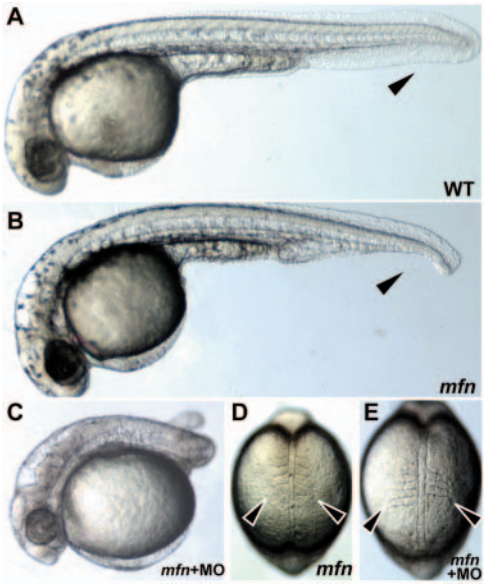

Fig. 4. Knockdown of Tsg function enhances dorsalization in *mini fin* (*tolloid*) mutants. (A) Wild-type and (B) *mfn* homozygous mutant embryo at 1 dpf. Arrowheads indicate normal and deficient ventral tail tissue. Injection of 12.5 ng MO5 into *mfn/mfn* embryos results in a moderate class 3 dorsalization (C). The increase in phenotypic strength is seen at the four-somite stage with the lateral expansion of somites in injected (E) *mfn* homozygotes (arrowheads) compared with uninjected (D).

Table 4. Enhancement of *mfn/mfn* dorsalization by sub-dorsalizing *tsg1* knockdown

	C3	C1	Wild type
Uninjected (*n*=131)*	0%	21%	79%
Injected *mfn/mfn* (*n*=113)†	76%	24%	0%
Injected *mfn*/+ or +/+ (*n*=239)†	5%	1%	94%

*Uninjected embryos from intercrosses of *mfn* heterozygotes. Only the *mfn* homozygotes displayed a C1 phenotype, as confirmed by genotypic analysis.
†12.5 ng MO5 injected per embryo from crosses of two *mfn* heterozygotes. Following morphological classification, all embryos were genotyped.

mfn mutants (Fig. 4B,D). These results indicate that Tsg1 ventralizing activity does not depend on the product of the *mfn* gene or the ability of Mfn to generate Chordin fragments. Additionally, this result uncovers a previously undetected role for Mfn/Tolloid in patterning dorsoanterior tissues, which remain unaltered in *mfn* mutants (Connors et al., 1999; Mullins et al., 1996), but are expanded by injection of *tsg1* MO at amounts insufficient to alter wild-type siblings.

Tsg does not require Chordin or Sizzled (Ogon) to function as a ventralizing factor

We tested the genetic interaction between Chordin and Tsg1 loss of function. In *Xenopus*, low levels of Tsg overexpressed simultaneously with Chordin lead to additive dorsalization (Chang et al., 2001; Ross et al., 2001; Scott et al., 2001), whereas higher levels of Tsg can release BMP from Chordin fragments and promote ventralization (Oelgeschlager et al., 2000; Ross et al., 2001). Thus, Tsg might function exclusively through Chordin, and consequently the *chordino* (*chordin*) mutation may be epistatic to Tsg loss of function. A zebrafish *chordino* null mutation causes a moderate ventralized phenotype, including a reduction in dorsoanterior neural and somitic mesodermal tissue, expansion of the ventrally derived blood and duplications in the ventral fin fold (Fig. 5A) (Hammerschmidt et al., 1996). The expected protein product of this mutant *chordin* allele lacks all its BMP-binding domains (Fisher and Halpern, 1999; Schulte-Merker et al., 1997). We

