Title
The opposing homeobox genes Goosecoid and Vent1/2 self-regulate Xenopus patterning

Journal
The EMBO Journal, 26(12)

ISSN
0261-4189

Authors
Sander, Veronika
Reversade, Bruno
De Robertis, EM

Publication Date
2007-06-20

DOI
10.1038/sj.emboj.7601705

Peer reviewed

The EMBO Journal (2007) 26, 2955–2965 | © 2007 European Molecular Biology Organization | Some Rights Reserved 0261-4189/07
www.embojournal.org

THE EMBO JOURNAL

EMBO open

The opposing homeobox genes *Goosecoid* and *Vent1/2* self-regulate *Xenopus* patterning

Veronika Sander, Bruno Reversade and EM De Robertis*

Howard Hughes Medical Institute and Department of Biological Chemistry, University of California, Los Angeles, CA, USA

We present a loss-of-function study using antisense morpholino (MO) reagents for the organizer-specific gene *Goosecoid* (*Gsc*) and the ventral genes *Vent1* and *Vent2*. Unlike in the mouse Gsc is required in *Xenopus* for mesodermal patterning during gastrulation, causing phenotypes ranging from reduction of head structures—including cyclopia and holoprosencephaly—to expansion of ventral tissues in MO-injected embryos. The overexpression effects of *Gsc* mRNA require the expression of the BMP antagonist *Chordin*, a downstream target of Gsc. Combined Vent1 and Vent2 MOs strongly dorsalized the embryo. Unexpectedly, simultaneous depletion of all three genes led to a rescue of almost normal development in a variety of embryological assays. Thus, the phenotypic effects of depleting Gsc or Vent1/2 are caused by the transcriptional upregulation of their opposing counterparts. A principal function of *Gsc* and *Vent1/2* homeobox genes might be to mediate a self-adjusting mechanism that restores the basic body plan when deviations from the norm occur, rather than generating individual cell types. The results may shed light on the molecular mechanisms of genetic redundancy.

The EMBO Journal (2007) **26,** 2955–2965. doi:10.1038/sj.emboj.7601705; Published online 24 May 2007
Subject Categories: development
Keywords: BMP; Chordin; embryonic induction; Goosecoid; Vent

Introduction

The isolation of the homeobox gene *Goosecoid* (*Gsc*) initiated the molecular exploration of the inductive activities of the Spemann organizer or dorsal lip of the blastopore (Cho *et al*, 1991). This venerable gene has been the subject of extensive studies in many organisms (reviewed in De Robertis, 2004). In *Xenopus*, overexpression studies showed that *Gsc* mRNA has axis inducing activities, promotes dorso-anterior migration of cells, and causes dose-dependent dorsalization of mesodermal tissues (Niehrs *et al*, 1993, 1994). Loss-of-function analyses indicated a requirement of Gsc for head formation in *Xenopus* by a variety of indirect methods such as antisense *Gsc* mRNA (Steinbeisser *et al*, 1995), antimorphic Goosecoids generated by the addition of epitope tags (Ferreiro *et al*, 1998), and fusion of the transcriptional activation domain of VP16 to the Gsc transcriptional repressor (Latinkic and Smith, 1999; Yao and Kessler, 2001). With the advent of antisense morpholinos (MOs) as powerful new tools for loss-of-function studies in *Xenopus* (Heasman, 2002), we decided to revisit the functional role of Gsc, and also of Vent1 and Vent2, two ventral homeobox genes that mediate part of the BMP activity during gastrulation (Onichtchouk *et al*, 1998).

This seemed worthwhile because knockout studies of Gsc in the mouse had shown no gastrulation phenotype, with death shortly after birth accompanied by a modest reduction in the midline of the base of the cranium (Rivera-Pérez *et al*, 1995; Yamada *et al*, 1995; Belo *et al*, 1998). Compound *Gsc*−/−; *HNF3*β/*FoxA2*+/− or *Gsc*−/−;*Dkk1*+/− mice showed severe disruptions of early embryonic patterning (Filosa *et al*, 1997; Lewis *et al*, 2006). Although *Gsc* knockout embryos gastrulate normally, *Gsc*−/− mouse nodes have a decreased neural inducing activity when transplanted into chick primitive streak embryos, indicating that the lack of gastrulation phenotype seen in *Gsc* mutant mice results from regulatory mechanisms that can compensate for the loss of this gene (Zhu *et al*, 1999).

