Short- and long-range functions of Goosecoid in zebrafish axis formation are independent of Chordin, Noggin 1 and Follistatin-like 1b

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The organizer is essential for dorsal-ventral (DV) patterning in vertebrates. Goosecoid (Gsc), a transcriptional repressor found in the organizer, elicits partial secondary axes when expressed ventrally in Xenopus, similar to an organizer transplant. Although gsc is expressed in all vertebrate organizers examined, knockout studies in mouse suggested that it is not required for DV patterning. Moreover, experiments in Xenopus and zebrafish suggest a role in head formation, although a function in axially mesoderm formation is less clear. To clarify the role of Gsc in vertebrate development, we used gain- and loss-of-function approaches in zebrafish. Ventral injection of low doses of gsc produced incomplete secondary axes, which we propose results from short-range repression of BMP signaling. Higher gsc doses resulted in complete secondary axes and long-range signaling, correlating with repression of BMP and Wnt signals. In striking contrast to Xenopus, the BMP inhibitor Chordin (Chd) is not required for Gsc function. Gsc produced complete secondary axes in chd null mutant embryos and gsc-morpholino knockdown in chd mutants enhanced the mutant phenotype, suggesting that Gsc has Chd-independent functions in DV patterning. Even more striking was that Gsc elicited complete secondary axes in the absence of three secreted BMP antagonists, Chd, Follistatin-like 1b and Noggin 1, suggesting that Gsc functions in parallel with secreted BMP inhibitors. Our findings suggest that Gsc has dose dependent effects on axis induction and provide new insights into molecularly distinct short- and long-range signaling activities of the organizer.

KEY WORDS: Goosecoid, Chordin, Noggin, Follistatin-like, Axis formation, DV patterning, Zebrafish, Organizer

INTRODUCTION

Formation of the vertebrate body plan depends on the organizer. Originally identified in amphibians (Spemann and Mangold, 1924) and later in fish (Ho, 1992; Oppenheimer, 1934; Saúde et al., 2000; Shih and Fraser, 1995), chick (Waddington, 1932) and mouse (Beddington, 1994), transplantation of dorsal organizer tissue to ventral regions of a host embryo leads to secondary axis formation. Whereas the transplanted organizer contributes predominantly to axial mesoderm, it also has the remarkable capacity to non cell-autonomously re-specify host cells from their original fates and recruit them to compose the remaining tissues of the secondary axis.

In zebrafish, the organizer/shield elicits a secondary axis when transplanted ventrally (Saúde et al., 2000; Shih and Fraser, 1996). By convention the shield marks dorsal but, because dorsal and anterior development are linked, dorsal and anterior tissues arise from the shield side of the embryo, whereas posterior and ventral structures arise from non-shield regions (Schier and Talbot, 2005). The shield contributes to axial mesoderm and makes smaller contributions to paraxial mesoderm, ventral neural tissue, and skin (Saúde et al., 2000; Shih and Fraser, 1995; Shih and Fraser, 1996).

In Xenopus and zebrafish, maternal Wnt signaling results in nuclear accumulation of β-catenin on the presumptive dorsal side of the embryo, where it activates expression of zygotic organizer genes. Nodal signaling is also involved in initial organizer formation (Erter et al., 1998; Feldman et al., 1998), whereas later in development both Nodal and Wnt are inhibitory to organizer function. Historically, the organizer was thought to express inducers of dorsal cell fates; however, it is now well established that a main function of the organizer is to repress factors secreted from ventral regions of the embryo (Niehrs, 2004).

Secreting ventralizing factors of the BMP, Wnt and Nodal families function in gradients in the early embryo and organizer-derived molecules attenuate the activity of these factors (Niehrs, 2004). Ventrally expressed Wnt8 restricts the size of the zebrafish organizer in the late blastula/early gastrula by regulating the expression of the transcriptional repressors Vox and Vent, whereas BMP signaling is required to maintain expression of these genes during late gastrulation (Ramel et al., 2005; Ramel and Lekven, 2004). Thus, Wnt and BMP act together to limit the organizer and to promote ventral development.

A distinctive feature of the organizer is its ability to influence distant cell fates as well as to generate axial tissues. Surprisingly, investigation of the interrelationship between these short- and long-range organizer activities has not been a major research focus. It is known that many genes implicated in organizer activity have highly overlapping functions, making it difficult to determine the precise roles that individual genes play. Here we investigate basic questions about organizer activity, including: its short- and long-range signaling functions, the extent to which they are linked and the mechanisms underlying the redundancy of organizer gene activity.