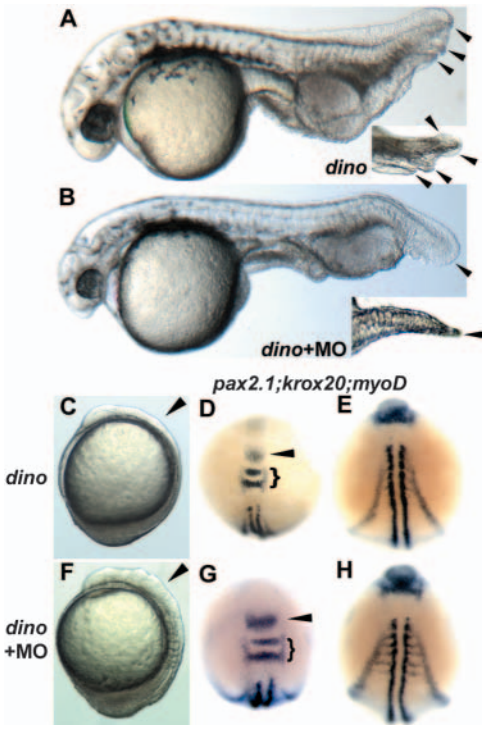

Fig. 5. Knockdown of Tsg function partially suppresses the *chordino* ventralization. (A) Uninjected *chordino* homozygote; arrowheads indicate multiple ventral fin folds, also seen in a slightly different view at higher magnification (inset). Appearance of multiple fin folds is suppressed by injection of 8 ng MO1 (B, inset shows dorsal view of tail of same embryo). (C-H) Injection of 25 ng MO5 into *chordino* homozygotes results in a substantial enlargement of dorsally derived tissues. Examination of live embryos at the six-somite stage showed reduced head neural tissue in C and suppression of the reduction in F (arrowheads). (C) Uninjected *chordino* homozygote; (F) injected *chordino* homozygote; lateral views, anterior is upwards. In situ analysis at the same stage with *pax2.1* (arrowhead), *krox20* (bracket) (D,G) and *myod* (E,H) showed that the somites, the MHB and rhombomeres 3 and 5 are increased in size in the injected (*n*=14/20) compared with uninjected mutants. (D,E) Uninjected mutants; (G,H) two views of the same injected mutant. In the injected mutant, the anterior neural and somitic mesoderm appears similar to wild type; however, the tail bud is still enlarged, indicating that the *dino* phenotype is not fully suppressed.

Table 5. Suppression of *dino* and *ogon* ventral fin fold duplication by sub-dorsalizing *tsg1* knockdown

	Fin fold duplication	Single fin fold
dino (*n*=180)	88%	12%
dino+MO1* (*n*=298)	13%	87%
ogon (*n*=149)	97%	3%
ogon+MO1* (*n*=155)	4%	96%

*8 ng MO1 per embryo.

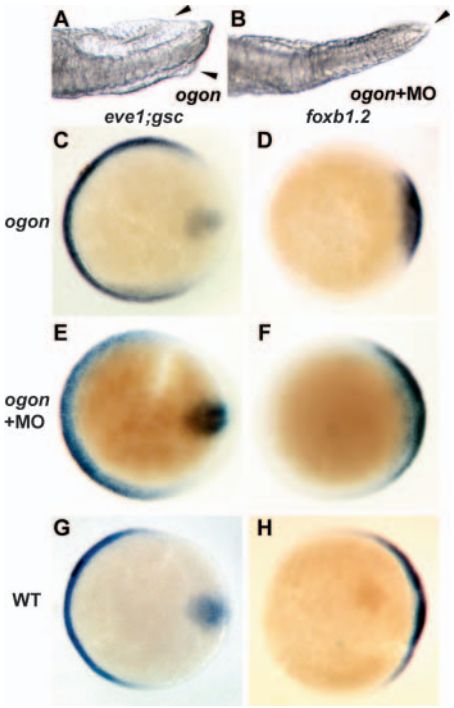

Fig. 6. Tsg knockdown partially suppresses the *sizzled* (*ogon*) ventralized phenotype. (A) Dorsal view of the tail of an *ogon* mutant at 24 hpf showing duplication of the ventral fin fold (arrowheads). (B) The fin fold duplication is suppressed by 8 ng MO1. (C-H) Whole-mount in situ hybridization at 80% epiboly (animal pole views, dorsal towards the right) shows ventral marker *eve1* expanded in uninjected mutants (C), whereas the dorsal marker *foxb1.2* is reduced (D). Expression of these markers in mutants injected with 25 ng MO5 in E (n=16/22) and F (n=15/20) is similar to that seen in wild type (G,H).

Table 6. *tsg1* overexpression

	C3	C2	C1	Wild type	Ventralized*
Wild type (n=198)†	8%	50%	18%	24%	0
+/+ or *dino*/+ (n=156)‡	0	27%	30%	43%	0
dino/*dino* (n=77)‡	0	18%	16%	5%	61%
ogon/*ogon* (n=583)§	7%	13%	53%	11%	16%

*Ventralized phenotype similar to uninjected mutant siblings except for partial loss of ventral fin fold tissue (see text).
†100-200 pg *tsg1* RNA injected per embryo from wild-type crosses.
‡75-150 pg *tsg1* RNA injected per embryo from crosses between *dino* heterozygotes.
§75-200 pg *tsg1* RNA injected per embryo from crosses between *ogon* homozygotes.

found that injection of 8 ng of MO1 into *chordino* mutants could suppress duplications of the ventral tail fin fold (Fig. 5B; Table 5), not supporting a mechanism in which *chordino* is epistatic to *tsg1*. Additionally, *chordino* null mutants injected with 25 ng of MO5 displayed more prominent dorsoanterior neural and somitic mesodermal tissue during early somitogenesis stages, compared with uninjected mutant siblings (Fig. 5C-H), indicating a partial suppression of these ventralized defects as well (compare with wild-type embryos; Fig. 2L,M). Thus, the endogenous ventralizing activity of Tsg1 does not rely exclusively on the presence of Chordin or Chordin fragments.

