Twisted gastrulation enhances BMP signaling through chordin dependent and independent mechanisms

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Summary

BMP signaling is modulated by a number of extracellular proteins, including the inhibitor Chordin, Tolloid-related enzymes (Tld), and the interacting protein Twisted Gastrulation (Tsg). Although in vitro studies have demonstrated Chordin cleavage by Tld enzymes, its significance as a regulatory mechanism in vivo has not been established in vertebrates. In addition, Tsg has been reported in different contexts to either enhance or inhibit BMP signaling through its interactions with Chordin. We have used the zebrafish gastrula to carry out structure/function studies on Chordin, by making versions of Chordin partially or wholly resistant to Tld cleavage and structure/function studies on Chordin, by making versions.

We find that depletion of the embryo for Tsg leads to decreased BMP signaling, and to increased levels of Chordin. Finally, we show that Tsg also enhances BMP signaling in the absence of Chordin, and its depletion can partially rescue the chordin mutant phenotype, demonstrating that important components of the BMP signaling pathway remain unidentified.

Key words: Chordin, Twisted gastrulation, BMP, Dorsoventral patterning, Zebrafish

Introduction

Bone morphogenetic protein (BMP) signaling is regulated by complex interactions of inhibitors, proteases and other proteins (Balemans and Van Hul, 2002). The BMP inhibitor Chordin was first identified in Xenopus as a factor capable of rescuing axis formation in ultraviolet treated embryos (Sasai et al., 1994), and was subsequently shown to bind BMPs and prevent them from activating their receptors (Piccolo et al., 1996). Many components of the BMP signaling pathway are functionally conserved in Drosophila, including short gastrulation (sog), a homolog of Chordin, and decapentaplegic (dpp), a homolog of a subset of the vertebrate BMPs (Francois and Bier, 1995; Holley et al., 1995; Schmidt et al., 1995).

Chordin contains four cysteine-rich (CR) domains, similar to those found in a number of extracellular matrix proteins (Sasai et al., 1994). High-affinity binding of Chordin for BMPs resides in its CR domains (Larraín et al., 2000); CR1 and CR3 have significant binding affinity as individual domains, although approximately one-tenth that of the full-length (FL) protein, and the biological activity of FL Chordin and its fragments roughly parallels their binding affinities. These data predict that cleavage of Chordin into smaller fragments would significantly reduce its biological activity as a BMP inhibitor.

tolloid (tld) was first identified as a zygotic Drosophila gene required for dorsoventral (DV) patterning, and homologous to vertebrate Bmp1 (Shimell et al., 1991). Tld cleaves Sog at two sites, presumably explaining its ability to enhance Dpp signaling by decreasing the levels of a Dpp inhibitor (Marques et al., 1997). In addition to Bmp1, several other Tld-related vertebrate enzymes have been identified. Some of these similarly cleave Chordin in vitro (Blader et al., 1997; Piccolo et al., 1997; Scott et al., 1999), and when misexpressed in the embryo can antagonize Chordin function (Goodman et al., 1998; Piccolo et al., 1997).

Direct genetic evidence for the importance of vertebrate Tld proteins in regulating Chordin has been less clear. The zebrafish DV patterning gene mini-fin (mfn; tll1 – Zebrafish Information Network) encodes a Tld-related enzyme (Connors et al., 1999), which cleaves Chordin in vitro (Blader et al., 1997). However, mfn mutants have no phenotype during gastrulation, when chordin expression is highest, and the mutation has not been correlated with abnormalities in Chordin cleavage in vivo. In mouse, the Bmp1 and Tll1 genes have each been knocked out (Clark et al., 1999; Suzuki et al., 1996), revealing functions in heart septation, body wall closure and collagen processing. However, none of these roles have been correlated with abnormalities in Chordin cleavage in the single mutant mice. Analysis of cells from single and double mutants has shown redundant function for the two enzymes in cleaving Chordin and other substrates (Pappano et al., 2003). Genetic analysis in vertebrates is clearly complicated by the fact that
multiple enzymes cleave Chordin, and redundancy masks the importance of this regulatory mechanism.

