Inactivation of mouse Twisted gastrulation reveals its role in promoting Bmp4 activity during forebrain development

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Summary

Twisted gastrulation (Tsg) is a secreted protein that regulates Bmp signaling in the extracellular space through its direct interaction with Bmp/Dpp and Chordin (Chd)/Short gastrulation (Sog). The ternary complex of Tsg/Chd/Bmp is cleaved by the metalloprotease Tolloid (Tld)/Xolloid (Xld). Studies in Drosophila, Xenopus and zebrafish suggest that Tsg can act both as an anti-Bmp and as a pro-Bmp. We have analyzed Tsg loss-of-function in the mouse. Tsg homozygous mutants are viable but of smaller size and display mild vertebral abnormalities and osteoporosis. We provide evidence that Tsg interacts genetically with Bmp4. When only one copy of Bmp4 is present, a requirement of Tsg for embryonic development is revealed. Tsg–/–;Bmp4+/– compound mutants die at birth and display holoprosencephaly, first branchial arch and eye defects. The results show that Tsg functions to promote Bmp4 signaling during mouse head development.

Key words: Twisted gastrulation, Bmp, Tgf, Crossveinless, Holoprosencephaly, Vertebral column

Introduction

Growth factors of the bone morphogenetic protein (Bmp) superfamily are major regulators of cell-cell signaling in animal development (Hogan, 1996; Massague and Chen, 2000). A gradient of Bmp activity establishes dorsoventral patterning in both vertebrate and invertebrate embryos. Studies in vertebrates and Drosophila have shown that Bmp/Dpp activity is regulated in the extracellular space by a network of secreted proteins including Short gastrulation (Sog)/chordin (Chd), Tolloid (Tld)/Xolloid (Xld) and Twisted gastrulation (Tsg) (De Robertis et al., 2000). The Drosophila Tsg mutation was isolated during classical genetic screens for embryonic cuticle defects due to its lack of the amnioserosa, the dorsal-most tissue of the fruit fly embryo (Wieschaus et al., 1984). The Drosophila Tsg product is necessary for peak Dpp/Bmp activity in the early Drosophila embryo, which is required to form the amnioserosa (Mason et al., 1994). Tsg contains two conserved cysteine-rich domains and was shown to bind both Bmp and Chd (Oelgeschläger et al., 2000). The Tsg N-terminal domain binds to Bmp and shares similarity with the cysteine-rich (CR) Bmp-binding modules of Chd, whereas the C-terminal domain is conserved only among the Tsg homologues (Oelgeschläger et al., 2000; Vilmos et al., 2001; Oelgeschläger et al., 2003a). In addition, Drosophila Tsg is highly diffusible and can act at a long distance from its site of expression (Mason et al., 1997).

Tsg has multiple biochemical activities. First, it promotes the formation of stable ternary Bmp/Chd/Tsg complexes (Oelgeschläger et al., 2000; Chang et al., 2001; Larrain et al., 2001; Scott et al., 2001). As this ternary complex prevents binding of Bmp to its cell surface receptors, in this aspect of its function Tsg behaves as a Bmp antagonist. Second, the stability of these inhibitory ternary complexes is controlled by the Tld metalloprotease, which cleaves Chd/Sog at specific sites (Marques et al., 1997; Piccolo et al., 1997). In the presence of Tsg, Chd/Sog is a better substrate for cleavage by the Tld enzyme (Larrain et al., 2001; Scott et al., 2001; Shimmi and O’Connor, 2003). By promoting Chd degradation in the presence of Tld, Tsg releases Bmp that is now able to signal through Bmp receptors. In this second aspect of its activity, Tsg functions to increase Bmp activity (Piccolo et al., 1997; Larrain et al., 2001; Shimmi and O’Connor, 2003).