We focused on the first organizer gene discovered, goosecoid (gsc), which encodes a homeodomain transcription factor (Blumberg et al., 1991). gsc is expressed in all vertebrate organizers examined, suggesting a fundamental role in organizer function (Blumberg et al., 1992; Blumberg et al., 1991; Izsisa-Belmonte et al., 1993; Schulte-Merker et al., 1994; Stachel et al., 1993). gsc has been most studied in Xenopus, where gain-of-function experiments demonstrated that Gsc induces secondary axes, similar to an organizer transplant (Cho et al., 1991; Niehrs et al., 1993; Sander et al., 2007). However, secondary axes were often incomplete, lacking head and notochord. Loss-of-function experiments using dominant-negative and
antsense gsc constructs led to either head or head and notochord reductions (Ferreiro et al., 1998; Steinbeisser et al., 1995; Yao and Kessler, 2001). Results of gsc morpholo oligonucleotide injections demonstrated that Gsc is required for head but not notochord formation (Sander et al., 2007). In addition, Sander et al. suggested that a major function of Gsc is to block the expression of the ventralizing transcription factors Vent1/2. It has also been shown that ectopic Gsc represses expression of the ventralizing factors XWnt-8 and BMP4 (Christian and Moon, 1993; Fainsod et al., 1994; Steinbeisser et al., 1995).

A gene that is activated by ectopic Gsc, presumably indirectly, is the organizer gene chd, which encodes an extracellular inhibitor of BMPs (Piccolo et al., 1996; Sasai et al., 1994). Xenopus Chd is an essential downstream effector of Gsc function (Sander et al., 2007). The Xenopus work suggests that Gsc plays an important role in organizer function. In sharp contrast, targeted gsc knockout in mouse had no effect on early DV patterning (Rivera-Perez et al., 1995; Yamada et al., 1995). However, in heterotypic transplantation experiments the neural-inducing properties of the mutant organizer were impaired (Zhu et al., 1999). Technical issues might explain some of the differences in the severity of these effects, whereas real differences between species could stem from the known redundancy in DV patterning mechanisms, which might vary between different organisms. In either case, there are many unanswered questions that warrant further investigation.

In zebrafish, gsc transcripts are present maternally, with zygotic expression beginning at the midblastula transition in the region of the future organizer (Schulte-Merker et al., 1994; Stachel et al., 1993). Typically, embryos with mutations that disrupt the organizer have little or no gsc expression and reduced dorsal structures (Feldman et al., 1998; Gritsman et al., 1999; Kelly et al., 2000; Koos and Ho, 1999; Nojima et al., 2004; Sampath et al., 1998; Schier et al., 1997; Yamanaka et al., 1998), whereas gsc expression is expanded in embryos dorsalized by lithium chloride treatment (Stachel et al., 1993). Thus gsc transcript levels correlate with organizer activity. In loss-of-function studies, injection of gsc morpholinos cause head truncations in a small fraction of embryos without affecting the notochord, whereas head defects occur at higher frequency when morpholinos against gsc and foxa3 were combined (Seiliez et al., 2006). As in Xenopus, ectopic zebrafish Gsc represses wnt8 (Seiliez et al., 2006).

To investigate the molecular basis of organizer activity, we examined Gsc function in zebrafish. Ventrinal injection of low doses of gsc mRNA produced partial secondary axes that reflect a short-range activity of the organizer, whereas higher doses led to complete secondary axes, mimicking the long-range signaling activity of the organizer. We propose that short-range signaling is accompanied by BMP repression and long-range signaling by BMP and Wnt repression. Surprisingly, Chd is not essential for Gsc function in zebrafish, unlike in Xenopus. In fact, Gsc exhibited organizer activity in the absence of three secreted BMP antagonists, suggesting that Gsc functions in a parallel pathway to BMP inhibitors.

MATERIALS AND METHODS

Zebrafish

Wild-type AB and chordin(−) embryos were obtained from the Zebrafish International Resource Center (ZIRC, Eugene, OR, USA). Adult chordin(−) were a gift from M. Mullins (University of Pennsylvania, Philadelphia, PA, USA). Embryos obtained by natural spawning were staged as described (Kimmel et al., 1995). Animals were treated in accordance with the policies of the local animal care committee.

Constructs and morpholinos

gsc and follistatin-like 1b (flsl1b) ORFs were cloned from cdna into pCS2+ (Rupp et al., 1994). mRNAs were transcribed using the SP6 mMESSAGE mMACHINE Kit (Ambion, Austin, TX, USA). mRNAs from chordin-pCS2+ and noggin-pCS2+ (nog1-pCS2+) were prepared as described (Dal-Pra et al., 2006; Miller-Bertoglio et al., 1997).

Morpholino (MO) sequences (5′ to 3′):

- gsc-MO: CAAGCGAAAGATGTGTGAGATTG (Open Biosystems, Huntsville, AL, USA) (Seiliez et al., 2006);
- chd-MO: ATCCACAGCACCTTCACTATCC (Gene Tools, Philomath, OR, USA);
- nog1-MO: GCGGGAAATCCATCCTTTGAATACTC (Gene Tools) (Dal-Pra et al., 2006);
- fsrlb-MO: CCATATTACAACCTACCTGGACTGG (Open Biosystems); and
- standard control: CCTCTAATGTAGTGAATTATA (Gene Tools).

gsc constructs

The gsc homeobox and 3′ sequence (codons 126 to 241) were cloned into pVP16-N and pENG-N (Kessler, 1997) to generate VP16-gsc (VP-gsc:HD) and engrailed-gsc (eng-gsc:HD), respectively. A Xeno\ncus construct consisting of the Xenopus gsc ORF with two minimal VP16 domains at the C-terminus was a gift from J. Smith (Latinkic and Smith, 1999). To create a zebrafish version (gsc-VP2), Xenopus gsc was removed and replaced with the zebrafish gsc ORF.