The Drosophila twisted gastrulation (tsg) gene is required for peak Dpp signaling in the dorsal embryo, and also cooperates with Sog to inhibit Dpp signaling (Mason et al., 1994; Mason et al., 1997). Vertebrate Tsg genes have been reported both to enhance (Oelgeschlager et al., 2000; Zakin and De Robertis, 2004) and inhibit (Blitz et al., 2003; Chang et al., 2001; Ross et al., 2001; Scott et al., 2001) BMP signaling. To reconcile these findings, a model has been proposed in which Tsg acts in two steps, first enhancing the binding of Chordin to BMP, then after cleavage helping to displace the Chordin fragments (Larrañ et al., 2001). According to this model, the amount of Tld activity determines the balance between these two counteracting functions. In vitro, Tsg increases cleavage of mouse Chordin at the two identified Tld cleavage sites and at an additional, intermediate site not used in the absence of Tsg (Scott et al., 2001). A similar cleavage of Sog occurs in the Drosophila embryo, generating a more potent Dpp inhibitor termed ‘Supersog’ (Yu et al., 2000). If such a cleavage occurs in vivo for the vertebrate protein, it would ascribe a positive role to both Tld and Tsg in Chordin regulation.

We assessed the role of Tld in regulation of Chordin in the zebrafish gastrula and examined the effect of Tsg on Chordin cleavage and function. Through alterations of conserved residues near the cleavage sites, we created Chordin mutants that retain BMP-inhibitory activity but are resistant to Tld cleavage at one or both sites. RNAs encoding wild-type and cleavage mutant (CM) Chordins were used to rescue zebrafish chordin/dino (din; chd – Zebrafish Information Network) mutant embryos. Prevention of cleavage at either site enhances the ability of Chordin to rescue din mutants, confirming the importance of cleavage as a regulatory mechanism. We also provide evidence that the protein of the downstream cleavage event is a stronger BMP inhibitor than the FL protein, suggesting a positive role for cleavage in Chordin regulation. We show that Chordin cleavage is extremely rapid in vivo, and that redundant enzymes cleave Chordin in mfn mutants. However, we find no evidence of alternative cleavage sites being used in either the wild-type or CM proteins. Furthermore, we show that endogenous Tsg decreases steady state Chordin in the embryo, reducing its effectiveness as a BMP inhibitor. We reassessed the tsg1 (tsga – Zebrafish Information Network) morphant phenotype, both in wild-type and din mutant backgrounds; we find that the predominant effect of Tsg in the zebrafish gastrula is to enhance BMP signaling, and that it can also do so independently of Chordin.

Materials and methods

Fish stock maintenance

Fish were cared for using standard methods (Westerfield, 1995). The din<sup>g250</sup> and mfn<sup>pm224a</sup> alleles were maintained through intercrossing of heterozygous or homozygous mutant fish. For all injection experiments, clutches of mutant embryos were obtained by intercrossing din<sup>−/−</sup> or din<sup>+/−</sup>; mfn<sup>+/−</sup> fish.

Site-directed mutagenesis

The QuickChange site-directed mutagenesis kit (Stratagene) was used to introduce mutations into the zebrafish chordin coding sequence, starting with a previously described injection construct in the pCS2+ vector (Miller-Bertoglio et al., 1997). The sequence surrounding the upstream cleavage site was altered to TTCTTCAAACACAGA, and surrounding the downstream cleavage site to ATGGTGAGGCGAA-CGGG (altered bases are in bold). All constructs were verified by sequencing.

Construction of epitope-tagged chordins

The six-copy Myc tag was excised from the pCS2+MT vector with ClaI and Xbal and inserted following the wild-type and CM-chordin coding sequences. To verify that the tagged RNAs gave rise to full-length, stable proteins, they were subjected to in vitro translation and the resulting proteins detected by western blotting for Myc (data not shown). In initial experiments, similar amounts of tagged and untagged RNAs rescued din mutants, showing that the Myc sequences did not adversely affect protein activity or stability (data not shown). Therefore, subsequent rescue experiments were performed with the C-terminal tagged constructs.

To construct N-terminal tagged chordin vectors, a linker encoding the first 29 amino acids of Chordin was synthesized and inserted into the BamHI site of pCS2+MT. Then the remainder of the wild-type and CM-chordin coding sequences were amplified by PCR and inserted into the EcoRI and XhoI sites downstream of the Myc tags. Although placement of the Myc tag at the N-terminus does somewhat destabilize the protein, these RNAs also rescue din mutants at levels comparable with the untagged RNAs, showing that the proteins are functional.

To construct the vector encoding the N+I fragment containing the mutation of the upstream cleavage site (N+I<sub>1</sub>), the sequence encoding amino acids 1 to 849 of CM<sub>1</sub> was amplified and inserted into the pCS2+MT vector between BamHI and ClaI.