In *Drosophila*, mutation of *D-gsc* is embryonic lethal, but, as in the mouse, also fails to show early phenotypes, with the main abnormalities being restricted to the invaginating foregut (Goriely *et al*, 1996; Hahn and Jaeckle, 1996). In zebrafish, a recent study from the Thisses' lab found that a Gsc MO caused head defects in 14% of the knockdown embryos, but, interestingly, together with a FoxA3 MO, which on its own resulted in no head abnormalities, led to defects ranging from cyclopia to anterior head deletions in 54% of the embryos (Seiliez *et al*, 2005).

Gsc is thought to promote dorsal endomesodermal development in *Xenopus*, while *Vent* genes expressed at the ventral side of the embryo mediate mesodermal patterning on the opposite side (reviewed by Niehrs, 2001). *Vent1* (also known as *PV.1*) and *Vent2* (also known as *Vox*, *Xom* or *Xbr-1*) are homeobox genes strongly induced by BMP4 on the ventral side of the embryo (Gawantka *et al*, 1995; Ault *et al*, 1996; Ladher *et al*, 1996; Onichtchouk *et al*, 1996; Papalopulu and Kintner, 1996; Schmidt *et al*, 1996). *Vent1* was the founding member of the *Bmp4* synexpression group, which includes other ventral genes such as *BAMBI* (BMP and Activin membrane-bound inhibitor), *Sizzled* (a ventrally expressed metalloproteinase inhibitor), *Bmp receptor 2*, *Twisted Gastrulation*

*Corresponding author. Howard Hughes Medical Institute and Department of Biological Chemistry, University of California, 675 Charles Young Drive South, Los Angeles, CA 90095-1662, USA.
Tel.: +1 310 206 1401; Fax: +1 310 206 2008;
E-mail: ederobertis@mednet.ucla.edu

Received: 24 January 2007; accepted: 5 April 2007; published online: 24 May 2007

(a BMP modulator), *Smad 6* and *7* (intracellular inhibitors of the BMP signaling pathway), as well as *Vent2* (Niehrs and Pollet, 1999; Karaulanov *et al*, 2004). Since their discovery a decade ago, Vent1 and Vent2 have been proposed to negatively cross-regulate Gsc on the dorsal side (Gawantka *et al*, 1995; Onichtchouk *et al*, 1996), presumably mediating the negative regulatory loop between BMP4 and Gsc in the mesoderm identified by Fainsod *et al* (1994). This inhibitory relationship between ventral and dorsal homeobox genes has been further strengthened by a number of *Xenopus* and zebrafish studies (Melby *et al*, 2000; Kawahara *et al*, 2000a, b; Imai *et al*, 2001).

In the present study, we have generated MOs that deplete Gsc, Vent1 and Vent2 in the *Xenopus laevis* subtetraploid species. The MOs target both pseudoalleles for each gene, and are expected to be generally useful for investigators working in *X. laevis*. It was found that Gsc is required for head development and dorsal–ventral (DV) patterning. In gain-of-function experiments, the dorsalizing effects of *Gsc* mRNA were found to be dependent on the expression of *Chordin* (*Chd*). Depletion of either Vent1 or Vent2 had little effect on their own, but in combination a marked expansion of dorsal tissues was observed. This was accompanied by expanded Gsc expression throughout the endomesoderm. In animal cap explants, Vent1/2 MO injection induced the expression of organizer markers such as *Gsc* and *Chd*. In ventral half-embryos, which normally develop as belly-pieces, significant amounts of neural and axial tissues developed. These dorsalizing effects of Vent1/2 MOs were blocked in Vent1/2/Gsc triple knockdowns. Unexpectedly, triple depleted embryos appeared to have normal DV and anterior–posterior (AP) pattern, as if Gsc, Vent1 and Vent2 were dispensable for embryogenesis. One exception was the blood marker *Scl* (*Stem cell leukemia* transcription factor), which was not restored in triple MO embryos. Evidently, additional mechanisms exist in the embryo that are sufficient for embryonic patterning in the absence of these three important homeobox genes. These findings provide insights into the redundant mechanisms operating in vertebrate development. Taken together, our data suggest that DV patterning is mediated in part by the reciprocal transcriptional repression of Gsc and Vent, whose balanced activities provide a self-adjusting safety net that ensures robust and reproducible embryonic development.