The opposing functions of the Tsg protein may explain why conflicting results have been reported in various microinjection assays. In Xenopus, overexpression of Tsg mRNA results in Bmp-promoting effects (Oelgeschläger et al., 2000; Oelgeschläger et al., 2003a; Oelgeschläger et al., 2003b). The opposite observation was made in zebrafish embryos, in which microinjection of zebrafish Tsg or Xenopus Tsg caused dorsalization (Ross et al., 2001; Oelgeschläger et al., 2003b). These different outcomes have been attributed to the different levels of the Tld protease in the two model embryos (Larrain et al., 2001). In zebrafish, endogenous levels of the Tolloid protease are low, as indicated by the weak phenotype of tolloid-mini-fin mutant embryos which are viable and lack the ventral tail fin (Connors et al., 1999). At these low Xolloid concentrations, microinjection of Tsg mRNA favors the formation of inhibitory Bmp/Chd/Tsg complexes. In the Xenopus embryo, high levels of Tld activity are present, Tsg promotes the cleavage of Chd, and ventralization is observed. However, when the endogenous Tld protease is inhibited by co-injection of dominant-negative Xld mRNA the opposite result, dorsalization, is seen (Larrain et al., 2001). These experiments suggest that the proteolytic cleavage of Chd...
constitutes the crucial molecular switch between the anti-Bmp and Bmp-promoting activities of Tsg.

Additional evidence on the dual activity of Tsg was provided by the study of point mutations that dissociate Bmp binding and Chd interaction (Oelgeschlager et al., 2003a). Mutations in the N-terminal domain of Tsg constitute the crucial molecular switch between the anti-Bmp and Bmp-promoting activities of Tsg. As indicated in Fig. 1. Mice carrying the mutation were mated and their progeny analyzed. Viable homozygous Tsg–/– mice are viable and have skeletal defects.

In situ hybridization and skeletal preparations

In situ hybridization on whole-mount or on cryostat sections was performed as previously described (Henrique et al., 1995). The probes used were: Bmp4 (Winnier et al., 1995), Dlx5 (Acampora et al., 1999), Fgf8 (Crossley and Martin, 1995), Hex (Bedford et al., 1993), Shh (McMahon et al., 1998) and Cerl (Belo et al., 1997). The Tsg probe was synthesized using the full-length cDNA cloned in pGEMTeasy, linearized with XhoI and transcribed with SP6 RNA polymerase. Newborns and E12.5 to E15.5 embryos were fixed in Bouin's solution, dehydrated, cleared and embedded in paraffin wax. Serial 7 μm sections were stained using Hematoxylin and Eosin or Mallory's Tetrachrome method (Bachiller et al., 2003). Alcian Blue and Alizarin Red skeletal staining (Belo et al., 1998), and Alcian Blue staining at 37°C in 1 mg/ml Xgal, 2 mM MgCl2, 0.1 M phosphate buffer pH 7.4, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide.

Results

Disruption of the Tsg locus

Targeted inactivation of the Tsg gene was performed by in-frame insertion of the lacZ reporter gene into the first exon of Tsg at the initiator methionine. This also resulted in the deletion of the first Tsg exon, which encodes the initial 40 amino acids of Tsg including the entire signal peptide (Fig. 1A). This mutation also caused the loss of stable wild-type Tsg transcripts when monitored by RT-PCR of mutant E13.5 embryonic cells (Fig. 1D). We conclude that the Tsg mutation represents a complete loss-of-function as it lacks the signal peptide and wild-type Tsg mRNA is not expressed.

Comparison of the expression of the Tsg mRNA and β-galactosidase (β-gal) activity in E6.5 to E8.5 embryos showed that the lacZ reporter gene was expressed at the same time and place as the endogenous Tsg gene (Fig. 1, compare E,E′–H,H′). Tsg expression was observed at E6.5 in the anterior visceral endoderm and forming primitive streak (Fig. 1E,G). As gastrulation continued and mesodermal wings formed, Tsg expression was observed in the entire mesodermal layer (Fig. 1F,F′,G,G′). At early headfold stage Tsg was expressed in head mesenchyme, gut endoderm and ventral neuroectoderm (Fig. 1I). At E8.5, Tsg was expressed in ventral neuroectoderm such as basal diencephalon (Fig. 1J), the endoderm of the gut, the posterior eye vesicle and the forming pharyngeal arches (Fig. 1H,H′). We conclude that the in-frame reporter gene faithfully recapitulates the endogenous Tsg expression pattern.