As a control for quantification of Myc-tagged Chordins, we made a construct encoding a GFP-Myc fusion protein with the signal peptide of Chordin added at its N terminus. This RNA was co-injected with tagged chordin RNAs, and the amount of its product used as an internal standard for quantification.

RNA and morpholino injections and phenotypic scoring of injected embryos

The sequence of tsg1-MO1 has been previously described (Ross et al., 2001). The non-overlapping MO5 (CCGCGAACCTCTGAGCT-GAGCAGAAC), the four-base mismatch to MO1 (CTCATGTTGAT-GAGCAGAAC), and the five-base mismatch to MO5 (CCCG-GAACCTCTGAGCAGAAC) were gifts from M. Mullins.

RNAs for injection were transcribed with the mMessage mMachine Sp6 kit (Ambion) from NotI linearized templates. RNAs were quantified by spectrophotometry and the amounts confirmed by agarose gel electrophoresis. For each injection experiment, the RNAs encoding different versions of Chordin were synthesized, purified and quantified in parallel. RNA injections were performed as previously described (Fishier and Halpern, 1999). On the following day, embryos were scored using standard phenotypic indicators of excess or decreased BMP signaling (Hammerschmidt et al., 1996; Mullins et al., 1996). For the rescue experiments, ‘ventralized’ embryos resembled din mutants (small brain and somites, excess blood in ventral tail, multiplicated fin folds), ‘rescued’ embryos appeared wild-type or mildly dorsalized (Class 1, or partially absent ventral fin fold) and ‘dorsalized’ embryos were those in Class 2-5 (more severe tail defects or truncations, tail curled on top of yolk, some or all somites expanded to encircle the yolk).

Some morphant embryos were fixed at 80% epiboly or 8 to 12 somite stages, and in situ hybridizations performed as previously described (Miller-Bertoglio et al., 1997), using bmp4, chd, eve1, gsc, gata2, krox20 (egr2b – Zebrafish Information Network) and myod as markers indicative of DV patterning.

RNA encoding Tsg1 was transcribed as above and co-injected to rescue the tsg1 morphant phenotype. The construct containing the Xenopus tsg1 coding sequence, with the signal peptide replaced with that of ECM protein BM40/SPARC, was a gift from M. O’Connor.
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injected until optimal rescue was achieved. CMAB RNA was successively lower amounts of each RNA were described previously (Fisher and Halpern, 1999). In initial experiments, successively lower amounts of each RNA were ventralized, and indicated RNAs and sorted the next day; all embryos injected with GFP indicated residues shaded in gray; on the bottom line are the changes introduced by mutagenesis (bold). (B) Embryos were injected with indicated RNAs and sorted the next day; all embryos injected with GFP. Immunoprecipitation and western blotting

Results

sequences surrounding the two Tld cleavage sites in Chordin are conserved in several vertebrate species, including zebrafish (Scott et al., 1999). We altered conserved residues at the two sites, to make them resistant to cleavage (Fig. 1A). RNAs encoding cleavage mutant Chordin with the first, second, or both, sites altered (CMA, CMB, and CMAB-chordin) were injected to rescue Chordin-deficient din−/− embryos (Fig. 1B,C) as described previously (Fisher and Halpern, 1999). In initial experiments, successively lower amounts of each RNA were injected until optimal rescue was achieved. CMAB RNA was ~10-fold more potent than wild-type chordin in the rescue assay, demonstrating the importance of Tld cleavage in vivo as a mechanism to limit Chordin activity. We predicted that partial cleavage products of Chordin would have reduced BMP-binding affinity, and therefore the partial cleavage mutants would be intermediate in efficacy between wild-type and CMAB. CMB was ~3-fold more potent than wild type, fitting this prediction. However, CMA was more potent than CMAB; a result we have repeated in several independent experiments and with multiple batches of RNA (see also Fig. 3A, Fig. 4A).