Results

Depletion of Goosecoid affects head development and DV patterning of the embryo

The homeobox gene *Gsc* marks the Spemann organizer (Figure 1A) and it also executes some of the functions of the organizer when overexpressed, such as induction of secondary axes, recruitment of neighboring cells into axial tissues, and patterning of dorsal mesodermal tissues (Niehrs *et al*, 1993). We designed a MO targeted against both pseudoalleles of the *X. laevis Gsc* gene, which should provide a better tool than the more indirect loss-of-function reagents used by previous workers (Figure 1B). Radial injection of Gsc MO into the vegetal pole of early two-cell stage embryos caused severe truncations of the head, indicated by the forebrain markers *Otx2* and *Six3*, the midbrain/hindbrain border marker *Engrailed2* (*En2*), and the hindbrain rhombomere 3 and 5 marker *Krox20*, as well as a complete loss of the eyes marked by *Six3* or *Rx2a* in about 40% of the embryos (Figure 1C–I). Depletion of Gsc also affected DV pattern, ventralizing the embryo, as illustrated by a moderate expansion of the ventral marker gene *Sizzled* (*Szl*; Figure 1G). Ventralization was cell-autonomous, because dorsal B1 blastomeres co-injected at the 32-cell stage with *lacZ* mRNA lineage tracer and Gsc MO adopted somite fates instead of notochord (Supplementary Figure 1) (Niehrs *et al*, 1993). In dorsal marginal zone (DMZ) explants, increasing concentrations of Gsc MO induced notochord, muscle, and ventral mesoderm tissues at the expense of prechordal plate in a dose-responsive manner (Supplementary Figure 2; Niehrs *et al*, 1994).

The loss of Gsc also affected AP axis patterning, as indicated by the reduced head and an altered pattern of *MyoD* expression, including a significant loss of *MyoD* at the tip of the tail (Figure 1I, arrow), whereas the expression of the spinal cord marker *HoxB9* appeared normal (Figure 1D). The phenotypes of Gsc MO injection were completely rescued by co-injection of *mouse Gsc* (*mGsc*) mRNA (Figure 1D and E). Less affected embryos had cyclopic eyes and lacked the mouth opening (Figure 1J and K). These results show that Gsc MO works as a specific tool for Gsc knockdown, and demonstrate that Gsc is required for head formation and patterning of the DV axis in the *Xenopus* embryo.

The effects of Goosecoid overexpression are mediated by Chordin

We next investigated downstream effectors of Gsc, in particular Chd, a secreted BMP antagonist expressed in the organizer (Sasai *et al*, 1994). We observed that expression of *Chd* at midgastrula was reduced 2.5-fold in Gsc-depleted embryos (Figure 2A–C). The opposite effect was seen in gain-of-function experiments, in which *Chd* expression was greatly expanded after injection of *mGsc* mRNA (Figure 2, compare panels D and E). These results indicate that Chd is indeed a downstream target of Gsc.

Ectopic *Gsc* expression leads to axis induction or dorsalization of the embryo (Cho *et al*, 1991; Niehrs *et al*, 1993). To test whether Chd is required for these effects we injected *mGsc* mRNA into one of the ventral blastomeres at four-cell stage. A range of dorsalized phenotypes was observed (Figure 2J): 35% of the embryos showed dorsalization with enlarged head structures, whereas 50% formed secondary axes, of which 12% had complete secondary eyes (marked by *Rx2a*) and notochords (marked by *Xenopus brevican*, *Xbcan*, an extracellular protein also expressed in rhombomeres 5 and 6 of the hindbrain; Sander *et al*, 2001) and cement glands (Figure 2F–H). Simultaneous injection of Chd MO (Oelgeschläger *et al*, 2003) together with *mGsc* mRNA lead to a rescue of normal development in 97% of co-injected embryos (Figure 2I and J). This indicates that Chd mediates the dorsalizing effects of Gsc.

Gsc is also known to rescue DV patterning in embryos ventralized by irradiation with ultraviolet (UV) light (Steinbeisser *et al*, 1993). Fertilized eggs treated with UV develop into ventral tissue and are devoid of organizer and neural gene expression (Figure 2K and M). As expected, injection of *mGsc* mRNA induced *Chd* expression (Figure 2L) and rescued the dorsal axis and anterior tissues,