Tsg–/– mice are viable and have skeletal defects

To address the function of Tsg in vivo, heterozygous animals were mated and their progeny analyzed. Viable homozygous mutants were recovered in Mendelian proportions (e.g., in F1 crosses, of 106 neonates 25 were +/+; 57 were +/− and 24 were
Both male and female Tsg<sup>–/–</sup> animals were fertile. Growth analysis of Tsg<sup>–/–</sup> mutants showed that they were of smaller size, weighed 10-20% less than littermates and displayed a short tail. Eighty percent of the Tsg<sup>–/–</sup> animals presented multiple kinks in the tail, which became more marked with age (Fig. 2A,B). X-rays of adult mice indicated that the caudal vertebrae of mutant mice were shorter than normal and had lower bone density than wild-type or heterozygous animals (Fig. 2B). In E14.5 Tsg<sup>–/–</sup> embryos, defective cartilage formation was observed in the dorsal neural arches of most cervical and some thoracic vertebrae (Fig. 2C,C'). This defect persisted after birth and was characterized by the incomplete growth and fusion (dyssymphysis) of the osseous vertebral neural arches (compare Fig. 2D with 2D').

Alcian Blue/Alizarin Red staining revealed the origin of Tsg<sup>–/–</sup> tail kinks. At birth, Tsg<sup>–/–</sup> tail vertebrae contained two symmetric centers of ossification, rather than the single axial center observed in wild-type or heterozygous embryos (Fig. 2E,E'). Two weeks later, if the two centers of ossification had grown at the same rate, they produced straight, but shorter vertebrae with vertebral bodies that adopted a bow tie shape (Fig. 2F,F'). In other Tsg<sup>–/–</sup> vertebrae, the two centers grew asymmetrically, producing hemi-vertebrae that led to misalignment of the articular surfaces and therefore to tail
kinks (Fig. 2G,G’). The two types of abnormal vertebrae could be found in the same individual, with the bow tie shape more frequent in the sacral region. We conclude that Tsg is not an essential gene for embryogenesis and that its inactivation causes dwarfism, vertebral abnormalities and osteoporosis detectable by X-rays.

**Defective chondrogenesis in the caudal region of Tsg–/– embryos**

We next analyzed the pathogenesis of the vertebral defects (Fig. 3). Formation of the vertebral column is the result of several inductive events. First, the notochord induces differentiation of somitic mesoderm into sclerotome. After migrating next to the notochord, sclerotomal cells form an unsegmented perichordal tube, which is then subdivided into alternate condensed and uncondensed areas of mesenchyme (Grüneberg, 1963; Theiler, 1988). The condensed areas give rise to the annulus or future intervetebral disc. The annulus separates into a fibrous outer part (annulus fibrosus) and an inner part of prechondrogenic mesenchyme (inner annulus), forming concentric rings around the notochord. The uncondensed areas give rise to the cartilaginous primordia of the vertebral bodies. Finally, the notochord disappears from the developing vertebral body (possibly extruded by the increasing pressure of the cartilage extracellular matrix) but remains at the center of the intervertebral disc, where it forms the nucleus pulposus (Grüneberg, 1963; Theiler, 1988; Aszodi et al., 1998).

At E12.5, the morphology of the somites, sclerotomes and areas of condensed and uncondensed mesenchyme was similar in wild-type and Tsg–/– animals (Fig. 3A,A’). However, by E15.5, the Tsg–/– vertebral column showed distinct abnormalities. In the wild-type tails, the round vertebral bodies formed a core of differentiated chondrocytes flanked by proliferating chondrocyte precursors (Fig. 3B). Notochordal cells were mostly excluded from the vertebral bodies and accumulated in the intervertebral regions to form the nucleus pulposus (Fig. 3B). In the Tsg–/– tail, the vertebral bodies were narrower and rectangular with fewer differentiated chondrocytes, while the intervertebral regions were expanded and had fewer proliferating chondrocyte precursors (Fig. 3B’). The notochordal cells were not resorbed from vertebral bodies, and a distinctive nucleus pulposus was not formed (Fig. 3B’). These observations indicated impaired chondrocyte differentiation. The decrease of vertebral body cartilage, increase in intervertebral tissue and persistence of the notochord was confirmed by Alcian Blue staining (Fig. 3C,C’).
that chondrocyte differentiation is impaired in Tsg−/− mutants. Tsg intervertebral region markers were expanded in annulus fibrosus of the intervertebral region (Fig. 3E). Both vertebral bodies were decreased (Fig. 3D; data not shown). We next asked whether Tsg and Bmp4 interact genetically. Homozygous null mutants for Bmp4 die around gastrulation (Winnier et al., 1995). Heterozygous Bmp4 animals are viable and display craniofacial malformations, microphthalmia, cystic kidney and preaxial polydactyly (Dunn et al., 1997). Matings were set up between Tsg+/−;Bmp4+/− and Tsg−/+;Bmp4+/− animals in B6SJL/F1 background. No additional phenotypes, compared with the ones already described, were detected in Tsg−/+;Bmp4+/− or Tsg−/+;Bmp4+/− littermates. However, Tsg−/−;Bmp4+/− neonates displayed severe head malformations (Fig. 4) with a 64% penetrance (seven out of 11 Tsg−/−;Bmp4+/−, n=77). In severe cases, a single nostril, lack of mouth opening, anophthalmia and low implantation of the external ears were observed (Fig. 4A,A′). Histological sections of the snout region showed the absence of nasal septum, lower mandible and tongue (Fig. 4B,B′), the latter two being derivatives of the first branchial arch. Similar phenotypes were observed at E15.5 (Fig. 5A′).