One possible explanation for the efficacy of CMA is that cleavage at the upstream site is prerequisite to downstream cleavage. To follow the processing, we injected chordin with Myc epitope tags added to the C terminus into din+ embryos, which were lysed and subjected to immunoprecipitation and western blotting (Fig. 1D, left blot). The mutations introduced to the Tld sites largely prevented cleavage as predicted, and cleavage at the two sites occurred independently. The major product from wild-type Chordin was consistent in size with the predicted C-terminal cleavage product. Quantitation of a similar blot showed that in early gastrulation, 6 hours after injection, 93% of the Myc tag was in the C-terminal fragment (data not shown), demonstrating robust, rapid cleavage activity. By contrast, CMAB gave rise to a single prominent band representing FL protein 20 hours after injection. A small amount of lower molecular weight protein could be seen; however, this did not seem to represent cleavage at the normal site, as it appears as a smear rather than a sharp band on longer exposure (data not shown). The C-terminal fragment was the predominant product from CMA, showing that cleavage at the downstream site is unaffected by mutation of the upstream site. Similarly, from CMB the major band was slightly smaller than FL protein, produced by cleavage at the upstream site. Interestingly, following injection of the same amounts of tagged RNAs and subjected to immunoprecipitation and western blotting with anti-Myc at 20 hours (C-terminal) or 5 hours (N-terminal) after injection. The 50 kDa band in the second blot, also seen in Fig. 3B, is present in negative control samples with no injected RNA. The position of molecular weight markers (kDa) and of bands corresponding to predicted cleavage products are indicated to the right of the blots.
RNA, more protein accumulated from CMB than from the other constructs, a result observed consistently in multiple experiments (see also Fig. 2A). This suggests that a sequence near the N terminus, removed by cleavage at the upstream site, destabilizes Chordin protein.

Although studies on mouse Chordin showed cleavage at an alternative site in the presence of Tsg (Scott et al., 2001), our examination of the C-terminal cleavage products failed to reveal any novel fragments. However, alternative cleavage might be evident only by examination of N-terminal fragments. Therefore, we constructed chordin RNAs with Myc epitope tags added to the N terminus, immediately following the signal peptide. When these RNAs were injected into din mutants, the major fragments corresponded to predicted cleavage products (Fig. 1D, right blot); the band at ~50 kDa is non-specific and also observed in negative control samples not injected with Myc-tagged RNA. In conclusion, we see no evidence of alternative cleavage products by examining proteins labeled at either the C or N terminus, even when cleavage is prevented at both of the normal Tld sites.

Because of the enhanced accumulation of labeled protein from CMB, we wanted to also compare the stability of the N-terminal fragments. They generally appeared less stable than C-terminal fragments, but we directly tested the effect of Myc tag position on stability. We injected two groups of embryos with the same amount of CMAB RNA, labeled at either the C or N terminus, and processed the samples in parallel. Similar amounts of labeled proteins were detected 5 hours after injection, but substantially less N-terminal labeled protein at 20 hours (data not shown). Therefore, we could not compare the stability of FL Chordin and the cleavage products over longer periods. However, over shorter time periods, there did not appear to be greater accumulation of labeled protein from CMAB than from CMAB (Fig. 1D and data not shown).

We examined the products of Chordin cleavage at several time points after injection. For wild-type Chordin, almost all detectable protein was in the form of the small C-terminal fragment 8 hours after injection (Fig. 2A). On longer exposure, small amounts of FL protein and I+C fragment were seen, and the amounts decreased slightly from 8-14 hours. For CMAB, a similar amount of FL protein was detected at the time points examined (Fig. 2A). For CMAB, significantly more total Myc tag was detected at all time points, as noted above (Fig. 2A). Although in the blot in Fig. 2A it is impossible to resolve the faint band representing FL protein in the CMAB samples, upon longer electrophoresis of additional samples, we verified that a similar amount of FL protein is present in embryos injected with CMAB, CMAB or wild-type chordin at 6 hours (Fig. 2C).

Over the period examined, we did not observe large differences in cleavage kinetics for the different forms of Chordin, but because of the rapidity of cleavage, we cannot exclude subtle differences.

Our results demonstrate robust endogenous Tld activity in the zebrafish embryo, even prior to gastrulation. The zebrafish DV patterning gene mfn encodes a Tld-related enzyme (Connors et al., 1999), shown to cleave Chordin in vitro (Blader et al., 1997). When wild-type chordin RNA was injected into mfn;din double mutants, an increase in the amount of FL protein was seen (Fig. 2B). However, the majority (>75%) of detectable Myc tag was still on the small C-terminal fragment, showing that redundant enzyme activity compensates for loss of mfn. We also observed slight increases in FL protein for both of the partial CM Chordin (Fig. 2C), indicating that Mfn does not show a strong preference for cleavage either site.