Similarly, we tested whether Tsg depends on Sizzled (Ogon) activity. At 24 hpf, *ogon* mutants display a mild ventralization, which includes multiple ventral fin folds and expanded blood precursors (Fig. 6A) (Hammerschmidt et al., 1996; Miller-Bertoglio et al., 1999; Solnica-Krezel et al., 1996). When injected with 8 ng MO1, however, *ogon* mutants displayed a single ventral fin fold, similar to the suppression seen in *chordino* mutants (Fig. 6B; Table 5). Additionally, injection of 20 ng of MO5 into *ogon* mutants suppressed the expansion of a ventral marker (*eve1*, Fig. 6C,E) and the restriction of a dorsal marker (*foxb1.2*, Fig. 6D,F) during gastrulation. These results indicate that Tsg1 can promote BMP signaling when the BMP antagonistic function of Ogon is deficient. Our observations that *tsg1* MO can partially suppress both the *chordino* and *ogon* mutant phenotypes is consistent with the dependence of Sizzled (Ogon) dorsalizing activity on the function of Chordin (Yabe et al., 2003).

tsg1 overexpression dorsalizes wild type, and suppresses *dino* and *ogon*

Surprisingly, like the *tsg1* knockdown phenotype, Tsg overexpression also dorsalizes the zebrafish embryo (see also Ross et al., 2001). We injected various amounts of *tsg1* mRNA into wild-type embryos and monitored DV patterning by in situ hybridization with *pax2.1*, *krox20* and *myoD* at the eight-somite stage. We found that 100-200 pg of *tsg1* mRNA caused a moderate, lateral expansion of all three markers (Fig. 7A-E), and mild to moderate dorsalized phenotypes at 1 day post fertilization (dpf) (Table 6; Fig. 7F-I). About half of the mildly dorsalized embryos (class 1 or class 2, Fig. 7G,H) also displayed small duplications in the fin fold (Fig. 7H inset), similar to overexpression of *Xenopus* Tsg mRNA in zebrafish (Oelgeschlager et al., 2003) and some weakly dorsalized mutants (Kramer et al., 2002). Higher amounts of *tsg1* mRNA caused more severely dorsalized phenotypes (not shown). Lower amounts had negligible effects. At no dose did we observe ventralized phenotypes.

We also injected *tsg1* mRNA into ventralized mutant embryos. We intercrossed *chordino* (*dino*) heterozygotes, injected their progeny with 75-150 pg *tsg1* mRNA, then scored and genotyped the embryos. The majority of homozygotes retained a ventralized phenotype (Table 6) and could be readily distinguished from wild-type and heterozygous siblings. Frequently, this class displayed a partial loss of proximal ventral fin fold tissue, but maintained multiple distal fin duplications (not shown), a phenotype seen in *dino*$^{-/-}$; *swirl*$^{+/-}$ mutants and in a small fraction of *dino* mutants (Wagner and Mullins, 2002; Hammerschmidt and Mullins, 2002). In addition, 39% of *dino* mutants exhibited either a partial or complete suppression of the ventralization or a dorsalized phenotype. Thirty-four percent of homozygous mutants displayed weakly dorsalized phenotypes, indistinguishable from those seen in wild-type or heterozygous injected siblings. Additionally, a small fraction (5%) of *dino* mutants were rescued to a nearly wild-type phenotype (Table 6), showing that overexpressed Tsg can suppress the absence of Chordin, consistent with overexpressed Tsg antagonizing BMP signaling. Similar results were seen with *sizzled* (*ogon*) mutants injected with 75-200 pg of *tsg1* mRNA (Table 6), which normally display a weakly ventralized phenotype.

