

# Goosecoid promotes head organizer activity by direct repression of Xwnt8 in Spemann's organizer

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## SUMMARY

Formation of the vertebrate body plan is controlled by discrete head and trunk organizers that establish the anteroposterior pattern of the body axis. The Goosecoid (Gsc) homeodomain protein is expressed in all vertebrate organizers and has been implicated in the activity of Spemann's organizer in *Xenopus*. The role of Gsc in organizer function was examined by fusing defined transcriptional regulatory domains to the Gsc homeodomain. Like native Gsc, ventral injection of an Engrailed repressor fusion (Eng-Gsc) induced a partial axis, while a VP16 activator fusion (VP16-Gsc) did not, indicating that Gsc functions as a transcriptional repressor in axis induction. Dorsal injection of VP16-Gsc resulted in loss of head structures anterior to the hindbrain, while axial structures were unaffected, suggesting a requirement for Gsc function in head formation. The anterior truncation caused by VP16-Gsc was fully rescued by Frzb, a secreted Wnt inhibitor, indicating that activation of

ectopic Wnt signaling was responsible, at least in part, for the anterior defects. Supporting this idea, Xwnt8 expression was activated by VP16-Gsc in animal explants and the dorsal marginal zone, and repressed by Gsc in Activin-treated animal explants and the ventral marginal zone. Furthermore, expression of Gsc throughout the marginal zone inhibited trunk formation, identical to the effects of Frzb and other Xwnt8 inhibitors. A region of the Xwnt8 promoter containing four consensus homeodomain-binding sites was identified and this region mediated repression by Gsc and activation by VP16-Gsc, consistent with direct transcriptional regulation of Xwnt8 by Gsc. Therefore, Gsc promotes head organizer activity by direct repression of Xwnt8 in Spemann's organizer and this activity is essential for anterior development.

Key words: *Xenopus*, Goosecoid, Wnt, Homeobox, Transcription, Repression, Spemann's organizer

## INTRODUCTION

The seminal experiments of Spemann and Mangold (Spemann and Mangold, 1924) demonstrated that a discrete group of cells, known as the organizer, communicates with neighboring cells and regulates their fate, thus establishing pattern within the early embryo. Embryological experiments in the frog, chick, mouse and fish have shown that transplanted organizer recruits host cells into neural and mesodermal tissues of ectopic axial structures. This activity of the organizer results from its ability to regulate the fate of adjacent tissue and modify the dorsoventral and anteroposterior pattern of the early embryo. The fate of the organizer is not altered in these experiments and the transplanted tissue differentiates as chordamesoderm, forming the notochord of the ectopic axis. These two features of the organizer, the non-autonomous influence on the fate of adjacent cells and the autonomous differentiation as axial mesoderm, are conserved in vertebrates. This conceptual framework has guided the efforts of many, resulting in the isolation of numerous secreted and nuclear factors that are expressed in the organizer. Consistent with the conserved function of the organizer, a conserved group of organizer-specific genes has been identified that are expressed

in all vertebrate organizers and are required to execute organizer function (Lemaire and Kodjabachian, 1996; Harland and Gerhart, 1997; Beddington and Robertson, 1999; Nieto, 1999; De Robertis et al., 2000).

The organizer is a non-homogenous cell population in terms of inductive signals, morphogenetic activities and cell fate. The studies of Mangold (Mangold, 1933) and Spemann (Spemann, 1938) demonstrated that the early organizer (tissue that involutes first) induced anterior structures and that the late organizer induced posterior structures, thus establishing the concept of distinct head and trunk organizers. Recent molecular models of organizer function suggest that head induction occurs in response to the combined inhibition of Wnt, Nodal and bone morphogenetic protein (BMP) activities, whereas trunk induction requires inhibition of BMP only (Glinka et al., 1997; Piccolo et al., 1999; reviewed in Niehrs, 1999). Consistent with separable head- and trunk-inducing activities of the organizer, multiple Wnt, Nodal and BMP inhibitors, including Noggin, Chordin, Follistatin, Frzb, Cerberus, Antivin and Dickkopf are expressed in discrete, overlapping domains of the organizer during gastrulation (De Robertis et al., 2000). Although most of these secreted factors bind to and antagonize the activity of Wnt and/or BMP proteins

produced by non-organizer cells, these factors do not repress Wnt or BMP transcription, and are therefore not sufficient to exclude Wnt and BMP expression from the organizer. As ectopic activation of the zygotic Wnt or BMP pathways inhibits organizer function, a second class of inhibitors may exist that repress the transcription of Wnt and BMP genes in Spemann's organizer. One candidate for such an organizer-specific transcriptional repressor is the homeobox gene Goosecoid (Gsc).

Gsc is a homeodomain transcription factor found across animal phyla, from hydra to human (Blum et al., 1994; De Robertis et al., 1994; Lemaire and Kodjabachian, 1996; Broun et al., 1999). During gastrulation of the vertebrate embryo, Gsc is expressed in organizer cells – Spemann's organizer in *Xenopus*, the zebrafish shield, and the node in mouse and chick – and the conservation of Gsc structure and expression suggests an important function in early development (Blumberg et al., 1991; Blum et al., 1992; Izpisua-Belmonte et al., 1993; Schulte-Merker et al., 1994). In *Xenopus*, Gsc expression peaks at the early gastrula stage in the dorsal mesendoderm that constitutes Spemann's organizer, and injection of ventral blastomeres with Gsc mRNA induces an ectopic trunk with an anterior limit at the hindbrain (Cho et al., 1991; Steinbeisser et al., 1993). Gsc binds DNA through a paired-type homeodomain and the presence of a conserved N-terminal heptapeptide (Goosecoid-Engrailed homology domain) suggests that Gsc functions as a transcriptional repressor; several studies have reported repressor activity in cell culture and embryos (Smith and Jaynes, 1996; Danilov et al., 1998; Ferreira et al., 1998; Mailhos et al., 1998; Latinkic and Smith, 1999). The expression of Gsc in Spemann's organizer suggests that Gsc represses target genes that would, if transcribed in dorsal mesoderm, interfere with organizer formation, function or differentiation. Consistent with this idea, overexpression of Gsc inhibits the expression of *Xwnt8* and *BMP4* (Christian and Moon, 1993; Fainsod et al., 1994; Steinbeisser et al., 1995), genes that are expressed in non-dorsal mesoderm, and antagonize organizer function and axis formation (Dale et al., 1992; Jones et al., 1992; Jones et al., 1996; Christian and Moon, 1993; Hemmati-Brivanlou and Thomsen, 1995; Hoppler et al., 1996; Fredieu et al., 1997; Hoppler and Moon, 1998; Tian et al., 1999).

Gain-of-function analysis in *Xenopus* suggests that Gsc inhibits the expression of factors (*Xwnt8* and *BMP4*) that are antagonistic to organizer function, thus promoting axis formation. However, loss-of-function analysis in the mouse has shown that Gsc-null embryos have no defects in axis formation and embryos survive to birth. Neonates die with craniofacial, tracheal and skeletal malformations (Rivera-Perez et al., 1995; Yamada et al., 1995; Yamada et al., 1997; Belo et al., 1998; Zhu et al., 1998), consistent with sites of Gsc expression during later development (Gaunt et al., 1993). Given that organizer expression of Gsc is absolutely conserved in vertebrates, the lack of an early phenotype suggests that functionally redundant genes are expressed in the gastrula embryo that compensate for the loss of Gsc function. In fact, redundancy of negative regulatory factors is a consistent feature of the vertebrate organizer, and multiple BMP and Wnt inhibitors are co-expressed with Gsc in the mouse gastrula embryo (Beddington and Robertson, 1999). Interestingly, when grafted to a chick embryo to assess inducing activity, a Gsc-null node was

severely impaired in comparison with a wild-type node (Zhu et al., 1999). Therefore, while the intact Gsc-null embryo probably employs regulative mechanisms to complete gastrulation normally, the loss of Gsc function does result in a detectable defect in mouse organizer function.

The role of Gsc in *Xenopus* development has been examined by attempting to disrupt or antagonize the function of endogenous Gsc. Expression of antisense Gsc RNA or 'activating' Gsc fusion proteins resulted in axial defects ranging from cyclopia to near complete inhibition of axis formation (Steinbeisser et al., 1995; Ferreira et al., 1998; Latinkic and Smith, 1999). The observed defects have been attributed to ectopic activation of a class of ventral genes, or the specific activation of Brachyury, but in none of these studies have the observed developmental defects been linked to a specific regulatory target of Gsc. For example, while Gsc can bind the Brachyury promoter and repress transcription (Artinger et al., 1997; Latinkic et al., 1997), ectopic expression of Brachyury does not result in axial defects (Artinger et al., 1997; Tada et al., 1997).