Our rescue data suggest that the N+I fragment of Chordin is a stronger BMP inhibitor than the FL protein. To test this directly, we constructed a version of chordin encoding the N+I fragment containing the mutations of the upstream cleavage site in CMAB (N+IA). We compared its efficacy in the rescue of din mutants to CMAB and CMAB-Chordin. The N+IA fragment was slightly more effective than CMAB (Fig. 3A), and both were more effective than CMAB. These CM constructs are useful for separating the effects of cleavage and binding, although they admittedly give rise to stable fragments not present endogenously. However, the N+I fragment is normally produced in the embryo from wild-type Chordin, and is present at steady-state levels comparable with the FL protein (Fig. 3B), suggesting that it could significantly contribute to BMP inhibition in the embryo.

It is possible that an additional protein participates in the binding and preferentially increases the affinity of the N+I fragment for BMPs. Although Tsg displaces the N+I fragment from BMP in vitro (Larraín et al., 2001), it has been shown to enhance the binding of other Chordin fragments and the FL protein (Chang et al., 2001; Larraín et al., 2001; Oelgeschlager et al., 2000; Ross et al., 2001; Scott et al., 2001). To test Tsg as a candidate for this additional protein, we depleted embryos of Tsg function using a previously described antisense
morpholino directed against *tsg1* (*tsg1-MO1*) (Ross et al., 2001). We rescued *din* mutants with each *chordin* RNA in the absence or presence of *tsg1-MO1*; in every case, the depletion of Tsg resulted in a greater percentage of rescued or dorsalized embryos (Fig. 4A). These data argue that Tsg does not preferentially enhance the binding of the N+I fragment, and further support a general role for Tsg in decreasing the effectiveness of Chordin as a BMP inhibitor.

To determine the mechanism of this effect, we compared the Chordin cleavage products in the absence and presence of *tsg1-MO1* (Fig. 4B). These experiments were first performed in *mfn;din* double mutants, to enhance the accumulation of FL protein and more readily reveal alterations in the ratio of cleavage products. For all versions of Chordin, more total protein was observed in the presence of *tsg1-MO1*, indicating that endogenous Tsg decreases Chordin levels (Fig. 4C). The

![Fig. 3. C-terminally truncated Chordin is a more effective BMP inhibitor.](image)
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Fig. 5. Tsg enhances BMP signaling and can act independently of Chordin. Wild-type or din–/– embryos were injected with the indicated amounts of tsg1-MO1, and photographed on the second day following injection (A-F) or fixed and processed for in situ hybridization (G-M). Some wild-type embryos injected with 3-4 ng of tsg1-MO1 (B,C) displayed a smaller brain and increased blood in the ventral tail (arrows). However, none displayed multiplication of the ventral fin fold, a prominent feature in ventralized din mutants (unfilled arrowhead in D). (E,F) Injection of tsg1-MO1 ameliorated some aspects of the din mutant phenotype; the excess development of blood was reduced (compare arrows in D-F), and in some embryos the multiplication of the fin fold was corrected (black arrowhead in F). However, anterior nervous system development was not rescued. All embryos in A-F are shown from the side, with anterior towards the left. (G-M) Embryos were injected with the indicated amounts of tsg1-MO1 or MO5, fixed at the 8- to 12-somite stage, and processed for in situ hybridization. (I,J) In wild-type embryos, we observed features of dorsalization: lateral expansion of krox20 expression (arrowhead) and myod expression. (L,M) Injection of tsg1-MO1 also dorsalized din–/– embryos. (N) Wild-type embryos injected with the indicated amounts of tsg1-MO1 were fixed and processed for in situ hybridization as above. They were sorted as having a narrowed krox20 expression domain (Narrow HB); as wild type; or as having a widened expression domain for both markers (Dorsalized). The number of embryos is beneath each bar. (O-D’) Wild-type embryos were injected with indicated amounts of tsg1-MOs, fixed at mid-gastrulation (80% epiboly) and processed by in situ hybridization for markers of DV patterning. Expression of chordin, a marker for dorsal ectoderm and mesoderm, was expanded in embryos receiving higher amounts of tsg1-MOs (S,W), and unchanged in embryos receiving a lower amount of MO1 (A’). By contrast, three different markers of ventral territories (bmp4, eve1 and gata2) were decreased (T-V, X,Z) or absent (there is a lack of eve1 in Y) in embryos receiving high amounts of either MO. In the most strongly dorsalized embryos, gsc expression, which marks the dorsal midline tissue, was expanded (unfilled arrowheads in Y). In embryos injected with 3 ng of tsg1-MO1, there was increased expression of gata2, a marker for ventral ectoderm and hematopoetic cells in the ventral mesoderm (D’); expression of the other markers of ventral territories were unchanged (B’,C’). Embryos in G-M are in dorsal view, with anterior towards the left. (G,H and K,L) Two views of a single embryo rotated to show the krox20 or myod expression. All embryos in O-D’ are shown from the animal pole, with dorsal towards the right; arrowheads indicate the lateral limits of dorsal or ventral markers.