Overall, these experiments show that overexpressed Tsg can

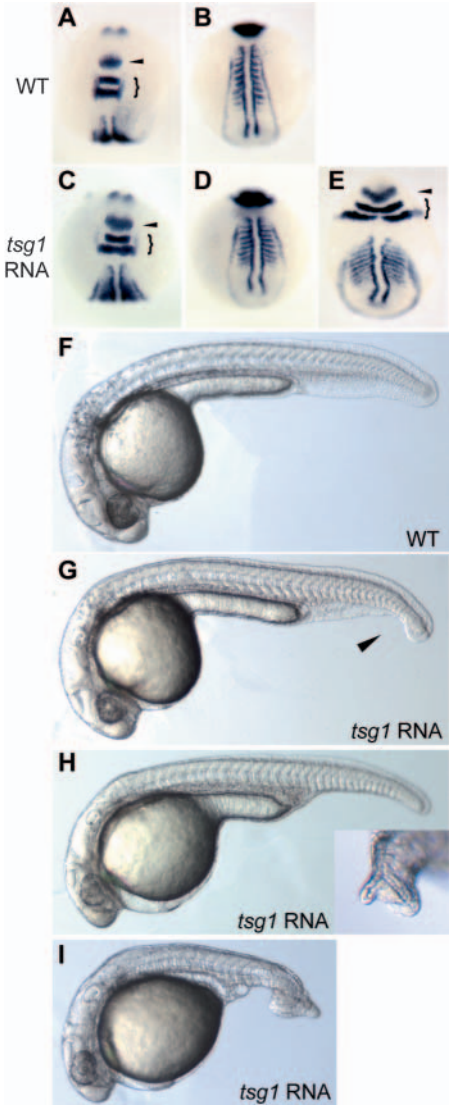

Fig. 7. Overexpression of *tsg1* RNA causes dorsalization. (A-E) Whole-mount in situ hybridization with *pax2.1* (arrowhead), *krox20* (brackets) and *myod* reveals expansion of all three markers in embryos injected with 200 pg *tsg1* RNA (C-E) relative to wild type (A,B). (B,D) More posterior views of embryos shown in A and C, respectively. (C,D) Mildly dorsalized and (E) moderately dorsalized embryos. (F-I) At 1 dpf, embryos injected with 100 pg *tsg1* RNA show a range of dorsalized phenotypes. (F) Uninjected sibling. (G) Class 1 dorsalization, arrowhead indicates gap in ventral tail fin. (H) Class 2 dorsalization, inset shows duplication in fin fold tissue seen in 50% of class 1 and class 2 embryos. (I) Class 3 phenotype.

antagonize ventral cell fate specification in wild-type and mutant backgrounds. However, our loss-of-function results indicate that endogenous Tsg1 promotes ventral cell fate specification. It is possible that Tsg1 acts as a crucial component of a BMP-promoting complex that cannot form in either the absence of Tsg1 or the presence of excess Tsg (see Discussion).

Discussion

Here, we demonstrate that a strong knockdown of Tsg1

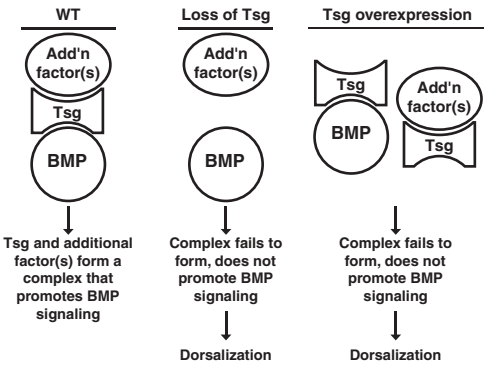

Fig. 8. Model of Tsg function.

dorsalizes the zebrafish embryo, indicating that in zebrafish Tsg1 functions predominantly as a ventralizing factor in DV patterning. We demonstrate a genetic interaction between *tsg* and *bmp2b* (*swirl*), consistent with Tsg promoting BMP signaling. Sub-dorsalizing knockdown of *tsg1* increases the severity of the *tolloid* (*mini fin*) mutant, suggesting that Tsg1 and Mini fin cooperate to pattern anterior tissue, a function not previously attributed to Mini fin (Tolloid). Furthermore, we show that Tsg functions as a ventralizing factor, in part, independently of the dorsalizing factors Chordin and Sizzled (Ogon). In contrast to the strong dorsalizations observed in null *bmp2b* (*swirl*), *bmp7* (*snailhouse*), MZ-*alk8* (*lost-a-fin*) and MZ-*smad5* (*somitabun*) mutants (Bauer et al., 2001; Kramer et al., 2002; Mintzer et al., 2001; Schmid et al., 2000), we found that the strongest *tsg1* knockdown phenotype is a moderately strong dorsalization of the embryo. As we do not know if Tsg1 function is completely knocked down, the moderately strong dorsalization may reflect the complete or partial role of Tsg1 in DV patterning and BMP signal modulation. Nevertheless, our results demonstrate a clear role in the zebrafish for Tsg1 as an agonist in BMP signaling during DV pattern formation.

A previous model of Tsg function suggests that full-length Chordin causes Tsg to act as a BMP antagonist, whereas the presence of Chordin fragments allows Tsg to exhibit pro-BMP behavior (Larrain et al., 2001). Thus, the activity of Tsg may depend on the cleavage status of Chordin. However, the partial suppression of the *chordin* mutant phenotype by Tsg1 knockdown indicates that endogenous Tsg1 acts as a ventralizing factor in a manner that does not rely entirely on Chordin. These results are consistent with the finding that an overexpressed mutant Tsg that cannot bind BMPs reduces ventral tissues to a greater extent than loss of Chordin alone (Oelgeschlager et al., 2003). Thus, Tsg1 may promote BMP signaling in part by inactivating BMP antagonists in addition to Chordin or Chordin fragments.

However, as *tsg1* knockdown only partially suppresses the *dino* ventralized phenotype and *tsg1* is not epistatic to *dino*, Tsg1 probably also inactivates Chordin in conjunction with Tolloid, as previous studies indicate (Larrain et al., 2001; Oelgeschlager et al., 2000). Moreover, as *dino* mutants display a range of phenotypes from a mild ventralization that is viable to a moderate ventralization that is lethal (Fisher and Halpern, 1999), it is possible that loss of *tsg1* affects the range of *dino* phenotypes, with the mild phenotype becoming predominant. This could be a direct effect of Tsg on other antagonists, as

discussed above, or it could be indirect by unknown mechanism(s) that also modulate the *dino* phenotype.