In an attempt to better define the role of Gsc in axial development, the molecular basis of the axial defects caused by 'activated' Gsc has been examined. We report that a VP16-Gsc fusion protein results in a specific loss of structures anterior to the midbrain-hindbrain region without perturbing development of the trunk. The loss of head structures was fully rescued by the Wnt inhibitor Frzb, suggesting that VP16-Gsc activates Wnt expression within the organizer, a condition previously shown to antagonize anterior development. Consistent with regulation of Wnt transcription, VP16-Gsc activated *Xwnt8* expression in animal explants without inducing other mesodermal markers. This ability of VP16-Gsc to activate *Xwnt8* suggested a role for Gsc in repressing *Xwnt8*, and in several assays of axis formation Gsc function was indistinguishable from that of Frzb. Finally, isolation and analysis of the *Xwnt8* promoter demonstrated that a region containing four Gsc-binding sites mediated a transcriptional response to Gsc and VP16-Gsc. Together, these observations indicate that transcriptional repression by Gsc is required to exclude *Xwnt8* transcription from Spemann's organizer and that this activity is essential for normal anterior development.

## MATERIAL AND METHODS

### Embryo culture and microinjection

Embryos were fertilized, injected and cultured; animal explants were prepared as described (Yao and Kessler, 1999) and stage determined according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Dorsal and ventral blastomeres were identified by pigmentation differences (Klein, 1987). In vitro transcribed RNA was synthesized from linear DNA templates using a Megascript kit (Ambion). For Activin-treatment of explants, Activin-containing oocyte supernatant was added to the culture medium at a 1/100 dilution as described (Yao and Kessler, 1999).

### Gsc fusions proteins and mutagenesis

Gsc fusion constructs were generated by PCR amplification of the Gsc homeodomain (residues 128-244) and subcloning into pCS2+ (Rupp et al., 1994) plasmids containing the VP16 activator or Engrailed repressor (Kessler, 1997; Fig. 1A). Gsc and VP16-Gsc mutagenesis was performed with the QuikChange mutagenesis kit (Stratagene)

using oligonucleotides complementary to bases 691-717 of Gsc with a single mismatch at position 703, resulting in a base change of A to G and a substitution of glutamate for lysine.

### Immunocytochemistry, in situ hybridization, $\beta$ -galactosidase detection and RT-PCR

For immunocytochemistry, embryos were processed as described (Sive et al., 2000) using monoclonal antibodies for muscle (12/101; Kintner and Brockes, 1984) or notochord (Tor 70.1; Bolce et al., 1992), HRP-coupled secondary antibodies and diaminobenzidine as the HRP substrate. For in situ hybridization, embryos were fixed and hybridized with antisense digoxigenin-labeled RNA probes as described (Sive et al., 2000). For lineage labeling, embryos were injected with  $\beta$ -galactosidase mRNA, fixed in MEMFA for 15 minutes and the chromogenic reaction performed as described (Sive et al., 2000) using the Rose-Gal substrate. For the RT-PCR assay, RNA extraction, cDNA synthesis, gel electrophoresis, and PCR conditions and primers were as described (Wilson and Melton, 1994).

### Isolation of the Xwnt8 promoter

A *Xenopus* genomic library (a gift from L. Zimmerman) was probed with a 603 bp *EcoRI-NcoI* fragment of Xwnt8, containing 54 bp of 5'UTR and 549 bp of coding region. One million plaques were screened in duplicate, rescreened through three rounds and six unique clones were obtained. A 5 kb fragment containing the 5' end of the Xwnt8 cDNA was sequenced and was found to include 3.8 kb of upstream sequence. For transcriptional analysis, 5' deletions of the 3.8 kb fragment were generated by PCR and the products were subcloned into the pGL3-Basic Luciferase reporter plasmid (Promega). Site-directed mutagenesis of the P3 consensus homeodomain binding sites was performed by PCR, using mismatch primers to replace the palindromic elements of each P3 site (TAATnnnATTA to GCGCnnnGCGC). The sequence of the Xwnt8 promoter has been deposited in GenBank (Accession Number, AF375658).

### Luciferase assay

Embryos were injected at the one-cell stage with Gsc or VP16-Gsc RNA in combination with Xwnt8 reporter plasmid (200 pg) and CMV-Renilla luciferase control plasmid (10 pg). Animal explants were prepared at the mid-blastula stage and 10 explants per sample were collected at the mid-gastrula stage for luciferase assay using the Dual-Luciferase Assay System (Promega). Explants were lysed in 50  $\mu$ l and 20  $\mu$ l of cleared lysate was sequentially assayed for firefly luciferase and Renilla luciferase activity. Renilla luciferase activity served as an internal control for normalizing the firefly luciferase activity. The results presented represent at least three independent experiments and were analyzed for statistical significance using Student's *t*-test.

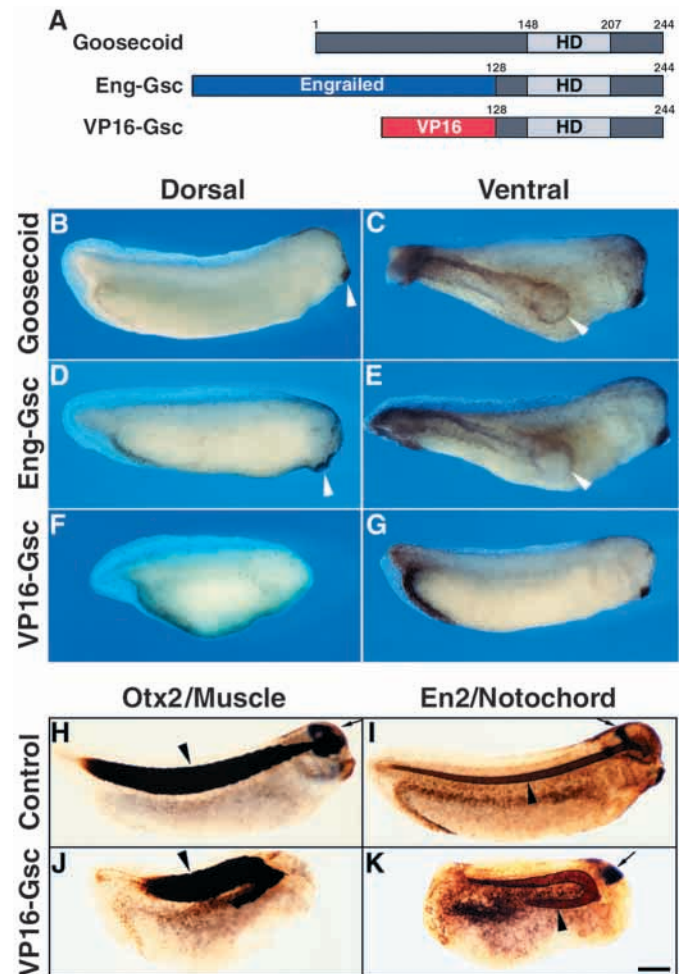
### Gsc DNA-binding assay

Gsc protein was produced in vitro using the TNT coupled transcription/translation system (Promega). The double-stranded probe encoded a single P3 site from the Xwnt8 promoter, shown in bold, and 8bp of flanking sequence (-462 ACATTAATGAG-ATTACTAA -442). For the binding reaction, 8  $\mu$ l of protein was added to 5  $\mu$ l of protein buffer (20 mM Tris-HCl (pH 7.5), 10% glycerol, and 50 mM KCl) in the presence of 0.7  $\mu$ g of pdIdC and incubated at room temperature for 15 minutes. This was followed by the addition of 10  $\mu$ l of probe buffer (20 mM Tris-HCl (pH 7.5), 10% glycerol, 50 mM KCl and 0.1% IGEPAL), 0.7  $\mu$ g of pdIdC and radiolabeled P3 site probe (3 ng at  $1 \times 10^8$  cpm/ $\mu$ g), and incubation at room temperature for 20 minutes. Competition assays were performed with the addition of unlabeled P3 site at a 300-fold excess. Samples were resolved on 4% native polyacrylamide gels in 0.25 $\times$  TBE at room temperature.