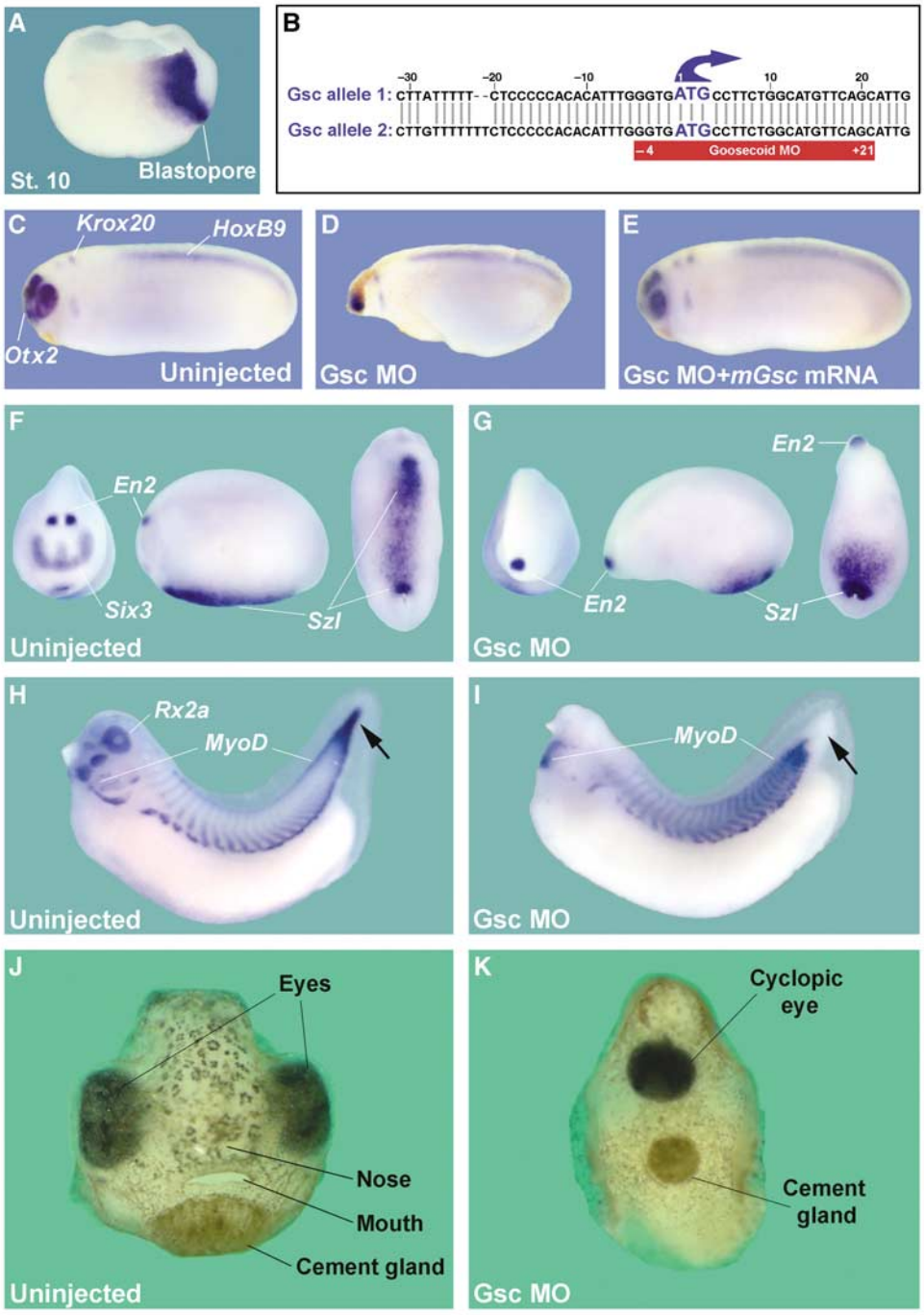

Figure 1 *Gsc* knockdown in *Xenopus* embryos causes loss of head structures and affects patterning of the AP and DV axes. (**A**) *Gsc* marks Spemann organizer endomesoderm at early gastrula. (**B**) Gsc MO targets both pseudoalleles of the *X. laevis Gsc* gene. (**C**–**I**) Gsc MO injection (136 ng total) causes loss of head structures, marked by *Otx2* (forebrain), *Six3* and *Rx2a* (forebrain and eyes), and *En2* (midbrain/hindbrain border) ($n = 106$; Supplementary Table I). Expression of the ventral marker *Szl* is reduced anteriorly and expanded posteriorly in the ventral blood island. (E) Co-injection of *mGsc* mRNA (200 pg total, radial injection) rescues the Gsc MO phenotype ($n = 78$). (H, I) Knockdown of Gsc reduces head size and affects patterning of the posterior somites, including loss of *MyoD* expression at the tip of the tail (arrows). (**J, K**) Moderately affected embryos survive until tadpole stage and have cyclopic eyes (indicating holoprosencephaly) and no mouth opening.

as shown by the expression of the pan-neural marker *Sox2* and the forebrain marker *Otx2* (Figure 2N). However, UV rescue by *mGsc* mRNA was completely blocked in Chd-depleted embryos (Figure 2O). Taken together, these results show that the effects of Gsc gain-of-function, which mimic the properties of the Spemann organizer, depend on the expression of its downstream target *Chordin*.