Tsg−/−;Bmp4+/− mutant embryos presented severe malformations of the prosencephalon. First, the telencephalon formed a single cavity (instead of two lateral vesicles), owing to the absence of the medial wall (Fig. 5C,C′). This malformation is known as holoprosencephaly. Second, the lateral ganglionic eminence or striatum, which gives rise to the basal nuclei (caudate, putamen and pallidum) of the telencephalon was completely absent (Fig. 5B′,C′). Third, the basal diencephalon was greatly thickened at the level of the hypothalamus (Fig. 5B′,B). Finally, after the initial formation of an optic vesicle, the retina and lens did not develop in Tsg−/−;Bmp4+/− embryos, despite the presence of an orbit and upper and lower eyelids (Fig. 5B′,B′ and data not shown). Cyclopia was not observed. The anophthalmia observed was more severe than the Bmp4+/− microphthalmia (Dunn et al., 1997). Because in Tsg−/− the main phenotype was observed in

The expression of Tsg (visualized by β-gal activity) and Bmp4 confirmed the expansion of the intervertebral region with respect to the vertebral bodies at E14.5 and E13.5 (Fig. 3D,D′,E,E′; data not shown). Tsg expression was observed in the notochord and in the condensed mesenchyme of the inner annulus (Fig. 3D; data not shown). Bmp4 was expressed in the annulus fibrosus of the intervertebral region (Fig. 3E). Both intervertebral region markers were expanded in Tsg−/−, while vertebral bodies were decreased (Fig. 3D′,E′). We conclude that chondrocyte differentiation is impaired in Tsg−/− mutants. As Bmp signaling is crucial for cartilage formation and several Bmp mutants display skeletal defects (Kingsley, 1994; Karsenty, 2000; Canalis et al., 2003), these observations could be considered consistent with a positive role for Tsg in Bmp signaling during vertebral differentiation.

Holoprosencephaly in Tsg−/−;Bmp4+/− mutants

We next asked whether Tsg and Bmp4 interact genetically. Homozygous null mutants for Bmp4 die around gastrulation

Fig. 3. Development of vertebral defects in Tsg mutant embryos. (A-A′) Hematoxylin and Eosin staining of sagittal sections of the tail of E12.5 wild-type and Tsg−/− embryos. (B-B′) Mallory’s tetrachrome staining of sagittal sections of E15.5 wild-type and Tsg−/− tails. (C-C′) Alcian Blue staining of E14.5 wild-type and Tsg−/− embryos. Note the smaller vertebral bodies and persistence of the notochord (n) in the mutant. (D-D′) β-gal staining of the tail region of E14.5 wild-type and Tsg−/− embryos showing expanded Tsg expression in intervertebral prechondrogenic mesenchyme and in the notochord of the mutant. (E-E′) Expression of Bmp4 at E14.5 in wild-type and Tsg−/− embryos. (E) Bmp4 is expressed in the intervertebral annulus fibrosus, which is expanded in the mutant (E′). cm, condensed mesenchyme; scl, sclerotome; s, somite; um, uncondensed mesenchyme; sp, spinal chord.
the tail vertebrae, we paid particular attention to enhancing or suppressing effects in the caudal region of Tsg−/−;Bmp4+/+ animals. However, we could not detect any differences with Tsg+/− embryos, indicating that the effects of Tsg on vertebral development may be caused by interactions with Bmps other than Bmp4. We also examined the kidney histologically, which can develop cysts in Bmp4+/−, and failed to find genetic interactions. We conclude that the reduction of Bmp4 gene dose by one half in Tsg−/−;Bmp4+/− embryos leads to holoprosencephaly, lack of basal ganglia and eyes.