## RESULTS

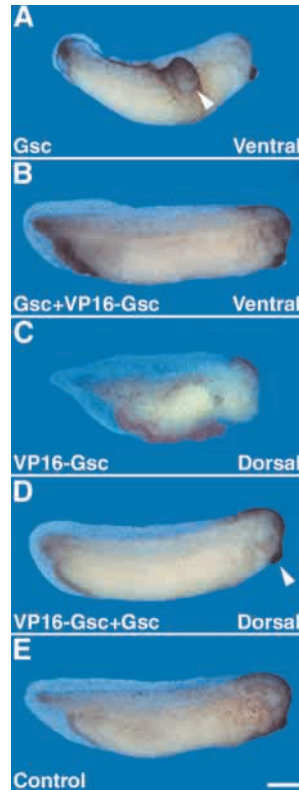
### Transcriptional repression by Goosecoid regulates anterior development

Gsc contains a C-terminal Paired-type homeodomain and an N-terminal heptapeptide active repression domain (Blumberg et al., 1991; Cho et al., 1991; Smith and Jaynes, 1996). The presence of this repression domain in all vertebrate Gsc proteins indicates that transcriptional repression of specific targets is a primary function of Gsc, although additional activities may be present. To address the role of the repression



**Fig. 1.** Transcriptional repression by Gsc regulates anterior development. (A) Schematic of the Gsc fusion constructs. A C-terminal region of Gsc (residues 128-244), containing the homeodomain (HD), was fused to the Engrailed repressor (residues 1-298) (Eng-Gsc) or the VP16 activator (residues 410-490) (VP16-Gsc). At the four-cell stage, one ventral (C,E,G) or two dorsal (B,D,F,J,K) blastomeres were injected with 150 pg of Gsc (B,C), 150 pg of Eng-Gsc (D,E) or 500 pg of VP16-Gsc (F,G,J,K) mRNA. See Table 1 for quantitation. In situ hybridization for Otx2 (H,J) or En2 (I,K) and immunocytochemistry with the muscle antibody 12/101 (H,J) or the notochord antibody Tor 70 (I,K) were performed to assess the axial and neural development of VP16-Gsc-injected or uninjected (Control) embryos. White arrowheads indicate partial secondary axis (C,E) or cement gland (B,D). Black arrowheads indicate muscle (H,J) or notochord staining (I,K). Black arrows indicate Otx2 (H) or En2 (I,K) staining. Scale bar: 0.5 mm.





**Fig. 2.** VP16-Gsc is a specific inhibitor of Gsc function. One ventral (A,B) or two dorsal (C,D) blastomeres were injected at the four-cell stage with 150 pg of Gsc (A), 500 pg of VP16-Gsc (C) or a combination of Gsc and VP16-Gsc (B,D). An uninjected embryo is also shown (E). Arrowheads indicate partial secondary axis (A) or cement gland (D). Scale bar: 0.5 mm.

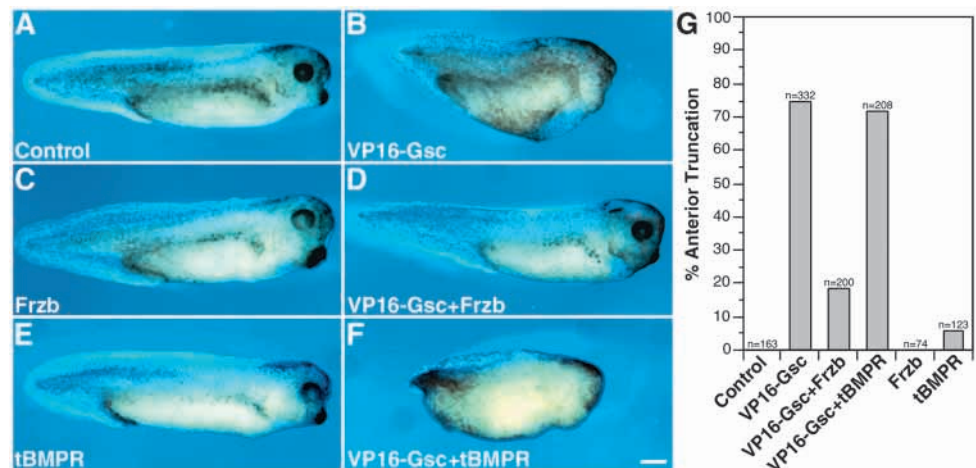
domain in axis induction by Gsc, the Engrailed repressor domain (Jaynes and O'Farrell, 1991; Han and Manley, 1993), containing the conserved heptapeptide sequence, was fused to the Gsc homeodomain and the activity of this fusion protein was examined (Fig. 1A). Similar to the activity of native Gsc (Cho et al., 1991; Steinbeisser et al., 1993), injection of a single ventral blastomere with Eng-Gsc mRNA induced an ectopic axis lacking head structures (Fig. 1C,E, Table 1). The anterior extent of the Eng-Gsc-induced axis was identical to that observed with Gsc. Dorsal injection of Gsc or Eng-Gsc had no effect on axis formation (Fig. 1B,D, Table 1). Given that the heptapeptide sequence is the only region, other than the homeodomain, that is conserved between Gsc and Engrailed (Smith and Jaynes, 1996), the results indicate that transcriptional repression by Gsc is responsible for axis induction. Consistent with this conclusion, the activity of a

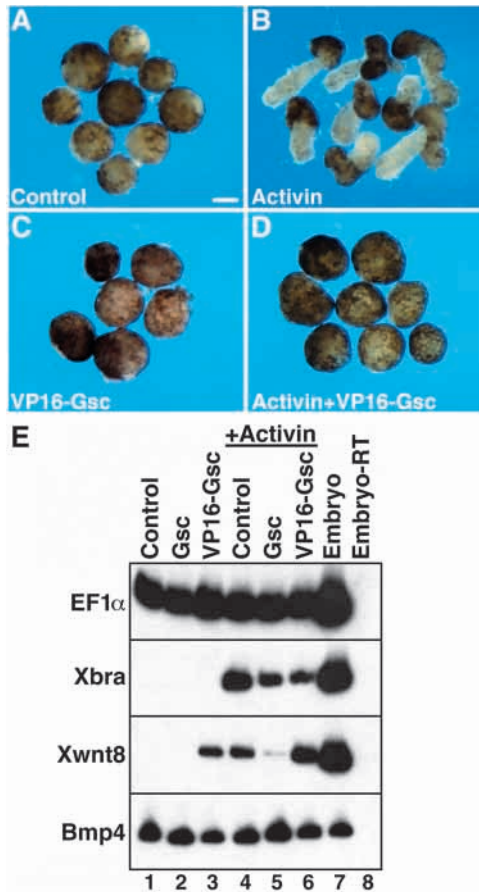
*Drosophila* Gsc ortholog was completely dependent on the heptapeptide sequence (Mailhos et al., 1998).

Previous studies have shown that conversion of a transcriptional activator to a repressor, or the converse, can generate a dominant form that antagonizes the endogenous protein (Conlon et al., 1996; Kessler, 1997; Onichtchouk et al., 1998). Using this approach, the function of endogenous Gsc was examined by fusing the VP16 activator domain (Sadowski et al., 1988; Triezenberg et al., 1988) to the Gsc homeodomain (Fig. 1A). Unlike native Gsc and Eng-Gsc, ventral injection of VP16-Gsc did not result in axis induction (Fig. 1G, Table 1). Injection of VP16-Gsc into both dorsal blastomeres at the four-cell stage resulted in embryos lacking head structures (Fig. 1F, Table 1). Immunocytochemistry and in situ hybridization were used to assess the axial and neural development of embryos with anterior truncations. While somitic muscle and notochord were present, indicating that axial development occurs, a marker of the midbrain-hindbrain boundary, En2 (Hemmati-Brivanlou et al., 1991), was expressed in the anteriormost tissue and the forebrain-midbrain marker Otx2 (Blitz and Cho, 1995; Pannese et al., 1995) was not detected (Fig. 1H-K). Therefore, dorsal injection of VP16-Gsc results in anterior truncation at the level of the midbrain, without inhibiting the development of axial mesoderm. Histological analysis confirmed the presence of normal axial organization and the absence of cement gland and eyes (data not shown). It is interesting to note that while the overall length of the embryos decreased, as expected with the loss of head structures, the length of axial mesodermal structures remained near normal, resulting in a ventral folding of anterior muscle and notochord in many cases (Fig. 1I,K). The defects observed are largely in agreement with previously reported injection of 'activated' Gsc or antisense RNA (Steinbeisser et al., 1995; Ferreiro et al., 1998; Latinkic and Smith, 1999).

The inhibition of head formation by VP16-Gsc is predicted to result from a specific block of endogenous Gsc function. However, it is possible that overexpressed VP16-Gsc may bind to targets not normally regulated by Gsc, resulting in interference with the function of other transcriptional regulators. The specificity of VP16-Gsc action was examined by co-expression of Gsc and VP16-Gsc in dorsal or ventral blastomeres. Axis induction in response to ventral injection of Gsc was blocked by co-injection with VP16-Gsc, confirming the ability of VP16-Gsc to antagonize Gsc function (Fig.

**Fig. 3.** Ectopic activation of Wnt signaling by VP16-Gsc inhibits head formation. At the four-cell stage two dorsal blastomeres were injected with 500 pg of VP16-Gsc (B), 100 pg of Frzb (C), 150 pg of tBMPR (E), VP16-Gsc and Frzb (D) or VP16-Gsc and tBMPR (F). An uninjected embryo is also shown (A). Quantitation is shown in G. Each bar represents the percentage of embryos with anterior truncations resulting from five independent experiments. *n*, total number of injected embryos. Scale bar: 0.5 mm.