We also did not observe products of cleavage at any site between the A and B sites, even when cleavage was prevented at those sites. When wild-type chordin was co-injected with tsg1 RNA to increase Tsg activity, we still observed no additional fragments, nor did we see a large decrease in total Chordin (Fig. 4D,E). This suggests that our failure to observe alternative cleavage was not due to insufficient Tsg.

Our data are inconsistent with previous reports that depletion of Tsg promotes BMP signaling (Blitz et al., 2003; Chang et al., 2001; Scott et al., 2001) and ventralizes the zebrafish embryo (Ross et al., 2001). To test the possibility that Tsg has different roles that predominate at different protein levels, we re-examined the tsg1 morphant phenotype, injecting different amounts of tsg1-MO1 into wild-type embryos. At a lower MO level, we observed some features suggestive of ventralization in the morphants (Fig. 5B,C,N), including a reduced anterior nervous system and blood...
accumulation in the ventral tail. However, the morphants did not have multiplicated ventral fin folds, which is a prominent feature of ventralized din and ogon mutants (Hammerschmidt et al., 1996). Importantly, at higher MO levels we observed primarily dorsalized phenotypes in the morphants (Fig. 5L,N), consistent with our rescue data and the effect of Tsg depletion on Chordin levels.

To verify our characterization of the morphant phenotypes, we analyzed tsgl morphants for markers indicative of DV patterning during gastrulation. We also performed injections with a second, non-overlapping MO targeting the tsgl 5’UTR (tsgl-MOS). At higher MO levels, we observed consistent expansion of dorsal markers and reduction of ventral markers, indicative of decreased BMP signaling, in embryos injected with either tsgl-MO (Fig. 5S-Z). Interestingly, at the lower MO level we did observe a consistent increase in the intensity of gata2 expression (Fig. 5D), although it was not accompanied by an increase in expression of other ventral markers or a decrease in expression of dorsal markers (Fig. 5A’-C’). This suggests a specific role for tsgl in limiting blood formation downstream of DV patterning, and may account for the increased blood we observe later in embryos injected with a low amount of MO.

As an additional control for the specificity of the morphant phenotypes, we co-injected tsgl-MO with tsgl RNA (data not shown). At the lower MO level, 1 pg of tsgl RNA rescued the phenotype of blood accumulation in the ventral tail, but did not correct the narrowing of the anterior nervous system, supporting our belief that it is non-specific. At the higher MO level, tsgl RNA also corrected the features of dorsalization. We also performed injections with mismatch control MOs, and observed none of the phenotypes produced with either high or low amounts of the specific MOs.

Another possible explanation for the disparate roles ascribed to Tsg is that it acts on Chordin to decrease its efficacy as a BMP inhibitor, and simultaneously inhibits BMP signaling through an independent mechanism. To test this hypothesis, we injected tsgl-MO1 into din mutants. Surprisingly, this depletion of Tsg partially rescued or even dorsalized din mutants (Fig. 5E,F,L,M). These results provide strong evidence that Tsg also can act independently of Chordin, but to further enhance rather than inhibit BMP signaling.

Discussion
Assessment of the importance of Chordin cleavage has been complicated in vertebrates by genetic redundancy. Therefore, we used a non-genetic approach, mutating conserved residues at the Tld sites in Chordin to render them resistant to cleavage. The altered Chordin was introduced into Chordin-deficient embryos and their effects on embryonic patterning analyzed. This approach allowed us to test directly the importance of Chordin cleavage, examine the cleavage products under various conditions, and determine the role of Tsg in regulating Chordin cleavage and efficacy in vivo. Although RNA injections are widely used in zebrafish embryos, and often to express mutated gene products, our study combines RNA rescue with structure/function correlations and the biochemical analysis of protein processing. Given the large number of zebrafish mutants with embryonic phenotypes that can be rescued by RNA injection, this general approach should have wide applicability in understanding signaling pathways in early development.