Microinjections

Initial gsc microinjections were done double blind. Approximately 4 pl of mRNA was injected into a single cell of 8-cell stage embryos as described (Bruce et al., 2003). gsc mRNA (12, 24 or 48 pg) and chd mRNA (200, lower concentrations had little effect, or 664 pg) were injected. gfp mRNA doses ranged from 130 to 340 pg. gsc constructs were injected at the following doses: 660 pg VP-gsc:HD, 24 pg eng-gsc:HD and 200-520 pg gsc-VP2. For subthreshold experiments gsc was injected at 4 pg and gsc-VP2 at 100 pg.

Approximately 100 pl of gsc, chd, nog1, fsrlb, and control MOs were injected into the yolk at the 1- to 2-cell stage. gsc-MO (330 pg) was injected and nonspecific phenotypes, which were not rescued by coinjection of gsc mRNA, included mild head necrosis and general developmental delay. The presence of the MO target site was confirmed by PCR and sequencing. chd-MO injected at 22 pg produced no phenotype and chd-MO injected at 100 pg ventralized embryos. nog1- and fsrlb-MOs (3 ng) were injected, producing results as described (Dal-Pra et al., 2006). Dal-Pra et al. observed a range of phenotypes falling into three phenotypic classes, whereas we observed phenotypes that fell predominantly into one class. This is likely to be due to the fact that we injected into the chd mutants, whereas Dal-Pra et al. primarily employed chd-MO.

Morpholino rescue experiments

Xenopus chd mRNA (Sasai et al., 1994) was used to rescue the effects of the chd-MO, as described (Nasevicius and Ekker, 2000). Other mRNAs were generated from constructs that either did not contain the MO binding site (gsc, fsrlb) or contained four silent mutations to prevent MO binding (nog1).

Genotyping

Genotyping of embryos from chordin(−) heterozygous parents was performed as described by Oegeschläger (Oegeschläger et al., 2003) and ZIRC.

Time-lapse

Shield stage embryos were mounted in 3% methylcellulose. Velocity (Improvision, Lexington, MA, USA) was used to acquire DIC and fluorescent images at 5-minute intervals.

In situ hybridization

In situ hybridizations were performed as described (Jowett and Lettice, 1994) using riboprobes to bmp2b and bmp4 (Martinez-Barbera et al., 1997), chd (Miller-Bertoglio et al., 1997), dkk (Hashimoto et al., 2000), dlx2a (Akimenko et al., 1994), flh (Telbot et al., 1995), shha (Krauss et al., 1993) and wnt8 (Kelly et al., 1995).
Immunohistochemistry
Embryos were fixed, blocked and incubated in primary antibody as described (Bruce et al., 2001). The peroxidase anti-peroxidase method was used. Anti-Ntl antibody was diluted 1:5000 (Schulte-Merker et al., 1992), goat anti-rabbit secondary antibodies and rabbit peroxidase anti-peroxidase tertiary antibodies were diluted 1:100 and 1:500, respectively (Jackson ImmunoResearch, West Grove, PA, USA).

Cell counts
Embryos between bud and 2-somite stages, stained with anti-Ntl antibody, were flat-mounted and photographed on a Zeiss Axiolmaier Z1 compound microscope using an Orca-ER camera (Hamamatsu, Bridgewater, NJ, USA). Stained nuclei visible in each focal plane, excluding the tailbud, were traced onto transparencies and counted.

RESULTS
gsc-overexpressing cells populate organizer derivatives
To examine the results of localized gsc overexpression, gfp mRNA or gfp together with gsc mRNA was injected into one cell at the 8-cell stage. Several doses were tested to determine the lowest dose (24 pg) of gsc mRNA that consistently produced secondary axes (see below). Because there is no direct correlation between the position of a cell at the 8-cell stage and the location of its descendants along the DV axis (Abdelilah et al., 1994; Helde et al., 1994; Kimmel and Law, 1985; Kimmel and Warga, 1987), clone location was documented relative to the shield and embryos were sorted into dorsal, dorsolateral, lateral, ventral and ventrolateral classes at 6 hours post-fertilization (hpf) (Fig. 1L). At 1 day post-fertilization (dpf), sorted embryos were scored for GFP-expressing cells in shield-derived structures: hatching gland, notochord, floor plate of the spinal cord and hypochord (Saúde et al., 2000; Shih and Fraser, 1995; Shih and Fraser, 1996). In striking contrast to gfp control clones, every gsc clone labeled one or more shield derivative, regardless of its initial location (see Table S1 in the supplementary material). This occurred in two ways: gsc-expressing cells contributed either to enlarged dorsal axial structures or to axial structures in a secondary axis. Thus, gsc-overexpressing cells populated tissues normally derived from the shield.