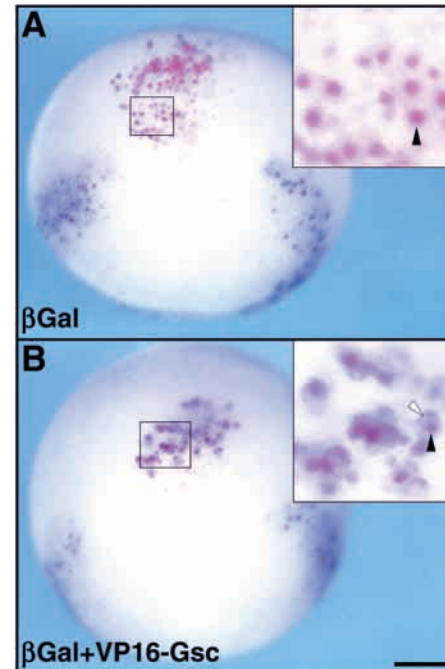




**Fig. 4.** VP16-Gsc activates Xwnt8 expression in animal explants. Animal explants were isolated from uninjected (A,B) or VP16-Gsc-injected embryos (C,D), and were cultured in the absence (A,C) or presence (B,D) of Activin protein. The morphology of explants was assessed at the tailbud stage. The expression of Xbra, Xwnt8 and BMP4 in response to Gsc or VP16-Gsc was examined by RT-PCR analysis at the gastrula stage (E). EF1 $\alpha$  is a control for RNA recovery and loading. Intact embryos (embryo) served as a positive control and an identical reaction without reverse transcriptase controlled for PCR contamination (embryo-RT). Scale bar: 0.2 mm.

2A,B). Consistent with this result, the anterior truncation resulting from dorsal injection of VP16-Gsc was completely rescued by co-injection of Gsc (Fig. 2C,D). Otx2, a homeodomain protein also expressed in Spemann's organizer (Blitz and Cho, 1995; Pannese et al., 1995), failed to rescue the effects of VP16-Gsc, further arguing for specificity of action (data not shown). The ability of Gsc to rescue head formation indicates that VP16-Gsc antagonizes the function of endogenous Gsc, and that Gsc function is required for anterior development. We note that at high doses (>1 ng) of VP16-Gsc, development of the trunk and axial mesoderm was inhibited, similar to that reported by Ferrerio et al. (Ferrerio et al., 1998), but these additional effects could not be rescued by Gsc co-injection (data not shown). Therefore, while the anterior truncation appears to result from specific inhibition of Gsc function, previously reported axial defects may result from interference with activities other than Gsc.

The function of VP16-Gsc presumably results from the delivery of the VP16 activator to specific transcriptional targets

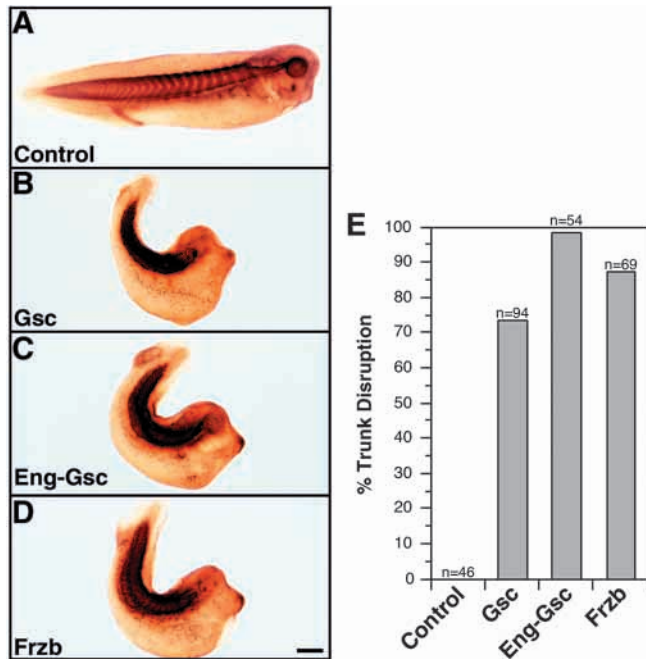


**Fig. 5.** Cell autonomous activation of Xwnt8 by VP16-Gsc. At the 16-cell stage, one dorsal marginal zone blastomere was injected with 750 pg of  $\beta$ -galactosidase ( $\beta$ -gal) mRNA (A) or a combination of  $\beta$ -gal and 500 pg of VP16-Gsc (B). Descendants of the injected cell were identified at the gastrula stage by the presence of  $\beta$ -gal, using the Rose-gal substrate. Activation of Xwnt8 by VP16-Gsc in the dorsal marginal zone and endogenous ventrolateral expression of Xwnt8 was detected by in situ hybridization with BM-purple substrate (A,B). Dorsal views of stage 10.25 embryos with endogenous Xwnt8 expression visible in lateral regions. Insets show higher magnification views of boxed regions. Black arrowheads indicate  $\beta$ -gal-positive nuclei (red) and the white arrowhead indicates cytoplasmic Xwnt8 in situ stain (purple). Scale bar: 0.25 mm; 80  $\mu$ m in the insets.

**Table 1. Effects of Goosecoid fusion proteins on axial development**

	Axis induction		Anterior truncation	
	n/N	%	n/N	%
Uninjected	0/140	0	0/126	0
Goosecoid	99/132	32	0/50	0
Eng-Gsc	72/93	77	0/45	0
VP16-Gsc	0/175	0	166/194	86
Goosecoid K197E	0/59	0	nd	
VP16-Gsc K197E	nd		0/49	0
Gsc-HD	0/17	0	0/27	0
Gsc $\Delta$ HD	0/23	0	0/31	0
VP16	0/37	0	0/33	0
Eng	0/40	0	0/57	0

At the four-cell stage, a single ventral blastomere (axis induction) or both dorsal blastomeres (anterior truncation) were injected with 150 pg of Goosecoid, Eng-Gsc, the Goosecoid homeodomain (Gsc-HD), Goosecoid with a deletion of the homeodomain (Gsc $\Delta$ HD), a Goosecoid homeodomain mutant (K197E) or the Engrailed repressor domain (Eng), or with 500 pg of VP16-Gsc, VP16-Gsc homeodomain mutant (K197E) or the VP16 activator domain (VP16). At the tailbud stage, embryos were scored for axis formation or loss of anterior structures. n, injected embryos with ectopic axis (axis induction) or loss of anterior structures (anterior truncation); N, total number of injected embryos; %, percent affected embryos; nd, not determined.



**Fig. 6.** Gsc inhibition of trunk formation. At the two-cell stage, both blastomeres were injected in the equatorial region with 100 pg of Gsc (B), Eng-Gsc (C) or Frzb (D). The presence of axial structures was examined by immunostaining with a muscle-specific antibody (12/101). Quantitation is shown in E. Each bar represents the percentage of embryos with reduction or absence of trunk resulting from three independent experiments. n, total number of injected embryos. Scale bar: 0.5 mm.

by the Gsc homeodomain, resulting in activation of genes normally repressed by Gsc. To confirm this mechanism, the requirement for specific DNA-binding for VP16-Gsc function was examined by mutating residue 50 of the homeodomain, a crucial residue for DNA-binding affinity and site selection (Hanes and Brent, 1989; Treisman et al., 1989; reviewed by Mann, 1995). For both Gsc and VP16-Gsc, replacing Lys197 with glutamate abolished biological activity (Table 1), indicating that VP16-Gsc activity is dependent on sequence-specific DNA binding. Furthermore, injection of the individual domains of VP16, Engrailed and Gsc that make up the fusion proteins, as well as a homeodomain deletion mutant of Gsc, had no effect on axis formation (Table 1).

#### Ectopic activation of Xwnt8 by VP16-Gsc inhibits head formation

The block of head formation by VP16-Gsc is likely due to the activation of a transcriptional target(s) normally repressed by Gsc in Spemann's organizer. Two candidate target genes for activation by VP16-Gsc are Xwnt8 and BMP4. Ectopic expression of BMP4 or Xwnt8 in dorsal blastomeres results in an anterior truncation similar to that observed with VP16-Gsc, and ventral injection of Gsc inhibits the expression of both BMP4 and Xwnt8 (Jones et al., 1992; Jones et al., 1996; Christian and Moon, 1993; Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995; Schmidt et al., 1995; Steinbeisser et al., 1995; Hoppler et al., 1996; Dosch et al., 1997; Hoppler and Moon, 1998). To address the possible role of Xwnt8 or BMP4 activation in the embryonic response to

VP16-Gsc, specific Wnt and BMP inhibitors were tested for the ability to rescue normal anterior development.