Tsg has been reported to enhance Tolloid proteolysis of Chordin (Larrain et al., 2001; Scott et al., 2001; Shimmi and O'Connor, 2003; Yu et al., 2000). We found that a sub-dorsalizing knockdown of Tsg1 in wild type exacerbates the mildly dorsalized phenotype of *mini fin* (*tolloid*) mutants (Fig. 5), suggesting a previously unknown role for Mini fin (Tolloid) in patterning rostral tissues in zebrafish (Connors et al., 1999; Mullins et al., 1996). There is evidence that additional Tolloid-related enzymes function during gastrulation (J. Xie and S. Fisher, personal communication), which may normally mask the loss of *mini fin* (*tolloid*) during these stages. If Tsg1 increases the rate of proteolysis of multiple Tolloid enzymes, then loss of Tsg could dorsalize the embryo by reducing the ability of multiple Tolloid factors to degrade their targets.

If endogenous Tsg1 promotes ventral cell fates by facilitating BMP signaling, why does Tsg1 overexpression dorsalize the embryo, reflecting a loss of BMP signaling? One possibility is that Tsg functions in a multi-component protein complex, binding both to a BMP ligand, as previously shown (Chang et al., 2001; Larrain et al., 2001; Oelgeschlager et al., 2000; Ross et al., 2001; Scott et al., 2001), and at least one other factor required to promote BMP signaling. Loss of Tsg would disrupt formation of this complex and result in decreased BMP signaling. Excess Tsg would bind independently to both free BMP ligands and the other factor in the complex, again preventing formation of the trimolecular complex and reducing BMP signaling. Thus, both the loss- and gain-of-function phenotypes would cause the same defect (Fig. 8). A similar phenomenon is also observed in the loss- and gain-of-function phenotypes of some Wnt planar cell polarity components and other genes (Gubb et al., 1999; Hiromi et al., 1993; Krasnow and Adler, 1994; Strutt et al., 1997; Tomlinson et al., 1997).

In the mouse, Tsg mutants do not display defects in DV patterning of the embryonic axis (Nosaka et al., 2003; Petryk et al., 2004; Zakin and De Robertis, 2004). However, they do exhibit several other defects consistent with possible roles in either promoting or inhibiting BMP signaling. For example, thymocytes from $Tsg^{-/-}$ animals show increased phospho-Smad1 levels, suggesting an increase in BMP signaling in the absence of Tsg (Nosaka et al., 2003). $Tsg^{-/-}$ mice also display defective skeletogenesis, similar to the effect of a dominant-negative BMP receptor (Nosaka et al., 2003; Zakin and De Robertis, 2004), indicating a possible defect in BMP signaling. Interestingly, a genetic interaction is also observed in the mouse, as we observe in zebrafish, between a *tsg* and BMP mutation. $Tsg^{-/-};Bmp4^{+/-}$ animals display holoprosencephaly and craniofacial defects, not seen in either *Tsg* mutant animals or *Bmp4* heterozygotes (Zakin and De Robertis, 2004). These results support a role for Tsg in promoting BMP signaling in head development in the mouse.

In contrast to our study, a recent loss of function study in *Xenopus* reports that MO-induced knockdown of Tsg causes ventralization, indicating that Tsg predominantly inhibits BMP signaling in *Xenopus* (Blitz et al., 2003). Tsg knockdown in *Xenopus* mildly restricts a small fraction of dorsally expressed genes during midgastrulation, with more substantial changes evident during neurula stages. These findings led to the conclusion that in *Xenopus* Tsg maintains the specification of dorsal cell fates, presumably after BMP signaling has patterned the early DV axis. We find that Tsg1 knockdown in zebrafish causes a moderately strong dorsalization in pattern formation during gastrulation. It is possible that Tsg acts oppositely at different stages and/or activity levels to affect the DV pattern of the embryo or functions in a nonconserved manner in these two organisms.

In *Drosophila*, Tsg also modulates BMP signaling during DV axial patterning. Tsg functions similarly to the Chordin ortholog Short gastrulation (Sog) in specifying the dorsal-most tissue, the amnioserosa, which requires the highest levels of BMP signaling in the fly embryo (Ashe and Levine, 1999; Francois et al., 1994; Mason et al., 1994; Ross et al., 2001; Zusman and Wieschaus, 1985). In addition to this pro-BMP activity, both Tsg and Sog exhibit anti-BMP activity in dorsolateral regions of the embryo (Ross et al., 2001). Current models suggest that Sog and Tsg bind to and transport BMP ligands toward dorsal regions of the embryo, where they are released from Sog by the activity of Tolloid, thereby generating the highest levels of BMP signal dorsally (Decotto and Ferguson, 2001; Eldar et al., 2002; Shimmi and O'Connor, 2003). In this model, the activity of Tsg relies on the presence of Sog and Tolloid. In vertebrates, there is no evidence for a role for Chordin in promoting gastrula BMP signaling, although it is possible it plays such a role at later stages in tail patterning (Hammerschmidt and Mullins, 2002; Wagner and Mullins, 2002). Thus, all aspects of how Sog/Chordin and Tsg function in DV patterning in vertebrates and invertebrates may not be conserved.