Analysis of ventral clones allowed us to examine Gsc function in isolation from other organizer factors. gsc-injected embryos with ventral/ventrolateral clones gave rise to secondary axes at high frequency, whereas dorsal/dorsolateral gsc clones and gfp control clones did not (see Table S2 in the supplementary material). As expected, in controls with ventral clones (Fig. 1A), GFP-positive cells were located posteriorly at the 1-somite stage and were absent from axial mesoderm (Fig. 1B) and, at 1 dpf, GFP-positive cells were located mainly in the myotomes of the trunk and tail (Fig. 1C). By contrast, gsc-injected embryos with ventral clones (Fig. 1D) often had two axes on opposite sides of an elongated yolk cell at bud stage, one of which was GFP labeled (Fig. 1E). Elongated yolk morphology is also seen with dorsolized embryos (Mullins et al., 1996). GFP-positive cells populated axial tissues of the secondary axis, including an apparent prechordal plate (Fig. 1E,F) and a secondary notochord (Fig. 1F). Secondary axes did not always contain notochord at bud stage (see Table S3 in the supplementary material) although they did by 1 dpf.

Two distinct axes were often no longer apparent by 1 dpf, and would have been missed if bud-stage embryos had not been examined. At 1 dpf, the severely abnormal embryo contained a broad head with two eyes (Fig. 1G) and a partially GFP-labeled notochord (Fig. 1H), thus the two axes moved together between bud and 1 dpf. When double axes were generated, they moved together in 36% (13/36) of cases, whereas in 58% (21/36) of cases the two axes remained distinct, although they shared a single posterior trunk and tail. Expression of the neural marker dlx2a (Akimenko et al., 1994) was often normal in secondary axes, indicating the presence of telenepcephalic and diencephalic tissue (Fig. 1J,K; see Table S4 in the supplementary material). Therefore secondary axes were essentially complete, containing notochord and anterior-most brain, indicating that gsc overexpression mimicked an organizer transplant.

To determine whether Gsc has a role in organizer function dorsally, where it is normally expressed, we examined embryos with dorsal/dorsolateral gsc clones and observed the effect on DV patterning. In controls (Fig. 2A), GFP was predominantly confined to shield derivatives at bud and at 1 dpf (Fig. 2B-D), consistent with previously described fate maps (Kimmel et al., 1990). The distribution of dorsal/dorsolateral gsc clones was similar to controls (Fig. 2E-H) and contrasted with results in Xenopus, in which dorsal gsc-overexpressing cells were excluded from axial tissues (Niehrs et al., 1993).

The single axes in gsc-injected embryos often had enlarged shield derivatives, but thinner posterior trunks and tails, suggesting the presence of excess dorsal/anterior tissue at the expense of ventral/posterior tissues. For example, the notochord domain of an embryo with a dorsolateral gsc clone was much larger than the control (see Figs S2B and S2F in the supplementary material). To quantify this observation, notochord nuclei were stained in late gastrula stage embryos using the No tail (Ntl) antibody (Schulte-Merker et al., 1992). There was a 40% increase in Ntl-positive nuclei in embryos with dorsal, dorsolateral, or lateral gsc clones (Fig. 2I).
A representative embryo, with a ventrolateral gsc clone, contained a secondary GFP-labeled notochord, whereas its associated myotomes were unlabeled (Fig. 3A-C). In another embryo, neural tissue of a secondary axis contained labeled and unlabeled cells (Fig. 3F-H). Unlabeled cells were morphologically normal and well integrated into the tissue. Thus, just like an organizer transplant, gsc-overexpressing cells recruited surrounding cells, causing them to change their fate and participate in secondary axis formation. This non cell-autonomous activity of Gsc is most likely to be indirect, as Gsc is a transcription factor.

We used a molecular readout to measure the distance over which Gsc exerts its non cell-autonomous effect by examining the expression of the organizer gene chd in embryos with ventral gsc clones. Double labeling of membrane GFP (to mark the clone) and chd showed that ectopic chd was induced at a distance of up to ten cell diameters away from the gsc clone (Fig. 3D). Signaling over this large distance constitutes long-range signaling, as defined by Chen and Schier for the zebrafish protein Nodal-related 1 (Chen and Schier, 2001). Thus, Gsc exhibits long-range signaling activity by a molecular criterion, which is probably responsible for the long-range organizer activity observed morphologically. Another potential explanation is that dorsal chd-expressing cells migrated towards the ventral gsc clone. We eliminated this possibility by labeling the shield with Rhodamine Dextran in embryos with ventral gsc clones and observing no movement of Rhodamine positive cells towards ventral gsc clones in live embryos (not shown).