**Comparison of the expression of Tsg and Bmp4**

In light of the phenotypes uncovered by genetic interactions between Tsg and Bmp4, we examined the expression of Tsg in comparison to that of Bmp4 and Chd, specifically in the affected structures (Fig. 6). At E8.0 Tsg, Bmp4 and Chd are expressed in adjacent domains in anterior regions. Tsg was diffusely expressed in ventral neuroectoderm, head mesoderm and foregut endoderm (Fig. 6A, Fig. 1I,J). At this stage Bmp4 was expressed in the surface ectoderm surrounding the neural folds and in extra-embryonic mesoderm (Fig. 6B,C) (Lawson et al., 1999; Fujiwara et al., 2002). Chd was expressed in the notochord, prechordal plate and dorsal endoderm (Fig. 6D) (Bachiller et al., 2003). The Tld proteases that regulate Chd activity have broad expression patterns, which include the neuroectoderm and extra-embryonic tissues (Scott et al., 1999). Tsg expression continued to be diffuse at later stages, with ventral brain, eye, branchial arches, limb buds and ventral posterior mesoderm expressing Tsg at higher levels (Fig. 6E,G, Fig. 1H,H′). Bmp4 was expressed in dorsal telencephalon, eye, proximal ectoderm of the first branchial arch, frontonasal mass, maxillary arch and limb buds, ventral-posterior mesoderm and allantois (Fig. 6F,H). Chd is expressed in pharyngeal endoderm at these stages, and at low levels in the first branchial arch (Stottmann et al., 2001; Bachiller et al., 2003). We conclude that the genetic interactions between Bmp4 and Tsg produce phenotypes in regions in which both genes are expressed in adjacent domains, such as the forebrain and the eye. However, other regions in which the expression domains show strong overlap, such as ventral mesoderm and limb buds, do not show additional phenotypes. Presumably in these regions other Bmps compensate for the decreased level of Bmp4. One region in which expression of Bmp4 overlaps with Tsg and produces dose-dependent phenotypes is the first branchial arch.

**First branchial arch defects in Tsg−/−;Bmp4+/− embryos**

The first branchial arch becomes apparent at E8.25 and then subdivides into the mandibular and maxillary components. After outgrowth and fusion at the midline, they give rise to many structures, such as the jaws, tongue and teeth. Some of these elements were absent or reduced in Tsg−/−;Bmp4+/− embryos (Fig. 4B′). Tsg is expressed in all branchial arches, whereas Bmp4 is expressed in the surface ectoderm of the maxillary and mandibular arches (Fig. 6F,H), in which phenotypes were observed. The hyoid arch does not express Bmp4 and was unaffected [in Chd mutants the hyoid arch and most of the hyoid bone are missing (Bachiller et al., 2003)]. We used two markers, Dlx5 and Fgf8, to follow the development of the first arch. Dlx5 is a homeobox gene expressed in the mandibular component of the first branchial arch and in the hyoid arch at E10.5 (Acampora et al., 1999) (Fig. 7A). Dlx5 is required for first branchial arch development and is a downstream target of Bmp4 signals (Miyama, 1999). In the Tsg−/−;Bmp4+/− embryo shown in Fig. 6A′, the mandibular arch, marked by Dlx5 expression, was absent on the right side and reduced on the left. Fgf8 is a member of the fibroblast growth factor family that is required for the patterning of the first branchial arch (Trumpp et al., 1999; Abu-Issa et al., 2002). Fgf8 is expressed in the rostral surface ectoderm of the first arch at E8.5 (Fig. 7B). In Tsg−/−;Bmp4+/− embryos the first arch was not distinguishable and Fgf8 expression was lost (Fig. 7B′). Thus, it appears that in the compound mutant the mandibular component of the first arch is reduced or fails to develop. We conclude that Tsg
and Bmp4 cooperate in the development of the first branchial arch.