The secreted Wnt inhibitor Frzb (Leyns et al., 1997; Wang et al., 1997a; Wang et al., 1997b) was used to examine the role of Wnt activity in anterior truncation by VP16-Gsc. While VP16-Gsc resulted in anterior truncation in 74% ( $n=332$ ) of injected embryos (Fig. 3B), embryos injected with the combination of VP16-Gsc and Frzb mRNAs displayed anterior defects in only 18% ( $n=200$ ) of the cases (Fig. 3D). At the dose used, dorsal expression of Frzb had no effect on anterior development (Fig. 3C), but was sufficient to inhibit the effects of co-injected pCS2-Xwnt8 DNA on anterior development (data not shown). In contrast, a truncated BMP2/4 receptor (tBMPR) that inhibits the activity of BMP2, 4 and 7 (Graff et al., 1994; Suzuki et al., 1994) failed to rescue head development when co-expressed with VP16-Gsc (Fig. 3F), even at doses of tBMPR sufficient to induce axis formation in ventral blastomeres (data not shown). The results suggest that inhibition of head formation by VP16-Gsc is due, at least in part, to the activation of Wnt expression in Spemann's organizer. Therefore, a primary function of endogenous Gsc may be the transcriptional repression of Xwnt8 or of other zygotic Wnts.

The potential regulation of Xwnt8 expression by Gsc and VP16-Gsc was examined in animal explants. At the one-cell stage, the animal pole was injected with Gsc or VP16-Gsc mRNA, and blastula-stage explants were cultured in the presence or absence of Activin protein. Although the morphology of animal caps expressing VP16-Gsc was indistinguishable from uninjected caps (Fig. 4A,C), activation of Xwnt8 expression by VP16-Gsc was detected at the gastrula stage by RT-PCR (Fig. 4E). VP16-Gsc did not induce Brachyury and did not alter the level of BMP4 expression. In Activin-treated caps, VP16-Gsc blocked morphogenesis and resulted in an elevation of Xwnt8 expression without affecting Brachyury or BMP4 expression (Fig. 4B,D,E). In contrast, Gsc had no effect on the morphology of untreated or Activin-treated explants (data not shown), did not induce Xwnt8 in untreated explants, but did repress the Activin-induced expression of Xwnt8 (Fig. 4E). The response of additional genes to VP16-Gsc was assessed, and expression of Frzb, Chordin, Xvent1 and Xvent2 was not affected by VP16-Gsc in animal explants (data not shown). Therefore, VP16-Gsc activated and Gsc repressed the expression of Xwnt8 in explants, without significantly affecting the expression of other gastrula markers. Furthermore, given the role of Wnt signaling in cell fate specification and morphogenesis, the inhibition of Activin-induced morphogenesis by VP16-Gsc is likely to be a consequence of the upregulation of Xwnt8 expression.

The potential regulation of Xwnt8 by Gsc in the marginal zone, the normal site of Xwnt8 expression, is more relevant to the analysis of Gsc function. Ectopic expression of Gsc or VP16-Gsc was directed to a limited region of the marginal zone by equatorial injection of a single blastomere at the 16-cell stage.  $\beta$ -galactosidase ( $\beta$ -gal) RNA was co-injected as a lineage marker to identify Gsc- or VP16-Gsc-expressing cells and determine their spatial relation to Xwnt8-expressing cells. Consistent with previous studies (Christian and Moon, 1993; Steinbeisser et al., 1995), Gsc-expressing cells in the lateral and ventral marginal zone did not express Xwnt8, and lineage labeling indicated a precise correlation between Gsc

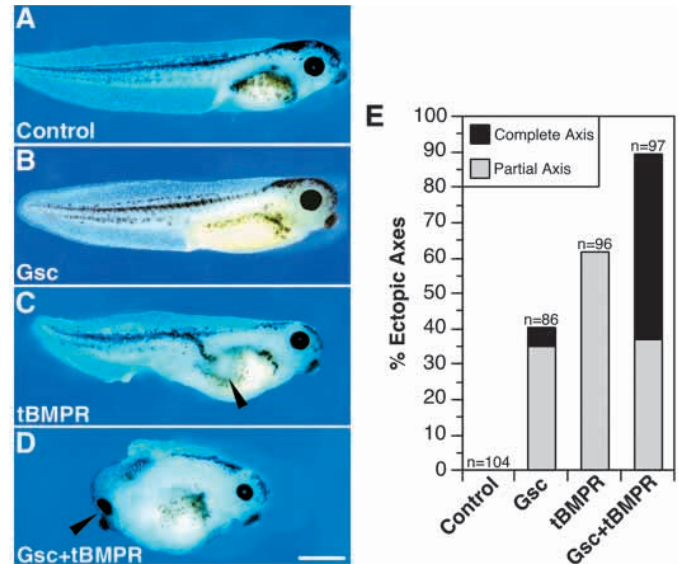


expression and Xwnt8 repression (data not shown). In dorsal cells, normally devoid of Xwnt8 transcripts, VP16-Gsc activated strong expression of Xwnt8 (Fig. 5B). Xwnt8 transcripts were detected only in  $\beta$ -gal-positive, VP16-Gsc-expressing cells and adjacent  $\beta$ -gal-negative cells did not express Xwnt8 (Fig. 5B). The results indicate that Gsc and VP16-Gsc act in a cell autonomous manner to regulate Xwnt8 expression, suggesting direct transcriptional regulation of Xwnt8. Furthermore, the reciprocal response of Xwnt8 to Gsc and VP16-Gsc, observed in both marginal and animal cells, supports a role for endogenous Gsc in repressing Xwnt8.

### Gsc functions as a Xwnt8 inhibitor to regulate head and trunk formation

A variety of natural and constructed inhibitors of Xwnt8 have been used to examine the function of zygotic Xwnt8 activity in axis formation (Frzb, Nxfz8, dnXwnt8, dnDsh) and ectopic expression of each resulted in a reduction or loss of trunk development (Hoppler et al., 1996; Sokol, 1996; Leyns et al., 1997; Rattner et al., 1997; Wang et al., 1997a; Deardorff et al., 1998). If Gsc does function as a transcriptional repressor of Xwnt8, then Gsc may alter axis formation in a manner similar to the Xwnt8 inhibitors. To assess this possibility, Gsc or Frzb was expressed throughout the marginal zone and the effect on axis formation was examined. At the two-cell stage, both blastomeres were injected in the equatorial region and axis formation was assessed at the tadpole stage (Fig. 6). As previously described, Frzb resulted in a severe reduction of trunk formation without inhibiting head formation (Fig. 6D). Gsc resulted in an identical reduction of trunk development in most injected embryos (Fig. 6B). The presence of axial structures was examined by immunostaining for somitic muscle and by histology. As seen with Frzb, somitic muscle was present, but reduced in Gsc-injected embryos and histology confirmed the presence of notochord and neural tissue (data not shown). Eng-Gsc had a similar effect on trunk formation (Fig. 6C), indicating that transcriptional repression by Gsc was responsible for the reduction of trunk formation. Therefore, when expressed throughout the marginal zone, Gsc was indistinguishable from a defined Xwnt8 inhibitor. Furthermore, induction of ectopic axes was observed infrequently (<5%), suggesting that the predominant effect of Gsc overexpression, under these experimental conditions, is inhibition of trunk development, not axis induction.

Niehrs and colleagues have shown that head structures can be induced by the simultaneous inhibition of zygotic BMP4 and Xwnt8 signaling (Glinka et al., 1997). While ventral expression of tBMPR alone induced an ectopic axis lacking head structures, co-expression with a dominant negative mutant of Xwnt8 (dnXwnt8) induced head as well as trunk. The ability of Gsc to mimic this effect of dnXwnt8 was examined by co-expression with tBMPR. At the four-cell stage, both ventral blastomeres were injected with tBMPR alone, or a mixture of tBMPR and Gsc, and the formation of ectopic axial structures was assessed. Injection of tBMPR induced ectopic trunk, but not head structures (Fig. 7C). In contrast, co-injection of tBMPR and Gsc resulted in head formation at high frequency (Fig. 7D) and histology confirmed the presence of pigmented eye, cement gland, well-organized neural structures, notochord and somites in the complete axes induced (data not shown). Ventral injection of Gsc alone