We next asked whether gsc had dose dependent effects on axis formation by injecting half the standard dose (12 pg). Partial secondary axes were produced that lacked heads and notochords and consisted only of neural and somitic tissue that was nearly completely GFP labeled (Fig. 3I-K). Only a very few unlabeled cells were recruited to the partial axis (Fig. 3K, insets), suggesting that short-range signaling occurred. Examination of ectopic chd expression in ventral gsc clones revealed that chd was primarily confined to the clone itself and only extended at most one or two cell diameters away (Fig. 3E). Thus, lower gsc doses acted predominantly cell-autonomously and were unable to elicit long-range signaling.

gsc also had dose dependent effects on DV gene expression at shield stage. At doses of gsc sufficient to induce complete secondary axes, we observed a reduction in wnt8, bmp2b and bmp4 expression and ectopic expression of the organizer genes chd, floating head (flh) and dickkopf 1 (dkk1) (Fig. 3L-O; Table 1). In embryos injected with a lower dose of gsc, chd was induced at similar frequencies but dkk1 (a Wnt signaling inhibitor) was induced only about half as often, and the frequency of wnt8 inhibition was also moderately but consistently reduced (Table 1). These results suggest that partial secondary axes produced by low gsc doses might result from inhibition of BMP signaling but insufficient inhibition of Wnt signaling. In addition, we noticed that at the standard gsc dose, wnt8 was always repressed in a relatively small domain, whereas chd was consistently induced in a large domain, that extended outside the gsc clone. This spatial arrangement mimics what is normally seen in the organizer, suggesting that Gsc is able to induce this organizer pattern in a dose dependent fashion.

**gsc overexpression has non cell-autonomous and dose dependent effects on cell fate**

The organizer is defined by its ability to re-specify and pattern surrounding cells. Therefore, recruitment of unlabeled cells into Gsc-induced secondary axes would demonstrate organizer activity.

**gsc overexpression induces secondary axes in chd null mutants**

In *Xenopus*, Gsc function is mediated entirely by Chd (Sander et al., 2007). To determine if Chd was required for Gsc function in zebrafish, Gsc activity was examined in the absence of Chd by

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**Fig. 2. Dorsal gsc overexpression induces excess dorsal tissue.**

(A-H) Merged images of live zebrafish embryo injected with gfp (A-D) or gsc (E-H) RNA. (A,E) Shield (arrowhead). (B,F) Bud, arrowheads indicate notochord. (C,D,G,H) 1 dpf. (I) Notochord cell counts with standard deviations, five embryos per treatment. (J) Bud, anterior to the top. Anti-Ntl staining is brown, injected RNA is listed in lower right. hypo, hypochord; peri, periderm.
overexpressing gsc in chd<sup>250</sup> null mutant embryos (Schulte-Merker et al., 1997). chd<sup>250</sup> mutants have expanded ventral tissues, most notably blood, blood island and tail fin, and have reduced anterior neural tissue, whereas gsc expression is normal in most mutants (Fisher et al., 1997; Hammerschmidt et al., 1996; Schulte-Merker et al., 1997). gsc was injected into one cell at the 8-cell stage and embryos were genotyped at the end of the experiment. At the standard gsc dose (24 pg), the majority of mutant embryos with ventral/ventrolateral clones (Fig. 4A) contained partial secondary axes with neural and somitic tissue but not notochord (Fig. 4D), similar to the overexpression of a low dose of gsc in wild-type embryos. However, these axes occasionally had well developed heads, separate from the endogenous head (Fig. 4B). Interestingly, the endogenous head was often more fully developed than in uninjected chd mutants (Fig. 4B), suggesting that ventral gsc injection could partially rescue the endogenous axis, indicative of a long-range effect.

Strikingly, doubling the gsc concentration (48 pg) resulted in complete secondary axes with GFP-labeled notochords (Fig. 4E-G). Thus, in the absence of chd, a higher concentration of gsc was needed to induce complete axes, suggesting that Chd facilitates, but is not essential for, Gsc-induced axis formation.

**chd and gsc overexpression are not equivalent**

Since Chd facilitates the ability of Gsc to induce secondary axes, we next addressed whether it was sufficient to induce complete secondary axes by overexpressing chd ventrally. Global chd overexpression in zebrafish dorsalizes embryos (Miller-Bertoglio et al., 1997) however, localized overexpression was not examined. To test the axis-forming ability of Chd in zebrafish, 200 pg of chd mRNA was injected into one cell at the 8-cell stage. Ventral/ventrolateral chd clones generated partial secondary axes that never contained head or notochord and that merged with the endogenous axis posteriorly. Morphologically, secondary axes contained neural tissue (Fig. 5C,D), most had ectopic myotomes (Fig. 5E,F), some had beating cardiac tissue (not shown) and ectopic or enlarged otic vesicles (Fig. 5G,H). Notably, these partial axes resembled those induced by low gsc doses. Chd did not induce ectopic Gsc (see Fig. S3 in the supplementary material). dll2a expression was not detected in the secondary neural tube and flh and wnt8 expression were normal at shield stage (not shown). Interestingly, secondary axes were typically well separated from the primary axis, in contrast to those seen following gsc overexpression. In addition, partial secondary axes were nearly completely GFP labeled, suggesting that chd did not have long-range inductive effects.