In zebrafish, Tsg1 could act as a BMP antagonist at other stages of development or under particular conditions that we did not detect in our studies. We do not know the nature of the tail vein edema phenotype observed in low level Tsg knockdown embryos (Fig. 1). It may reflect its role as a BMP antagonist, as *chordin* mutants exhibit a similar edema, although in conjunction with other ventralized defects, which we do not detect in *tsg1* morphants. It is likely that the timing, location and/or levels of expression of Tsg, possibly with other factors, are crucial in determining whether Tsg functions to promote or antagonize BMP signaling in different developmental contexts. In zebrafish, the mechanism by which Tsg promotes BMP signaling, and the identity of any additional Tsg-interacting factors, remains unclear. Further work will be required to determine how Tsg acts in relation to other BMP modulating factors, in order to elucidate the mechanism by which Tsg promotes BMP signaling.

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References

Ashe, H. L. and Levine, M. (1999). Local inhibition and long-range enhancement of Dpp signal transduction by Sog. *Nature* **398**, 427-431.

Akimenko, M.-A., Ekker, M., Wegner, J., Lin, W. and Westerfield, M.

(1994). Combinatorial expression of three zebrafish genes related to distal-less: part of a homeobox gene code for the head. *J. Neurosci.* **14**, 3475-3486.

Bauer, H., Lele, Z., Rauch, G. J., Geisler, R. and Hammerschmidt, M. (2001). The type I serine/threonine kinase receptor Alk8/Lost-a-fin is required for Bmp2b/7 signal transduction during dorsoventral patterning of the zebrafish embryo. *Development* **128**, 849-858.

Blitz, I. L., Cho, K. W. and Chang, C. (2003). Twisted gastrulation loss-of-function analyses support its role as a BMP inhibitor during early Xenopus embryogenesis. *Development* **130**, 4975-4988.

Chang, C., Holtzman, D. A., Chau, S., Chickering, T., Woolf, E. A., Holmgren, L. M., Bodorova, J., Gearing, D. P., Holmes, W. E. and Brivanlou, A. H. (2001). Twisted gastrulation can function as a BMP antagonist. *Nature* **410**, 483-487.

Chin, A. J., Chen, J. N. and Weinberg, E. S. (1997). *Bone morphogenic protein-4* expression characterizes inductive boundaries in organs of developing zebrafish. *Dev. Genes Evol.* **207**, 107-114.

Connors, S. A., Trout, J., Ekker, M. and Mullins, M. C. (1999). The role of *tolloid/mini fin* in dorsoventral pattern formation of the zebrafish embryo. *Development* **126**, 3119-3130.

De Robertis, E. M. and Sasai, Y. (1996). A common plan for dorsoventral patterning in Bilateria. *Nature* **380**, 37-40.

De Robertis, E. M., Larrain, J., Oelgeschlager, M. and Wessely, O. (2000). The establishment of Spemann's organizer and patterning of the vertebrate embryo. *Nat. Rev. Genet.* **1**, 171-181.

Decotto, E. and Ferguson, E. L. (2001). A positive role for Short gastrulation in modulating BMP signaling during dorsoventral patterning in the Drosophila embryo. *Development* **128**, 3831-3841.

Detrich, H. W., Kieran, M. W., Chan, F. Y., Barone, L. M., Yee, K., Rundstadler, J. A. and Zon, L. I. (1995). Intraembryonic hematopoietic cell migration during vertebrate development. *Proc. Natl. Acad. Sci. USA* **92**, 10713-10717.

Dick, A., Hild, M., Bauer, H., Imai, Y., Maifeld, H., Schier, A. F., Talbot, W. S., Bouwmeester, T. and Hammerschmidt, M. (2000). Essential role of Bmp7 (snailhouse) and its prodomain in dorsoventral patterning of the zebrafish embryo. *Development* **127**, 343-354.

Eldar, A., Dorfman, R., Weiss, D., Ashe, H., Shilo, B. Z. and Barkai, N. (2002). Robustness of the BMP morphogen gradient in Drosophila embryonic patterning. *Nature* **419**, 304-308.

Fisher, S. and Halpern, M. E. (1999). Patterning the zebrafish axial skeleton requires early chordin function. *Nat. Genet.* **23**, 442-446.

Francois, V., Solloway, M., O'Neill, J. W., Emery, J. and Bier, E. (1994). Dorsal-ventral patterning of the Drosophila embryo depends on a putative negative growth factor encoded by the short gastrulation gene. *Genes Dev.* **8**, 2602-2616.

Gubb, D., Green, C., Huen, D., Coulson, D., Johnson, G., Tree, D., Collier, S. and Roote, J. (1999). The balance between isoforms of the prickle LIM domain protein is critical for planar polarity in Drosophila imaginal discs. *Genes Dev.* **13**, 2315-2327.

Hammerschmidt, M. and Mullins, M. C. (2002). Dorsoventral patterning in the zebrafish: bone morphogenetic proteins and beyond. In *Pattern Formation in Zebrafish,* Vol. 40 (ed. L. Solnica-Krezel), pp. 219-255. Heidelberg, Germany: Springer-Verlag.