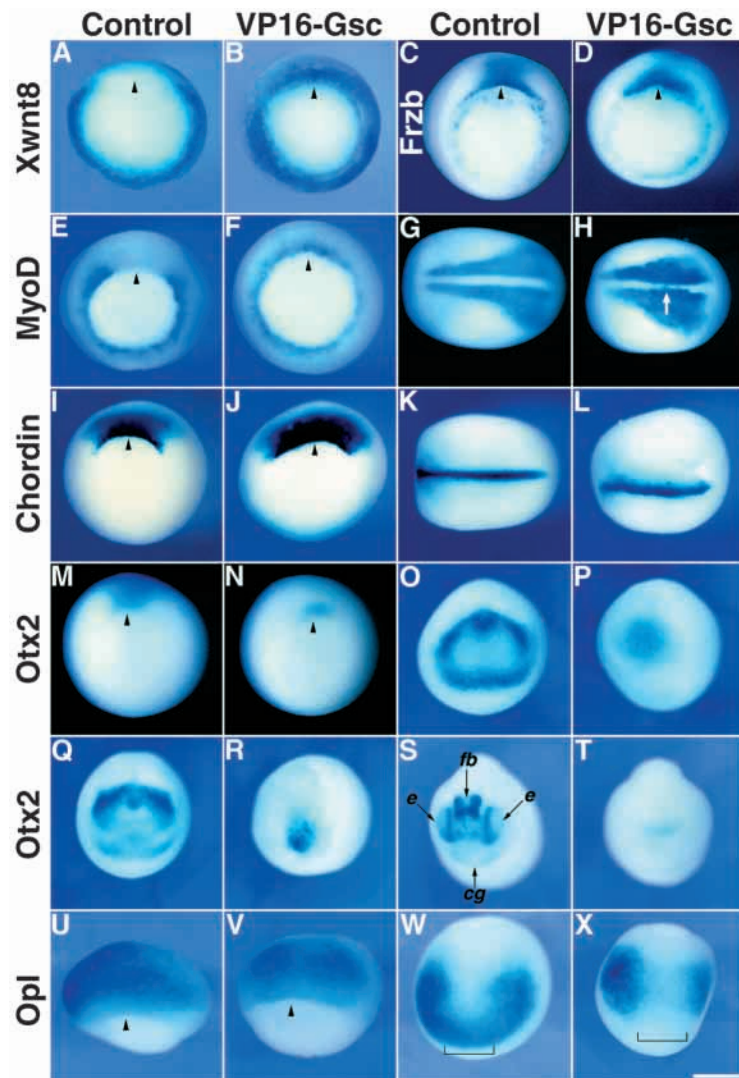


**Fig. 7.** Complete axis induction by co-expression of Gsc and a truncated BMP receptor. At the four-cell stage, two ventral blastomeres were injected with 100 pg of Gsc (B), 250 pg of a truncated BMPR (tBMPR) (C) or a combination of both (D), and axis induction was assessed at the late tadpole stage. Arrowhead indicates partial axis (C) or ectopic eye in a complete secondary axis (D). Quantitation is shown in E. Each bar represents the percentage of embryos with ectopic axes resulting from four independent experiments. Dark bar, complete secondary axes; light bar, partial secondary axes; *n*, total number of injected embryos. Scale bar: 1.0 mm.

induced trunk structures at a low frequency (35%) and the presence of head structures was rare (4%) (Fig. 7B). These results further support a role for Gsc in repressing Xwnt8 expression and promoting anterior development.

### VP16-Gsc alters gene expression at the early gastrula stage

VP16-Gsc may interfere with Gsc function in the head organizer during gastrulation or in the prechordal plate during neurulation, or possibly at both sites of expression. While VP16-Gsc can induce Xwnt8 at the gastrula stage, a detailed gene expression analysis was undertaken to better understand how anterior truncations arise in VP16-Gsc-injected embryos. VP16-Gsc was injected into both dorsal blastomeres at the four-cell stage and embryos were collected at the gastrula, neurula and tailbud stages for whole-mount in situ hybridization. Dorsal injection of VP16-Gsc induced an expansion of Xwnt8 into the organizer region at the early gastrula stage (Fig. 8B). Similarly, MyoD (Hopwood et al., 1989) expression expanded into the organizer region in response to VP16-Gsc (Fig. 8F). At the early neurula stage, scattered MyoD-positive cells were present at the dorsal midline (Fig. 8H), consistent with the role of Xwnt8 in promoting somitic mesoderm formation (Hoppler et al., 1996). VP16-Gsc resulted in a near complete loss of Otx2 expression (Blitz and Cho, 1995) in the organizer at the gastrula stage and later in the anterior mesoderm and neuroectoderm (Fig. 8N,P,R,T), indicating that VP16-Gsc perturbs anterior patterning at the onset of gastrulation. However, VP16-Gsc did



**Fig. 8.** VP16-Gsc alters gene expression at the gastrula and neurula stages. At the four-cell stage, two dorsal blastomeres were injected with 500 pg of VP16-Gsc. Embryos were fixed at stage 10.5 (early gastrula), 12.5 (late gastrula), 15 (mid-neurula) or 20 (late neurula). Gene expression was detected by in situ hybridization of uninjected (Control) and VP16-Gsc-injected embryos. VP16-Gsc caused an expansion of Xwnt8 (B) and MyoD (F) into the dorsal marginal zone at stage 10.5, and MyoD-positive cells were present at the midline (white arrow) at stage 15 (H). Frzb and Chordin expression in the organizer was not affected at stage 10.5 (D,J) and Chordin expression in the chordamesoderm was normal at stage 15 (L). Otx2 was reduced or absent at all stages examined (N,P,R,T). Opl expression in the prospective neural plate was not affected by VP16-Gsc at stage 10.5 (V), but was absent from the most anterior domain of the neural plate (bracket) at stage 12.5 (X). Black arrowheads indicate the dorsal blastopore lip. The black arrows indicate the primordia of the forebrain (fb), eyes (e) and cement gland (cg) (S). Vegetal views are shown in A-F,I,J,M,N, dorsal view is shown in G,H,K,L,U,V with anterior on the left in G,H,K,L and anterior view is shown in O-T,W,X. Scale bar: 0.5 mm.

not significantly alter the expression of other organizer genes, including Frzb (Leyns et al., 1997; Wang et al., 1997a; Fig. 8D) and Chordin (Sasai et al., 1994; Fig. 8J), indicating that anterior defects do not result from inhibition of organizer formation or from complete loss of organizer function. In

addition, Chordin expression was near normal in the chordamesoderm at the neurula stage (Fig. 8L). Neural induction, indicated by the expression of Opl in the prospective neural plate of the early gastrula (Kuo et al., 1998), was not affected by VP16-Gsc (Fig. 8V). However, Opl expression was absent from the most anterior region of the neural plate in the late gastrula (Fig. 8X), consistent with the absence of Otx2 expression and the VP16-Gsc-induced defects in head formation. The results indicate that VP16-Gsc activates Xwnt8 in the organizer, leading to expansion of MyoD into the organizer domain and reduction of Otx2 in the early gastrula. Therefore, VP16-Gsc perturbs gene expression at the onset of gastrulation and these early effects likely result in anterior truncation.

### Gsc repression of the Xwnt8 promoter

To directly address the regulatory interaction of Xwnt8 and Gsc, Xwnt8 transcriptional regulatory sequences were isolated and responsiveness to Gsc was examined. A *Xenopus* genomic library was screened with a probe containing the 5' end of the Xwnt8 cDNA and a clone containing 5 kb of the Xwnt8 gene, including 3.8 kb of upstream sequence, was isolated and used for further analysis. A TATA element was identified 36 bp upstream of the Xwnt8 transcriptional start site, confirming the presence of a minimal promoter in this genomic fragment. Sequence analysis also identified four regions with high similarity to a consensus Paired-type homeodomain binding site (P3 site) within 650 bp of the transcription start site (Fig. 9A). The P3 consensus site is a palindromic sequence (TAATnnnATTA) that is bound by Paired-type homeodomain proteins, such as Gsc, with high affinity (Wilson et al., 1993). Therefore, these four P3 sites within the Xwnt8 promoter are strong candidates for elements that mediate a transcriptional response to Gsc.

To assess the regulation of the Xwnt8 promoter by Gsc, the 3.8 kb upstream fragment and 5' deletion mutants, containing the TATA element and transcriptional start site, were subcloned into a promoterless luciferase plasmid (pGL3-Basic), and analyzed for responsiveness to Gsc and VP16-Gsc. One-cell stage embryos were injected with Gsc or VP16-Gsc, and a Xwnt8 reporter plasmid with an internal control plasmid (pRLCMV). Animal explants prepared at the blastula stage were collected at the mid-gastrula stage for analysis of luciferase activity. Xwnt8 reporter plasmids that contained the four P3 sites (−628 or larger) were strongly responsive to VP16-Gsc and Gsc (Fig. 9B and data not shown). The −628 reporter was activated 7.9-fold by VP16-Gsc and was repressed 3.5-fold by Gsc when compared with the −628 reporter in the absence of co-injected mRNA. The −202 reporter, which lacks the P3 sites, was unresponsive to VP16-Gsc or Gsc. The results indicate that a proximal region of the Xwnt8 promoter (−628 to −202) that contained four P3 sites was sufficient for responsiveness to VP16-Gsc and Gsc. The importance of the P3 sites for the response of the −628 reporter was examined by site-directed mutagenesis of all four P3 sites (Fig. 9A). The −628 reporter with inactivating mutations at