Chordin cleavage is an important regulatory mechanism in the zebrafish gastrula
Previously we have shown that chordin RNA injected into embryos at the one-cell stage is detectable by in situ hybridization for ~10 hours (Fisher and Halpern, 1999). Therefore, at 5 hours after injection there is still RNA available for new protein synthesis. However, at this time point the large majority of Chordin has been cleaved, demonstrating robust endogenous Tld activity even prior to gastrulation. Our data further show that cleavage at the A and B sites occurs independently and that the kinetics of cleavage are not significantly different for wild-type and CM Chordin.

The unexpectedly mild phenotype of the zebrafish mutant mfn has led to speculation that, at least in zebrafish, Chordin cleavage does not play an important role in DV patterning during gastrulation (Connors et al., 1999; Oelgeschlager et al., 2003; Zakin and De Robertis, 2004). We show instead that redundant enzyme activity compensates for loss of mfn. Both during and after gastrulation, the majority of Chordin protein is cleaved in mfn mutants, although measurably less than in wild type. In mouse, the Tld-related Bmp1 and Tll1 gene products function redundantly to cleave Chordin and other substrates (Pappano et al., 2003). The mfn gene is most closely homologous to Tll1 (Scott et al., 1999); we have identified zebrafish ESTs corresponding to a second ortholog of Tll1, which is strongly expressed in the early embryo (J.X. and S.F., unpublished). The product of this gene is a likely candidate for at least some of the Tld activity present in mfn mutants.

Positive role for Tld in Chordin regulation
Although mutating either cleavage site increased the efficacy of Chordin as a BMP inhibitor, the effect of alterations at the two sites was not equal. Prevention of cleavage at both sites resulted in a stable FL protein ~10 times more effective as a BMP inhibitor. However, by preventing cleavage at the upstream site, we created a C-terminally truncated Chordin fragment that was even more effective than the stable FL protein. Another protein may participate in Chordin-BMP binding in vivo, selectively increasing the effectiveness of the truncated Chordin. Our data indicate that Tsg is unlikely to play this role, as it decreases the efficacy of all cleavage forms of Chordin. In fact, although there is evidence from a number of in vitro binding studies that Tsg enhances the binding of FL Chordin to BMP (Larraín et al., 2001; Oelgeschlager et al., 2000; Scott et al., 2001), our data indicate that this is not its predominant role in vivo. If it were, then the efficacy of CMAB would decrease in embryos depleted of Tsg.

The N+I fragment may be more stable than the FL protein, as we observed for the I+C fragment. It is difficult to assess this because of the destabilizing effect of N-terminal Myc epitopes. However, at several time points there appeared to be comparable levels of labeled protein accumulated from the CM A and CMAB constructs (see Fig. 1D; data not shown), making this unlikely. We favor the possibility that CR4, which has little BMP binding affinity or biological activity on its own (Larraín et al., 2000; Scott et al., 2001), actually decreases the overall binding affinity of the FL protein.

There is evidence in Drosophila for an alternative cleavage
product of Sog with enhanced Dpp inhibitory activity, whose creation is promoted by Tsg (Yu et al., 2000). Tsg also enhances Chordin cleavage at an intermediate site in vitro, suggesting that a parallel event occurs for the vertebrate proteins (Scott et al., 2001). However, we see no evidence of alternative cleavage products, either when cleavage is prevented at both of the normal Tld sites or when tsgl is overexpressed by RNA injection. We cannot rule out that this cleavage event takes place in a small region of the embryo, or in specific tissues later in development. However, the necessary components (Chordin, Tld enzymes, and Tsg) are all present in the gastrula, and we should be able to detect fragments present even at less than 1% of the total label on our western blots. Therefore, we conclude that such cleavage is not likely to play a significant role in the gastrula.

Tld cleavage might also play a positive role if Chordin fragments have novel activities, independent of BMP binding. However, several lines of evidence argue against this. In particular, the epistasis between dino and the BMP mutants swirl and snailhouse has been examined (Wagner and Mullins, 2002). That study confirmed that the BMP mutant phenotypes are epistatic to dino, and importantly discovered no additional phenotypes in the double mutants, as would be expected if Chordin or its fragments had functions independent of BMP. We also find that CMAB-Chordin is capable of fully rescuing the dino phenotype, which would not be the case if the fragments had independent functions.