### Table 1. DV markers at shield stage in gsc-injected embryos

<table>
<thead>
<tr>
<th>Injected mRNA</th>
<th>wnt8</th>
<th>bmp2b</th>
<th>bmp4</th>
<th>chd</th>
<th>flh</th>
<th>dkk1</th>
</tr>
</thead>
<tbody>
<tr>
<td>gfp</td>
<td>13/71 (18%) reduced</td>
<td>2/24 (9%) reduced</td>
<td>2/38 (5%) reduced</td>
<td>2/46 (4%) ectopic</td>
<td>0/60 ectopic</td>
<td>0/12 (uninjected) ectopic</td>
</tr>
<tr>
<td>gsc (24 pg) + gfp</td>
<td>19/30 (63%) reduced</td>
<td>23/30 (77%) reduced</td>
<td>24/38 (63%) reduced</td>
<td>78/80 (98%) ectopic</td>
<td>54/62 (87%) ectopic</td>
<td>11/12 (92%) ectopic</td>
</tr>
<tr>
<td>gsc (12 pg) + gfp</td>
<td>14/30 (47%) reduced</td>
<td>n.d.</td>
<td>n.d.</td>
<td>88/93 (95%) ectopic</td>
<td>n.d.</td>
<td>13/25 (52%) ectopic</td>
</tr>
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</table>

Ventral markers appear less strongly affected than dorsal markers. This is likely to be a technical issue, as dorsal marker probes typically produced stronger signals than ventral marker probes, making these embryos easier to score. n.d., not defined.
Tripling the concentration of chd (664 pg) also produced partial secondary axes (not shown). Therefore, no chd dose tested could elicit a complete secondary axis. Thus, Chd neither had organizer properties, nor did it generate axial mesoderm, the primary organizer derivative.

**gsc overexpression induces secondary axes in the absence of three BMP inhibitors**

The ability of Gsc to induce secondary axes in chd mutant embryos could be explained by the presence of other, compensatory, BMP inhibitors. The BMP inhibitors Noggin1 (Nog1) and Follistatin-like 1b (Fstl1b) were reported to be zygotically expressed and to function redundantly with Chd (Dal-Pra et al., 2006; Fürthauer et al., 1999). As shown previously, coinjection of nog1- and fstl1b-MOs into chd mutant embryos enhances the mutant phenotype (see Fig. S3 in the supplementary material) (Dal-Pra et al., 2006). mRNA rescue experiments demonstrated morpholino specificity (see Fig. S4 in the supplementary material). When gsc was injected ventrally into embryos injected with nog1- and fstl1b-MOs, both partial (Fig. 5I,J) and complete (Fig. 5K,L) secondary axes were observed. Therefore, Gsc induced complete secondary axes when all known zygotic BMP antagonists expressed in the early embryo were reduced or eliminated. In addition, the head of the endogenous axis was partially rescued in embryos with ventral gsc clones, further demonstrating the ability of Gsc to trigger long-range effects (not shown).

A caveat to these results is that the morpholino knockdowns might have been incomplete. However, in wild-type embryos, ventral overexpression of nog1 induced only partial secondary axes, lacking notochords, whereas ventral overexpression of fstl1b had no effect (not shown), similar to previous results in which these two genes were globally overexpressed (Dal-Pra et al., 2006). Crucially, ventral coinjection of chd, fstl1b and nog1 mRNAs in wild-type embryos also induced only partial secondary axes (Fig. 5M,N). These data indicate that Gsc can function in the absence of all three secreted BMP inhibitors.

**Distinct mechanisms underlie long- and short-range signaling**

We next addressed whether Gsc is normally involved in DV patterning. No gsc mutant exists, and maternal gsc expression raises the possibility that maternal protein is present. For these reasons, we initially took a dominant-negative approach to block Gsc function. Gsc is a transcriptional repressor in other systems (Danilov et al.,...
1998; Ferreiro et al., 1998; Latinkic and Smith, 1999; Mailhos et al., 1998; Smith and Jaynes, 1996). Therefore, we made two activator constructs designed to antagonize endogenous Gsc function. VP-gscHD contains the gsc homeodomain (HD) fused to the VP16 transcriptional activator (Kessler, 1997) and is predicted to bind to and activate transcription of Gsc target genes. The second construct, gsc-VP2, contains the entire gsc coding region plus two minimal VP16 domains and, in Xenopus, the equivalent construct blocks Gsc function by binding to Gsc target genes without activating their transcription (Latinkic and Smith, 1999). Thus, gsc-VP2 should produce a phenotype more akin to Gsc loss-of-function than VP-gscHD. We also generated a repressor construct, consisting of the gsc HD fused to the engrailed repressor domain (eng-gscHD) (Kessler, 1997), that should mimic endogenous Gsc function. Similar constructs were originally used in Xenopus (Kessler, 1997; Latinkic and Smith, 1999) and our zebrafish versions performed as expected in xenopus embryos (see Fig. S5 in the supplementary material and not shown).