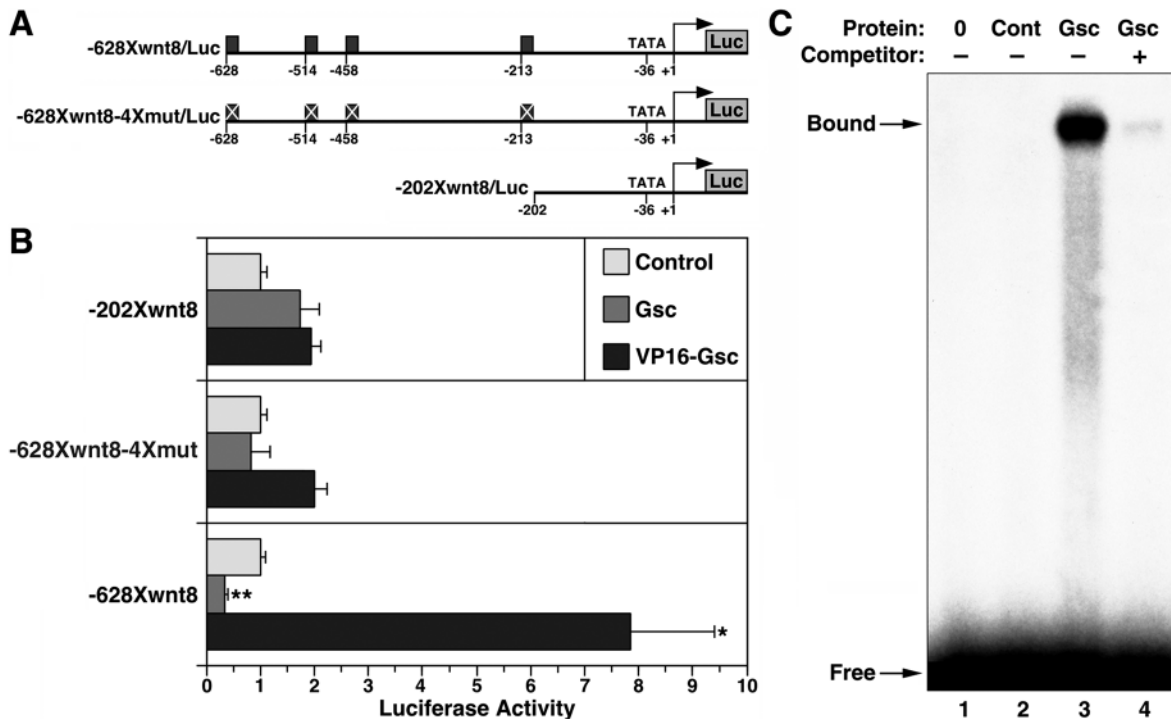


each P3 site responded weakly to VP16-Gsc (2.0-fold activation) and Gsc (1.25-fold repression), indicating that the P3 sites are necessary for the response to Gsc and VP16-Gsc (Fig. 9B). Therefore, the P3 consensus sites are required to mediate transcriptional repression of the Xwnt8 promoter by Gsc.

The presence of P3 consensus sites in the Xwnt8 promoter argues for direct regulation by Gsc. To confirm the direct binding of Gsc to the Xwnt8 promoter, *in vitro* translated Gsc protein was incubated with a double-stranded DNA probe encoding one of the four P3 sites (−462 to −442). A DNA-protein complex was observed with Gsc protein, but not with a control *in vitro* translation reaction (Fig. 9C). The addition of excess, unlabeled P3 probe prevented complex formation, indicating that Gsc specifically binds to the P3 site. Direct binding of Gsc to the other three P3 sites was examined and in each case complex formation was observed (data not shown). The results demonstrate that Gsc binds with high affinity to the P3 consensus sites present in the Xwnt8 promoter, consistent with direct repression of Xwnt8 transcription by Gsc.

## DISCUSSION

In this study we show that Gsc is a transcriptional repressor that promotes anterior development by direct repression of Xwnt8 transcription within Spemann's organizer. Expression of VP16-Gsc, a dominant activating form of Gsc, in the organizer produces anterior truncations without perturbing axial mesoderm formation or organizer gene expression. VP16-Gsc activates Xwnt8 transcription, and the ectopic expression of Xwnt8 in the organizer is responsible, at least in part, for the observed defects in head formation. Conversely, native Gsc rescues the anterior truncation produced by VP16-Gsc and inhibits endogenous Xwnt8 expression. Consistent with an ability to inhibit Xwnt8 expression, Gsc misexpression inhibits trunk formation and promotes head formation. The results suggest that Gsc represses Xwnt8, and the exclusion of Xwnt8 transcription from Spemann's organizer is required for normal anterior development. Supporting this conclusion, four Gsc-binding sites were identified in the Xwnt8 upstream regulatory sequences and these sites can mediate direct transcriptional repression by Gsc. As discussed below, the



**Fig. 9.** Direct regulation of Xwnt8 transcription by Gsc. (A) Xwnt8 promoter-luciferase reporter plasmids containing 628 bp of upstream sequence (−628Xwnt8/Luc), 628 bp with site-directed mutations at each P3 site (−628Xwnt8-4Xmut/Luc) or 202 bp (−202Xwnt8/Luc). Four Paired-type homeodomain binding sites (P3 consensus sequences) present in the −628Xwnt8/Luc reporter are indicated by dark gray boxes, and the TATA element (−36) and transcriptional start site (+1) are also indicated. (B) The animal pole was injected at the one-cell stage with 200 pg of the indicated reporter plasmid and 10 pg of the internal control (pRLCMV), in combination with 500 pg of Gsc or 1000 pg of VP16-Gsc mRNA and animal explants were analyzed for luciferase activity at the gastrula stage. Mean luciferase activity was determined by normalizing to the activity of each reporter plasmid in the absence of co-injected RNA (control), and the standard error is shown for each sample. Statistical analysis confirmed the significance of the response of −628Xwnt8/Luc to Gsc and VP16-Gsc (\*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ). The data presented are the average of three independent experiments. (C) Binding of Gsc to the Xwnt8 promoter was examined by electrophoretic mobility shift assay. *In vitro* translated Gsc protein was incubated with a radiolabeled probe encoding a single P3 site (−458). Gsc bound strongly to the P3 site (lane 3) and formation of this Gsc-DNA complex (bound) was competed by an excess of unlabeled P3 site (lane 4). Complex formation was not observed with the addition of a control *in vitro* translation reaction (cont) lacking Gsc protein (lane 2) or in the absence of any added protein (lane 1). Unbound P3 site is also indicated (free).

results suggest that Gsc promotes head organizer activity by direct repression of Xwnt8 transcription.

### Negative regulation of Xwnt8 and BMP4 by Gsc

In the early gastrula, Xwnt8 expression in the marginal zone is complementary to the Gsc expression domain (Christian et al., 1991), and overexpression of Gsc in ventral mesoderm blocks Xwnt8 expression (Christian and Moon, 1993). These observations suggest that Gsc may repress Xwnt8 in the organizer and our studies support this hypothesis. VP16-Gsc induced ectopic expression of Xwnt8, resulted in axial defects identical to zygotic overexpression of Xwnt8 and these axial defects were rescued by the Wnt inhibitor Frzb. In contrast, Gsc repressed Xwnt8 expression and enhanced head formation, identical to the effects of inhibiting zygotic Wnt function (Hoppler et al., 1996; Sokol, 1996; Leyns et al., 1997; Wang et al., 1997a; Dearnforff et al., 1998). The reciprocal effects of VP16-Gsc and Gsc implicate Gsc in repression of Xwnt8 transcription and in head organizer function. The identification of multiple Gsc-binding sites in the Xwnt8 promoter supports these conclusions and demonstrates the potential for direct repression of Xwnt8 transcription.

Like Xwnt8, BMP4 expression in the gastrula marginal zone is complementary to Gsc and misexpression of Gsc in the marginal zone blocks BMP4 expression (Dale et al., 1992; Jones et al., 1992; Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995; Schmidt et al., 1995). In addition, the anterior truncation caused by VP16-Gsc is similar to a subset of phenotypes resulting from BMP4 overexpression (Dale et al., 1992; Jones et al., 1992; Jones et al., 1996; Hemmati-Brivanlou and Thomsen, 1995). However, the interactions of Gsc with BMP4 and Xwnt8 differ in several important ways. VP16-Gsc did not induce ectopic BMP4 expression and therefore, a reciprocal response to Gsc and VP16-Gsc, observed for Xwnt8, is not observed for BMP4. In animal explants, Gsc did not repress BMP4 or the Vent genes downstream of BMP4, suggesting that additional factors, present in the marginal zone, are required for Gsc inhibition of BMP4. For example, if Gsc were a direct repressor of BMP4 transcription, Gsc-expressing animal explants should undergo neural induction and this has not been observed. Overall, these results argue that direct repression of Xwnt8 transcription is a predominant role of Gsc, while regulation of BMP4 may be an indirect effect.