Hammerschmidt, M., Pelegri, F., Mullins, M. C., Kane, D. A., van Eeden, F. J. M., Granato, M., Brand, M., Furutani-Seiki, M., Haffter, P., Heisenberg, C.-P. et al. (1996). *dino* and *mercedes*, two genes regulating dorsal development in the zebrafish embryo. *Development* **123**, 95-102.

Heasman, J. (2002). Morpholino oligos: making sense of antisense? *Dev. Biol.* **243**, 209-214.

Hiromi, Y., Mlodzik, M., West, S. R., Rubin, G. M. and Goodman, C. S. (1993). Ectopic expression of seven-up causes cell fate changes during ommatidial assembly. *Development* **188**, 1123-1135.

Hogan, B. L. (1996). Bone morphogenic proteins: mulitifunctional regulators of vertebrate development. *Genes Dev.* **10**, 1580-1594.

Holley, S. A. and Ferguson, E. L. (1997). Fish are like flies are like frogs: conservation of dorsal-ventral patterning mechanisms. *BioEssays* **19**, 281-284.

Joly, J. S., Joly, C., Schulte-Merker, S., Boulekbache, H. and Condamine, H. (1993). The ventral and posterior expression of the zebrafish homeobox gene *eve1* is perturbed in dorsalized and mutant embryos. *Development* **119**, 1261-1275.

Kishimoto, Y., Lee, K. H., Zon, L., Hammerschmidt, M. and Schulte-Merker, S. (1997). The molecular nature of zebrafish *swirl*: BMP2 function is essential during early dorsoventral patterning. *Development* **124**, 4457-4466.

Kramer, C., Mayr, T., Nowak, M., Schumacher, J., Runke, G., Bauer, H., Wagner, D. S., Schmid, B., Imai, Y., Talbot, W. S. et al. (2002). Maternally supplied smad5 is required for ventral specification in zebrafish embryos prior to zygotic Bmp signaling. *Dev. Biol.* **250**, 263-279.

Krasnow, R. E. and Adler, P. N. (1994). A single frizzled protein has a dual function in tissue polarity. *Development* **120**, 1883-1893.

Krauss, S., Johansen, T., Korzh, V. and Fjose, A. (1991). Expression pattern of zebrafish pax genes suggests a role in early brain Regionalization. *Nature* **353**, 267-270.

Larrain, J., Oelgeschlager, M., Ketpura, N. I., Reversade, B., Zakin, L. and de Robertis, E. M. (2001). Proteolytic cleavage of Chordin as a switch for the dual activities of Twisted gastrulation in BMP signaling. *Development* **128**, 4439-4447.

Mason, E. D., Konrad, K. D., Webb, C. D. and Marsh, J. L. (1994). Dorsal midline fate in *Drosophila* embryos requires *twisted gastrulation*, a gene encoding a secreted protein related to human connective tissue growth factor. *Genes Dev.* **8**, 1489-1501.

Miller-Bertoglio, V., Fisher, S., Sanchez, A., Mullins, M. C. and Halpern, M. E. (1997). Differential regulation of *chordin* expression domains in mutant zebrafish. *Dev. Biol.* **192**, 537-550.

Miller-Bertoglio, V., Carmany-Rampey, A., Furthauer, M., Gonzalez, E. M., Thisse, C., Thisse, B., Halpern, M. E. and Solnica-Krezel, L. (1999). Maternal and zygotic activity of the zebrafish ogon locus antagonizes BMP signaling. *Dev. Biol.* **214**, 72-86.

Mintzer, K. A., Lee, M. A., Runke, G., Trout, J., Whitman, M. and Mullins, M. C. (2001). Lost-a-fin encodes a type I BMP receptor, Alk8, acting maternally and zygotically in dorsoventral pattern formation. *Development* **128**, 859-869.

Mullins, M. C. (1998). Holy Tolloido: Tolloid cleaves Sog/Chordin to free DPP/Bmps. *Trends Genet.* **14**, 127-129.

Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., Brand, M., van Eeden, F. J. M., Furutani-Seiki, M., Granato, M., Haffter, P., Heisenberg, C.-P. et al. (1996). Genes establishing dorsoventral pattern formation in the zebrafish embryo: the ventral specifying genes. *Development* **123**, 81-93.

Nasevicius, A. and Ekker, S. C. (2000). Effective targeted gene 'knockdown' in zebrafish. *Nat. Genet.* **26**, 216-220.

Nguyen, V. H., Schmid, B., Trout, J., Connors, S. A., Ekker, M. and Mullins, M. C. (1998). Ventral and lateral regions of the zebrafish gastrula, including the neural crest progenitors, are established by a *bmp2b/swirl* pathway of genes. *Dev. Biol.* **199**, 93-110.

Nosaka, T., Morita, S., Kitamura, H., Nakajima, H., Shibata, F., Morikawa, Y., Kataoka, Y., Ebihara, Y., Kawashima, T., Itoh, T. et al. (2003). Mammalian Twisted gastrulation is essential for skeleto-lymphogenesis. *Mol. Cell. Biol.* **23**, 2969-2980.

Odenthal, J. and Nüsslein-Volhard, C. (1998). *fork head* domain genes in zebrafish. *Dev. Genes Evol.* **208**, 245-258.