Ventral injection of eng-gsc(HD) (24 pg) into zebrafish often produced complete secondary axes with GFP-positive cells located in organizer derivatives (Fig. 6A,B), demonstrating that Gsc functions as a transcriptional repressor in zebrafish. As predicted, ventral injection of VP-gsc(HD) had no effect, even at high doses (660 pg, not shown). To test whether VP-gsc(HD) could inhibit Gsc function, we examined its ability to block secondary axis formation by Gsc and found, surprisingly, that it could not (not shown). However, VP-gsc(HD) blocked eng-gsc(HD) from eliciting complete secondary axes, leading to partial axes instead (Fig. 6H,I). This suggested that VP-gsc(HD) could block the long-range organizer activity of eng-gsc(HD), producing an effect similar to injection of a low gsc dose.

Unexpectedly, ventral injection of gsc-VP2, at a moderate dose (200 pg) that was predicted to block Gsc function, occasionally produced partial secondary axes that were almost completely labeled by GFP (Fig. 6C,D). High concentrations (520 pg) also produced partial secondary axes. Thus, complete axes could not be induced at every dose tested, again mimicking the effect of low dose gsc. An analysis of chd expression in gsc-VP2-injected embryos revealed that ectopic chd was transiently induced, presumably accounting for the infrequent partial secondary axes observed (Fig. 6E-G and see Table S5 in the supplementary material). Ectopic expression of Xenopus gsc-VP2 (Latinkic and Smith, 1999) or dominant-negative Xenopus myc-tagged gsc (Ferreiro et al., 1998) also occasionally produced partial secondary axes in zebrafish (not shown). Significantly, we never observed partial secondary axes in Xenopus using either the frog or zebrafish constructs.

These findings suggest that constructs designed to block Gsc function in Xenopus could partially mimic the short-range function of Gsc in zebrafish. To confirm this, gsc was injected at a subthreshold dose (4 pg). This gsc dose had no effect alone but dorsalized embryos (6/7, 86%) when co-injected with a reduced dose of gsc-VP2 (100 pg), suggesting a synergistic effect.

Gsc and endogenous dorsal specification
Since the dominant-negative constructs failed to fully block Gsc function, we used gsc morpholinos instead (Seiliez et al., 2006). gsc-MO had mild ventralizing effects, as shown previously (Seiliez et al., 2006). However, morphologically, most embryos appeared normal (Fig. 7E). To further investigate the extent to which Gsc requires Chd and to explore potential synergistic effects, we used a fixed concentration of gsc-MO in combination with chd250 mutant embryos and two different doses of chd-MO to generate embryos possessing no, low or intermediate levels of Chd (Nasevicius and Ekker, 2000). Morpholino control experiments confirmed that the effects were specific (see Fig. S4 in the supplementary material).

Firstly, gsc-MO was injected into chd250 mutants. gsc-MO exacerbated the chd250 phenotype, leading to a more severe reduction in head and notochord (Fig. 7D,H,L,P). Thus, endogenous Gsc plays a role in DV patterning that is independent of Chd, consistent with our overexpression results. We then examined the effect of gsc-MO on embryos in which Chd levels were reduced, using concentrations of chd-MO that produced either a less severe phenotype than that of chd250 mutants (Fig. 7C) or no phenotype at all (Fig. 7B). At both concentrations of chd-MO, coinjection of gsc-MO resulted in more severely ventralized embryos with further reduced heads and enlarged ventral structures (compare Fig. 7F to 7B and Fig. 7G to 7C). Ntl antibody staining was used to examine the notochord and in situ hybridization for dlx2a was used to examine the anterior brain. Consistent with the increased ventralization observed by external morphology, we found that notochord and anterior neural structures were reduced when gsc-MO was added to chd-depleted embryos (Fig. 7I-P; Table 2 and not shown).

Analysis of DV markers at shield stage revealed no obvious changes in bmp2b, chd, or flh expression in gsc-MO injected embryos. bmp2b and flh expression were normal in chd-MO and double gsc-MO/chd-MO injected embryos (not shown). A slight dorsal expansion of wnt8 in a small percentage of gsc-MO injected
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Table 2. Notochord morphology assessed by anti-Ntl staining at 1 dpf

<table>
<thead>
<tr>
<th>Injected mRNA</th>
<th>Normal</th>
<th>Thin posteriorly</th>
<th>Small gaps posteriorly</th>
<th>Large gaps posteriorly</th>
<th>Absent from tail</th>
<th>Absent posterior to yolk extension</th>
<th>Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninjected (n=111)</td>
<td>111 (100%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gsc-MO (n=81)</td>
<td>81 (100%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chd-MO-I (n=71)</td>
<td>71 (100%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gsc-MO + chd-MO-I (n=81)</td>
<td>14 (17%)</td>
<td>50 (62%)</td>
<td>17 (21%)</td>
<td></td>
<td>5 (11%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chd-MO-h (n=47)</td>
<td>2 (4%)</td>
<td>34 (72%)</td>
<td>6 (13%)</td>
<td></td>
<td>8 (17%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gsc-MO + chd-MO-h (n=37)</td>
<td>17 (46%)</td>
<td>8 (22%)</td>
<td>12 (32%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chdtt250 mutants (n=23)</td>
<td>16 (70%)</td>
<td>5 (21%)</td>
<td>2 (9%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gsc-MO + chdtt250 mutants (n=22)</td>
<td>9 (41%)</td>
<td>3 (14%)</td>
<td>2 (9%)</td>
<td></td>
<td>7 (32%)</td>
<td>1 (4%)</td>
<td></td>
</tr>
</tbody>
</table>

chd-MO-I, low concentration of morpholino; chd-MO-h, high concentration of morpholino.
Gsc and long-range signaling