### Does Gsc function in the head organizer or trunk organizer?

In classical experiments, Mangold (Mangold, 1933) and Spemann (Spemann, 1931; Spemann, 1938) described the region-specific activities of the organizer that confer anteroposterior pattern on the embryonic axis. The organizer appears to consist of two domains, a vegetally positioned head organizer and an animally positioned trunk organizer, that exhibit differences in gene expression and inducing properties (Vodicka and Gerhart, 1995; Lemaire and Kodjabachian, 1996; Zoltewicz and Gerhart, 1997). The distinct functions of the head and trunk organizer are thought to correspond to specific subsets of organizer factors that inhibit the BMP, Wnt or Nodal signaling pathways. In current models, trunk induction requires the inhibition of BMP signals, while head induction requires the simultaneous inhibition of BMP, Wnt and Nodal signals

(Glinka et al., 1997; Piccolo et al., 1999; Thisse et al., 2000). As a potential repressor of Wnt and BMP transcription in the organizer, Gsc may contribute to the head- and/or trunk-inducing activities of the organizer. Our results show that one important role of Gsc is the direct repression of Xwnt8, a function that promotes head-organizer activity.

Gsc was originally described as a factor with trunk-organizer activity, consistent with the ability to inhibit BMP4 expression in the marginal zone (Cho et al., 1991; Steinbeisser et al., 1993). Our studies describe a distinct head-organizer activity of Gsc that results from direct repression of Xwnt8 transcription. Can these distinct activities of Gsc be incorporated into a coherent model of Gsc function? In considering the trunk-organizer activity of Gsc it is important to note that BMP4 is regulated by a positive feedback loop with BMP4 inducing Vent genes that then activate BMP4 transcription (Jones et al., 1992; Metz et al., 1998; Blitz et al., 2000; Schuler-Metz et al., 2000). While the inability of Gsc to inhibit BMP4 or Vent expression in animal explants argues against direct repression, Gsc can induce Chordin expression in ventral mesoderm, and disruption of the BMP4 feedback loop by Chordin may lead to trunk induction (Sasai et al., 1994; Piccolo et al., 1996). In this indirect mechanism, Chordin is upregulated but not overexpressed in response to Gsc, resulting in ventral levels of Chordin mRNA that are no higher than dorsal levels. This may account for the fact that when overexpressed, Gsc is a less effective trunk inducer than Chordin or a truncated BMP receptor.

As an inhibitor of both Xwnt8 and BMP4 expression, ventral injection of Gsc might be predicted to induce head structures at high frequency, but head induction is observed infrequently and only when high doses of Gsc are injected into multiple ventral blastomeres (Cho et al., 1991; Steinbeisser et al., 1993). The weak head-inducing activity of Gsc may simply reflect an insufficient BMP inhibition function. On the other hand, the inability of Gsc to induce head structures at high frequency may be due to a failure to inhibit Nodal signals along with BMP4 and Xwnt8. Piccolo et al. have shown that ventral activation of the Nodal antagonist, Cerberus, is dependent on inhibition of Wnt and BMP signaling, and Gsc inhibition of BMP4 may be insufficient to attain the levels of Cerberus required to inhibit Nodal signaling (Piccolo et al., 1999). It is possible that in the minority of Gsc-injected embryos that do form ectopic head structures, BMP4 inhibition has reached a threshold necessary for Cerberus activation. Supporting this interpretation, co-injection of Gsc and a truncated BMP receptor, a more potent BMP4 inhibitor, results in head formation at high frequency. In summary, our results indicate that Gsc promotes head-organizer function by repressing Xwnt8 transcription and also suggest that Gsc may contribute to the trunk organizer by indirect inhibition of BMP4.

Previous studies of Gsc have employed antisense RNA or activating Gsc fusion proteins to disrupt or antagonize the function of endogenous Gsc (Steinbeisser et al., 1995; Ferreira et al., 1998; Latinkic and Smith, 1999). While our phenotypic analysis of VP16-Gsc-injected embryos is largely consistent with these other studies, the conclusions we reach are distinct. In agreement with our results, antisense Gsc RNA caused a reduction or loss of head structures, without perturbing trunk formation, and resulted in an expansion of Xwnt8 expression (Steinbeisser et al., 1995). Several different activating forms of

Gsc have been shown to result in axial defects ranging from cyclopia to near complete inhibition of axis formation (Ferreiro et al., 1998; Latinkic and Smith, 1999), but the observed defects have not been linked to a direct regulatory target of Gsc. One study concluded that Xbra upregulation was responsible for the anterior truncation observed (Latinkic and Smith, 1999). Although Gsc can bind the Xbra promoter (Artinger et al., 1997; Latinkic et al., 1997), the fact that Xbra overexpression causes no axial defect (Artinger et al., 1997; Tada et al., 1997) argues against this conclusion. In addition, downregulation of endogenous Xbra is not dependent on Gsc, suggesting that Xbra is not an endogenous target of Gsc (Papin and Smith, 2000). Ferreiro et al. have reported the loss of both head and trunk structures, expansion of BMP4, Vent and Xwnt8 expression, and inhibition of organizer gene expression (Ferreiro et al., 1998). In our experiments, VP16-Gsc resulted in anterior truncation without upregulation of BMP pathway genes or downregulation of organizer genes. We note that inhibition of trunk and axial mesoderm formation was observed at substantially higher doses of VP16-Gsc, but these additional defects were not rescued by Gsc co-injection. Therefore, while specific inhibition of Gsc function results in anterior truncation, the previously reported trunk defects may result from interference with activities other than Gsc.

### Potential redundancy of multiple Wnt inhibitors in the Gsc null mouse

Analysis of the mouse embryo has demonstrated the existence of separate head and trunk organizers, with the head organizer derived from extra-embryonic endoderm (AVE, anterior visceral endoderm) and the trunk organizer derived from the epiblast (node) (reviewed by Beddington and Robertson, 1998; Beddington and Robertson, 1999). Several regulatory genes, including *Lim1*, *Otx2*, *HNF3 $\beta$* , *Nodal*, *Gsc*, *Cerberus-related 1* (*Cerr1*) and *Frzb1*, are expressed in the AVE (Beddington and Robertson, 1999), and embryos mutant for *lim1* or *otx2* completely lack head structures, while the trunk is largely unaffected (Acampora et al., 1995; Matsuo et al., 1995; Shawlot and Behringer, 1995; Ang et al., 1996). *Gsc* is expressed in both the AVE and the node and while a *Gsc* loss-of-function does not disrupt gastrulation or neurulation, embryos die soon after birth with craniofacial, tracheal, and skeletal malformations (Rivera-Perez et al., 1995; Yamada et al., 1995; Yamada et al., 1997; Belo et al., 1998; Zhu et al., 1998). Given the conservation of *Gsc* expression in the vertebrate gastrula, the absence of early defects suggests that functionally redundant genes may compensate for the loss of *Gsc*. Alternatively, *Gsc* may play no role in the gastrula, but several observations argue against this conclusion. Embryos heterozygous for *HNF3 $\beta$*  and homozygous for *Gsc* have severe defects anterior to the midbrain-hindbrain boundary, demonstrating a genetic interaction between these genes in head formation (Filosa et al., 1997). Furthermore, ablation and node transplantation studies of *Gsc*-null embryos demonstrate a requirement for *Gsc* function in the mouse gastrula (Zhu et al., 1999; Camus et al., 2000). Therefore, while the intact *Gsc*-null embryo can compensate and complete gastrulation normally, the loss of *Gsc* function does result in detectable defects in organizer function and anterior development.

Redundancy of negative regulatory factors is a consistent feature of the vertebrate embryo, and multiple BMP and Wnt

inhibitors are expressed in the mouse gastrula (Beddington and Robertson, 1999). Several Wnt inhibitors, including *Cerr1*, *Dickkopf* and *Frzb1*, are co-expressed with *Gsc* in the head-and/or trunk-organizer regions of the mouse embryo and these secreted factors presumably bind to and inhibit the activity of secreted Wnt proteins to promote axial development. In light of our analysis of *Xenopus Gsc*, we propose that the secreted Wnt inhibitors are sufficient to compensate for the loss of *Gsc* function, thus accounting for the absence of axial phenotypes in the *Gsc*-null embryos. Therefore, we speculate that inactivation of *Gsc* and one of the several secreted Wnt inhibitors would result in a double mutant embryo with defects in axial development arising at the gastrula stage.

In conclusion, our analysis of *Gsc* in the *Xenopus* embryo suggests a function for *Gsc* that differs from the prevailing view. In contrast to the trunk-inducing activity that has been described, our results suggest that *Gsc* directly represses *Xwnt8* transcription in the organizer, thus promoting head organizer activity and proper development of the head. Further study in the zebrafish, chick and mouse systems will determine whether the Wnt repression function of *Gsc* that we describe here plays a conserved role in vertebrate head formation.

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