Oelgeschlager, M., Larrain, J., Geissert, D. and de Robertis, E. M. (2000). The evolutionarily conserved BMP-binding protein Twisted gastrulation promotes BMP signalling. *Nature* **405**, 757-763.

Oelgeschlager, M., Reversade, B., Larrain, J., Little, S., Mullins, M. C. and de Robertis, E. M. (2003). The pro-BMP activity of Twisted gastrulation is independent of BMP binding. *Development* **130**, 4047-4056.

Oxtoby, E. and Jowett, T. (1993). Cloning of the zebrafish *krox-20* gene (*krx-20*) and its expression during hindbrain development. *Nucleic Acids Res.* **21**, 1087-1095.

Petryk, A., Anderson, R. M., Jarcho, M. P., Leaf, I., Carlson, C. S., Klingensmith, J., Shawlot, W. and O'Connor, M. B. (2004). The mammalian twisted gastrulation gene functions in foregut and craniofacial development. *Dev. Biol.* **267**, 374-386.

Ross, J. J., Shimmi, O., Vilmos, P., Petryk, A., Kim, H., Gaudenz, K., Hermanson, S., Ekker, S. C., O'Connor, M. B. and Marsh, J. L. (2001). Twisted gastrulation is a conserved extracellular BMP antagonist. *Nature* **410**, 479-483.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schmid, B., Furthauer, M., Connors, S. A., Trout, J., Thisse, B., Thisse, C. and Mullins, M. C. (2000). Equivalent genetic roles for bmp7/snailhouse and bmp2b/swirl in dorsoventral pattern formation. *Development* **127**, 957-967.

Schulte-Merker, S., Lee, K. J., McMahon, A. P. and Hammerschmidt, M. (1997). The zebrafish organizer requires *chordino*. *Nature* **387**, 862-863.

Scott, I. C., Blitz, I. L., Pappano, W. N., Maas, S. A., Cho, K. W. and Greenspan, D. S. (2001). Homologues of Twisted gastrulation are

extracellular cofactors in antagonism of BMP signalling. *Nature* **410**, 475-478.

Shimmi, O. and O'Connor, M. B. (2003). Physical properties of Tld, Sog, Tsg and Dpp protein interactions are predicted to help create a sharp boundary in Bmp signals during dorsoventral patterning of the Drosophila embryo. *Development* **130**, 4673-4682.

Solnica-Krezel, L., Stemple, D. L., Mountcastle-Shah, E., Rangini, Z., Neuhauss, S. C. F., Malicki, J., Schier, A. F., Stanier, D. Y. R., Zwartkruis, F., Abdelilah, S. et al. (1996). Mutations affecting cell fates and cellular rearrangements during gastrulation in zebrafish. *Development* **123**, 67-80.

Stachel, S. E., Grunwald, D. J. and Myers, P. Z. (1993). Lithium perturbation and goosecoid expression identify a dorsal specification pathway in the pregastrula zebrafish. *Development* **117**, 1261-1274.

Strutt, D. I., Weber, U. and Mlodzik, M. (1997). The role of RhoA in tissue polarity and Frizzled signaling. *Nature* **387**, 292-295.

Thomsen, G. H. (1997). Antagonism within and around the organizer: BMP inhibitors in vertebrate body patterning. *Trends Genet.* **13**, 209-211.

Tomlinson, A., Strapps, W. R. and Heemskerk, J. (1997). Linking Frizzled and Wnt signaling in Drosophila development. *Development* **124**, 4515-4521.

Wagner, D. S. and Mullins, M. C. (2002). Modulation of BMP activity in dorsal-ventral pattern formation by the Chordin and Ogon antagonists. *Dev. Biol.* **245**, 109-123.

Weinberg, E. S., Allende, M. L., Kelly, C. L., Abdelhamid, A., Murakami, T., Andermann, P., Doerre, G., Grunwald, D. J. and Riggleman, B. (1996). Developmental regulation of zebrafish *MyoD* in wild-type, *no tail*, and *spadetail* embryos. *Development* **122**, 271-280.

Yabe, T., Shimizu, T., Muraoka, O., Bae, Y. K., Hirata, T., Nojima, H., Kawakami, A., Hirano, T. and Hibi, M. (2003). Ogon/Secreted Frizzled functions as a negative feedback regulator of Bmp signaling. *Development* **130**, 2705-2716.

Yu, K., Srinivasan, S., Shimmi, O., Biehs, B., Rashka, K. E., Kimelman, D., O'Connor, M. B. and Bier, E. (2000). Processing of the Drosophila Sog protein creates a novel BMP inhibitory activity. *Development* **127**, 2143-2154.

Zakin, L. and de Robertis, E. M. (2004). Inactivation of mouse Twisted gastrulation reveals its role in promoting Bmp4 activity during forebrain development. *Development* **131**, 413-424.

Zusman, S. B. and Wieschaus, E. F. (1985). Requirements for zygotic gene activity during gastrulation in Drosophila melanogaster. *Development* **111**, 359-371.