Onset of head defects in Tsg<sup>−/−</sup>;Bmp4<sup>+/−</sup> embryos

Several signaling centers have been implicated in specific steps of forebrain development during embryogenesis. The anterior visceral endoderm (AVE) plays an early role, and later on the prechordal plate (PCP), the anterior neural ridge (ANR) and the midbrain-hindbrain isthmus further refine the pattern (Rubenstein et al., 1998; Beddington and Robertson, 1999). To analyze the onset of the holoprosencephaly in Tsg<sup>−/−</sup>;Bmp4<sup>+/−</sup> embryos, specific markers expressed in these signaling centers were analyzed between E7.5 and E8.5. Fgf8 is expressed in the ANR, the isthmus, the pharyngeal endoderm and the tailbud before the embryo turns (Crossley and Martin, 1995; Abu-Issa et al., 2002; Garel et al., 2003) (Fig. 7C), and in the commissural plate, the first branchial arch in addition to the isthmus and the tailbud after the turning of the embryo (Fig. 7B). No differences were found in the initial expression of Fgf8 in the Tsg<sup>−/−</sup>;Bmp4<sup>+/−</sup> mutants (Fig. 7C'), but at E8.5 the commissural plate and corresponding staining were missing (asterisk in Fig. 7B'). Shh is a secreted signaling factor expressed in the embryonic midline (notochord and prechordal plate) (Chiang et al., 1996). At E8.5, Shh expression is also seen in the basal plate of the telencephalon, diencephalon and mesencephalon (McMahon et al., 1998) (Fig. 7D). In Tsg<sup>−/−</sup>;Bmp4<sup>+/−</sup> embryos, Shh expression was lost in the basal telencephalon and diencephalon but persisted in the basal mesencephalon at E8.5 (Fig. 7D'). Six3 expression marks the developing telencephalic territory in the neural plate (Oliver et al., 1995) and was moderately reduced in mutant embryos at E8.0 (data not shown). During gastrulation, the AVE appeared normal in Tsg<sup>−/−</sup>;Bmp4<sup>+/−</sup> embryos, as judged by the specific marker Cer1 at E6.5 (Belo et al., 1997) (Fig. 7E,E'). The anterior endoderm and node marker Hex was normal at E7.5, a stage in which it marks both primitive and definitive endoderm (Thomas et al., 1998) (Fig. 7F,F').

We conclude that Tsg inactivation combined with half the normal dose of Bmp4 results in the loss of Shh and Fgf8 expression in the basal telencephalon and diencephalon. Although the absence of these important signaling molecules could suffice to explain the observed holoprosencephaly, a small decrease of the forebrain territory was already observed at the neural plate stage. Formation of the AVE and ANR appeared normal in the compound mutant embryos. The holoprosencephaly phenotype in Tsg<sup>−/−</sup>;Bmp4<sup>+/−</sup> embryos arose around E8.0, concomitantly with the loss of Shh and Fgf8 expression. The results indicate that Tsg is required for the proper function of Bmp4 in the formation of the telencephalic vesicles.

Discussion

The secreted factor Tsg exhibits multiple biochemical activities in the Bmp pathway. Depending on the context, Tsg results in Bmp-promoting or anti-Bmp effects. We have examined Tsg loss of function by targeting the Tsg gene in the mouse and by analyzing the genetic interaction between the Tsg and Bmp4 loci. Tsg inactivation resulted in viable homozygous mutants of reduced size displaying minor skeletal malformations. However, when in addition one copy of the Bmp4 gene was removed, the Tsg<sup>−/−</sup>;Bmp4<sup>+/−</sup> mutants displayed significant defects in forebrain, eye and branchial arch formation. The strong enhancement of Bmp4 haploinsufficiency in Tsg<sup>−/−</sup>
mutants supports the view that Tsg promotes Bmp4 activity in vivo.