Increasing the gsc dose ventrally mimics both the short- and long-range activities of the organizer, resulting in complete secondary axes, in which GFP-labeled cells are predominantly confined to organizer derivatives. Large-scale recruitment of unlabeled cells indicates that long-range signaling occurred. A demonstration of long-range signaling at the molecular level is that ectopic chd was induced at a distance from gsc clones; however, as discussed below, chd cannot mediate the long-range signal. We and others have observed that complete secondary axes move towards and often merge with the primary axis. By contrast, we found that partial secondary axes did not merge, suggesting that long-range signals are responsible for this phenomenon.

Analysis of DV marker expression demonstrated that high gsc doses resulted in inhibition of both BMP and Wnt signaling. In Xenopus, low levels of BMP and Wnt signaling are required dorsally for notochord formation and BMP, and Wnt signaling must be repressed ventrally for ectopic notochord induction (Yasuo and Lemaire, 2001). In zebrafish, overexpression of wnt8a or inhibition of the Wnt antagonist dkk1 results in head truncations (Seiliez et al., 2006), whereas overexpression of dkk1 and deletion of wnt8 produces expanded brain and axial mesoderm (Hashimoto et al., 2000; Lekven et al., 2001). Although axial mesoderm is unaffected in BMP mutants, when both wnt8 and bmp2b function are removed, axial mesoderm expands (Ramel et al., 2005). Thus, the ability of Gsc to induce complete secondary axes ventrally and increase notochord cell number dorsally is probably the result of combined inhibition of Wnt and BMP signaling. Gsc directly represses wnt8 transcription in Xenopus and we propose that this is likely to be the case in zebrafish as well (Fig. 8). Gsc might also directly inhibit expression of the ventralizing factors ved/vox/vent as it does in Xenopus (Sander et al., 2007).

What is the molecular identity of the long-range signal(s)? Induction of head and notochord was always accompanied by cell recruitment, indicative of long-range signaling. However, it is notable that we could see evidence of long-range signaling in the absence of notochord induction. Ventral injection of gsc into chd mutants at the dose that gave rise to partial secondary axes often resulted in more fully developed endogenous heads. We also found that ventral overexpression of dkk1 led to enlarged endogenous heads, suggesting that it could be the long-range signal, although dkk1 did not elicit complete secondary axes when overexpressed ventrally (our unpublished data). We also tested whether coinjection of dkk1 and chd ventrally could elicit complete secondary axes and found that it could not (our unpublished data). One possibility is that a combination of known factors is required, for example, Dkk1 might act in combination with other Wnt signaling inhibitors. Alternatively, an unknown factor(s) might be involved (Fig. 8, Factor Y). A recent study showed that implanted cells from early zebrafish gastrula and pharyngula cell lines could induce secondary axes by inducing organizer gene expression in host tissue, but this was not mediated by known organizer inducers, including Nodals, Bozozok, and FGFs, leading the authors to suggest that an unknown secreted factor was responsible (Hashiguchi et al., 2008). Clearly, additional experiments are required to identify these factors and to characterize their interactions.

Gsc does not require Chd

Xenopus Gsc function relies entirely on Chd (Sander et al., 2007). Furthermore, Xenopus Chd is required for complete secondary axis induction by ventral organizer transplantation (Oelgeschläger et al., 2003). Thus, it appears that Xenopus Chd can mediate both short-
and long-range organizer activities. We present evidence suggesting that Chd is not required for Gsc function in zebrafish. Gsc induces complete secondary axes in chd−/− null mutant embryos and injection of gsc-MO further ventralized chd−/− mutants, suggesting that Gsc has Chd-independent functions in DV patterning. Our database searches have not provided evidence for a second chd gene in zebrafish, nor is chd maternally expressed (Miller-Bertoogli et al., 1997). Moreover, our data suggest that in zebrafish, Gsc and Chd operate in parallel rather than in a simple linear pathway. We also showed that Gsc does not require two other zygotic BMP inhibitors, Nog1 and Fstl1b. Although Gsc might require other BMP inhibitors for its function, we have eliminated the known zygotic BMP inhibitors that are expressed at the appropriate time and place.

Conclusions

Our findings suggest that Gsc has dose dependent effects on axis induction and provide new insights into molecularly distinct short- and long-range signaling activities of the organizer. In addition, we showed that Gsc functions in parallel to three secreted BMP inhibitors. Our work also suggests that Gsc function in Xenopus and zebrafish is different. Thus, despite the conservation of organizer genes among vertebrates, divergent functions have evolved for key organizer genes in different species.

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goosecoid and DV patterning