**Tsg decreases steady-state levels of Chordin**

To test the effect of Tsg on Chordin efficacy in our system, and the dependence of the effect on cleavage by Tld at either site, we performed rescue experiments with wild-type and CM-chordin RNAs in the absence or presence of tsgl-MO. In every case, the rescue was more effective under conditions of lowered Tsg, suggesting that Tsg normally acts to suppress Chordin function in the zebrafish embryo. To determine the molecular basis of this effect, we examined Chordin cleavage products resulting from these experiments. The consistent effect of Tsg depletion was to increase steady-state Chordin levels. It has been previously shown that Tsg has the net effect of destabilizing Chordin (Oelgeschlager et al., 2003) and that Drosophila Tsg has a similar effect on Sog (Shimmi and O’Connor, 2003), consistent with our data. However, proposed mechanisms for this destabilization have invoked Tld cleavage. We see a similar effect on levels of wild-type and CMAB-Chordin in tsgl morphants, suggesting that this effect is in part independent of Tld cleavage. We do observe a low level of cleavage or degradation of CMAB-Chordin, although it appears not to represent cleavage at the normal site, it may still be mediated by Tld. To resolve this question definitively would require elimination of all Tld activity from the zebrafish gastrula, a challenge given the genetic redundancy.

**Endogenous Tsg enhances BMP signaling through Chordin dependent and independent mechanisms**

Contradictory roles have been ascribed to Tsg in modulating vertebrate BMP signaling. However, Tsg has been reported in zebrafish embryos to cooperate with Chordin to inhibit BMPs (Ross et al., 2001). To reconcile our data with these published results, we performed additional Tsg depletions with different levels of tsgl-MOs. By injecting lower amounts, we did produce features suggestive of a ventralized phenotype. However, we did not see multiplicated ventral fin folds in any of the morphants, although this is a sensitive indicator of increased BMP signaling and is a consistent feature of the ventralized din and ogon mutants (Hammerschmidt et al., 1996). We did observe narrowing of the anterior nervous system, but morpholinos can induce non-specific toxic effects in zebrafish (Heasman, 2002), including widespread cell death and neural degeneration (Braat et al., 2001; Lele et al., 2001). In support of this possibility, increased apoptosis occurs in the brains of tsgl morphants (Little and Mullins, 2004) and is not seen in ventralized din mutants (Fisher et al., 1997). Interestingly, we did observe increased expression of gata2 in embryos receiving a lower dose of MO. Increased gata2 expression was also previously reported in tsgl morphants, and cited as evidence of their ventralization (Ross et al., 2001). However, the increase is apparently downstream of alterations in DV patterning, and may point to a specific role for tsgl in limiting formation of blood or ventral ectoderm. Injection of higher levels of tsgl-MOs dorsalized the morphants, which we confirmed by examination of multiple markers during gastrulation. Our results show that endogenous Tsg enhances BMP signaling in vivo, in part by destabilizing Chordin.

Several previous studies of the effects of Tsg in the embryo relied on RNA overexpression. In our hands, tsgl RNA dorsalizes both wild-type and din−/− embryos, showing that the effect does not depend on Chordin (data not shown). This result is apparently at odds with our analysis of tsgl morphants, and might suggest a normal role for Tsg in inhibiting BMPs. However, Tsg binds BMPs with an affinity comparable with that of individual Chordin CR domains (Chang et al., 2001; Oelgeschlager et al., 2000; Scott et al., 2001). We speculate that, when overexpressed, Tsg can bind BMPs sufficiently to prevent receptor activation, although this does not reflect its normal function. Interestingly, mutated versions of Tsg which do not bind BMPs hyperventralize the zebrafish embryo (Oelgeschlager et al., 2003), consistent with the possibility that the dorsalization seen with overexpression is due to direct BMP binding.

Many proteins in addition to Chordin contain repeated CR domains, and it has been proposed that Tsg could also interact with some of these (Garcia Abreu et al., 2002; Oelgeschlager et al., 2003). We tested the possibility that Tsg decreases the efficacy of Chordin while simultaneously inhibiting BMP signaling through another interaction. However, depletion of Tsg in din mutants ameliorated features of the mutant phenotype and even dorsalized the mutants. Thus, endogenous Tsg enhances BMP signaling both in the presence and absence of Chordin, either through direct interaction with BMPs or in conjunction with other unidentified modulating proteins. Although we cannot rule out that Tsg inhibits BMP signaling under some circumstances, we show that it does not do so in the zebrafish gastrula.

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Chordin cleavage and Twisted gastrulation in zebrafish

References