**Tsg is not essential for embryogenesis**

Tsg has been conserved in evolution between *Drosophila* and the vertebrates and is known to bind in vitro several Bmps, such as Bmp2, Bmp4 and Bmp7, as well as the Bmp antagonist Chd (Oelgeschläger et al., 2000; Chang et al., 2001; Scott et al., 2001). Nosaka et al. (Nosaka et al., 2003) recently generated a larger deletion of the *Tsg* gene that resulted in a similar phenotype to the one described here. In addition, they reported a lethality of up to 50% of the mutants in the first month, which we did not observe. This discrepancy may be due to a difference in genetic backgrounds (inbred C57BL/6 versus the hybrid B6SJL/F1 background used here). Mouse Tsg had been cloned as a gene that is differentially expressed in the thymus (Graf et al., 2001; Graf et al., 2002). Nosaka et al. (Nosaka et al., 2003) identified severe deficiencies in thymocyte cell number and differentiation in *Tsg–/–* mice, which were interpreted to indicate an increase in Bmp signaling. The thymus phenotype was not analyzed here. As discussed in the introduction, Tsg has both Bmp-promoting and anti-Bmp properties, depending on the presence of Chd and Tld. Tsg facilitates the formation of ternary complexes with Bmp and Chd and in this way inhibits Bmp signaling through its cognate receptors (De Robertis et al., 2000) until Chd is cleaved by Tld (Larrain et al., 2001). The presence of Tsg facilitates proteolytic cleavage of Chd by Tld, promoting Bmp activity. Microinjection of antisense morpholino oligos in zebrafish and *Xenopus* has provided evidence that Tsg can inhibit Bmp signaling and that it cooperates with Chd in this function, presumably by interfering with ternary complex formation (Ross et al., 2001; Blitz et al., 2003). Tsg mutant proteins that are unable to bind Bmp4 can still bind to Chd and other CR-containing proteins and have potent Bmp-promoting activity (Oelgeschläger et al., 2003a). The multiple functions of Tsg made it particularly important to analyze its function in defined genetic situations.

Despite its strong evolutionary conservation, Tsg was not required for vertebrate embryonic development. In *Drosophila*, *Tsg* mutation results in the loss of the amnioserosa a tissue that requires highest Bmp/Dpp activity (Mason et al., 1994). Mutation of *Drosophila Tsg* results in the lack of peak phosphorylation of the transcription factor Mad (Ross et al., 2001) and in the inability of Dpp to diffuse in the embryo (Eldar et al., 2002). A second Tsg gene, *Drosophila Tsg2* (L. Marsh, communication to FlyBase FBgn000394) is affected in the *crossovessoins* (*cv*) mutant. *cv* causes a loss of the posterior crossveins (Bridges, 1920), a structure that requires maximal Bmp signaling in the *Drosophila* wing. In addition, a third *Tsg* homolog was identified, *Drosophila Tsg3*, which only contains the C-terminal half of the protein (Vilmos et al., 2001). *Drosophila Tsg3* maps close to *shrew*, a gene also required to achieve highest signaling levels in the Bmp/Dpp dorsoventral signaling gradient (Vilmos et al., 2001; Ferguson and Anderson, 1992). Although the mouse genome has been sequenced, genes with small exons can escape a BLAST search. It is conceivable that additional *Tsg* homologues may exist in the mouse genome; in *Xenopus* a divergent *xTsg2* cDNA with biological activities similar to those of *Xenopus* Tsg has been recently isolated (M. Oelgeschläger, U. Tran and E.M.D.R., unpublished).

**Tsg function in skeletal development**

The loss of *Tsg* in mouse resulted in vertebral abnormalities. The neural arches of the cervical and thoracic vertebrae fail to fuse at the midline and kinked tails were observed. We examined the pathogenesis of the vertebral defects in *Tsg*...
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Tsg interacts with Bmp4

The phenotypes of Tsg−/− and Bmp4+/− were indistinguishable from wild-type. (A–A′) Frontal view of E10.5 embryos stained with Dlx5. Insets show lateral views. The right mandibular component of the first arch is missing in the compound mutant (arrow). hy, hyoid arch; md, mandibular component of the first pharyngeal arch. (B–B′) Lateral view of E8.5 and E8.25 embryos hybridized with Fgf8. (B) At E8.5 Fgf8 is expressed in the proximal ectoderm of the first pharyngeal arch (a1), isthmus (is), commissural plate (cp) and tailbud (tb). (B′) Expression in the pharyngeal arch and the commissural plate (indicated by an asterisk) is missing in the mutant. (C–C′) At E8.25, Fgf8 is present in the anterior neural ridge (anr), isthmus, pharyngeal endoderm (en) and tailbud; no differences in Fgf8 expression were observed at this stage. (D–D′) At E8.5 Shh is expressed in the basal mesencephalon (bm), basal diencephalon (bd) and basal telencephalon (bt) and the notochord. Expression in the basal diencephalon and basal telencephalon is missing in the mutant (asterisk). (E–E′) Expression of mouse Cer1 in the anterior visceral endoderm (ave) of wild-type and Tsg−/−;Bmp4+/− embryos at E6.5. (F–F′) Expression of Hex in the anterior endoderm (ae) and node (no) of wild-type and Tsg−/−;Bmp4+/− embryos at E7.5.
$Tsg^{+/-};Bmp4^{+/-}$ animals did not survive beyond birth and presented additional phenotypes to those already found in either $Tsg^{+/-}$ or $Bmp4^{+/-}$ animals (Fig. 5). First, the forebrain presented reduced telencephalic vesicles that fused into a single ventricular cavity, the defining characteristic of holoprosencephaly (Muenke and Beachy, 2000). Second, eye vesicles were formed initially but degenerated, although the orbits and eyelids still differentiated. This phenotype suggests an enhancement of the $Bmp4^{+/-}$ phenotype since microphthalmia can result from $Bmp4$ haploinsufficiency (Dunn et al., 1997). Recently, anophthalmia has been described in mutant mice with hypomorphic alleles of $Bmp4$ (Kulessa and Hogan, 2002) and in $Cre-Foxg1;Bmp4^{loxP-lacZ-neo}$ mutants that lack $Bmp4$ expression in the telencephalon (Hebert et al., 2002; Hebert et al., 2003). $Bmp4$ and $Tsg$ are strongly expressed in the eye region (Figs 1 and 6). In addition, $Bmp4$ is essential for lens induction (Furuta and Hogan, 1998) and lenses were not observed in compound mutants. Third, the lateral ganglionic eminence of the basal telencephalon did not form. Fourth, the floor of the diencephalon was greatly thickened a site of co-expression for $Bmp4$ (Furuta et al., 1997) and $Tsg$ (Fig. 1J).

Finally, the mandibular component of the first branchial arch was hypoplastic or absent in the compound mutants. Both $Bmp4$ and $Tsg$ are expressed in the developing branchial arch (Fig. 6). A first branchial arch phenotype was described in $Cre-Foxg1;Bmp4^{loxP-lacZ-neo}$ mutants (Hebert et al., 2003). Of all the phenotypes of $Tsg^{+/-};Bmp4^{+/-}$ animals, the only one not previously reported in $Bmp4$ mutations was the holoprosencephaly. It may appear surprising that $Cre-Foxg1;Bmp4^{loxP-lacZ-neo}$ mutants that lack $Bmp4$ in the forebrain do not have this phenotype. However, our results show that the onset of the holoprosencephaly occurs earlier than the recombination induced by $Cre-Foxg1$ mice (Hebert et al., 2003). It is thus likely that $Bmp4$ is required for the development of the telencephalic vesicles prior to E8.5, which would not have been revealed in the conditional Cre-Lox approach (Hebert et al., 2003). In conclusion, $Tsg^{+/-};Bmp4^{+/-}$ mutants present phenotypes similar to those observed in $Bmp4$ hypomorphic and loss-of-function mutants.

$Tsg$, $Bmp4$ and holoprosencephaly (HPE)

The human $Tsg$ gene maps close to the HPE locus 4 on chromosome 18p11.3 (Graf et al., 2001). However no mutations in the human $Tsg$ gene could be detected in familial cases of HPE at locus 4 (M. Muenke and E.M.D.R., unpublished observations). It has been proposed in the multi-hit hypothesis that sporadic HPE may result from mutations in more than one gene (Ming and Muenke, 2002). This is what is observed in our study, in which HPE requires mutations in more than one gene (Ming and Muenke, 2002). This is what is observed in our study, in which HPE requires mutations in more than one gene (Ming and Muenke, 2002). This is what is observed in our study, in which HPE requires mutations in more than one gene (Ming and Muenke, 2002). This is what is observed in our study, in which HPE requires mutations in more than one gene (Ming and Muenke, 2002). This is what is observed in our study, in which HPE requires mutations in more than one gene (Ming and Muenke, 2002).

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