Goosecoid requirement in Activin-treated animal caps

We next addressed the requirement for Gsc in a sensitized ectodermal explant (animal cap) assay. Treatment with Activin leads to dose-dependent induction of mesodermal cell fates, which in animal caps assays result in explant elongation and neural induction. Animal caps from control and Gsc MO-injected embryos were excised at blastula,

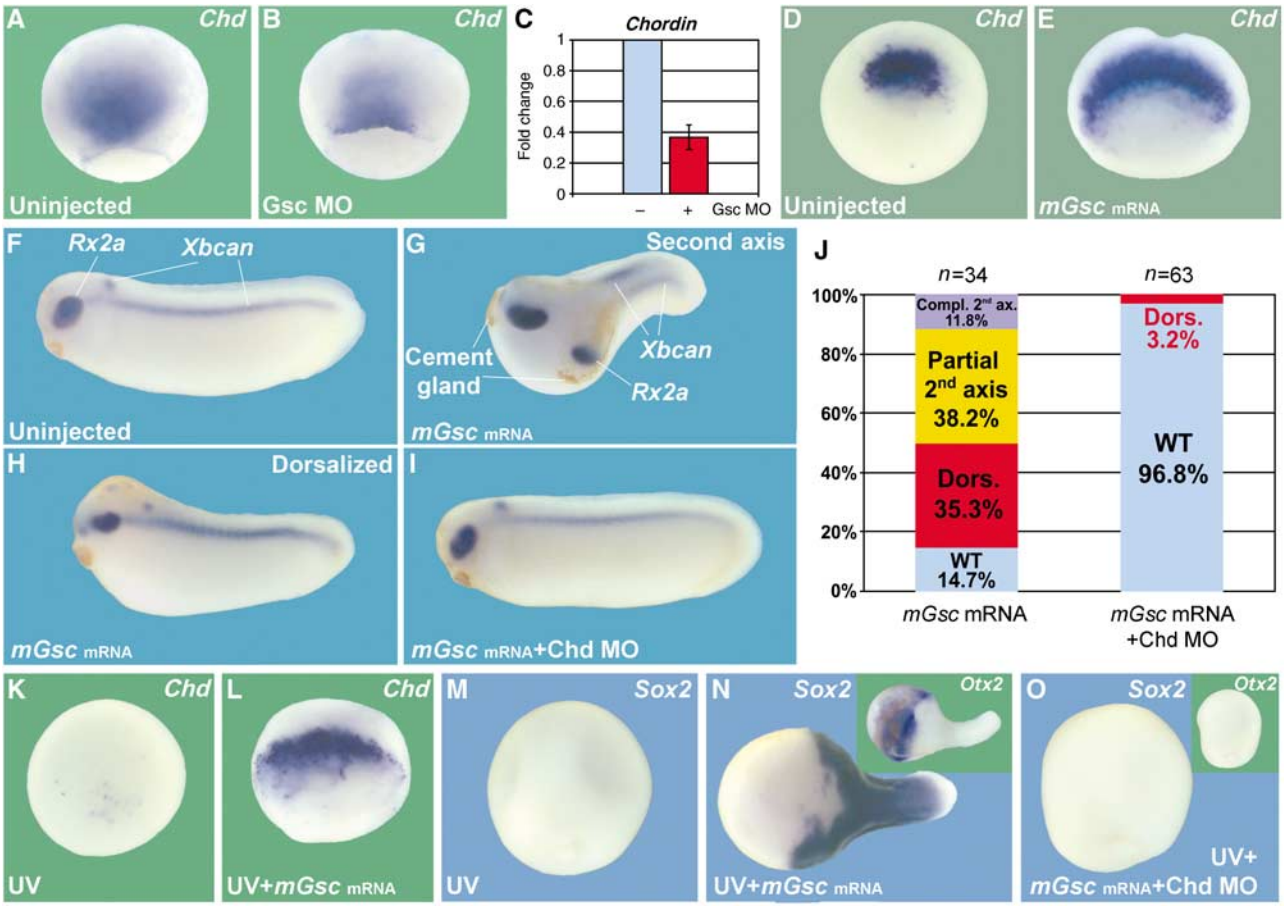

Figure 2 The dorsalizing effects of *mGsc* mRNA injection require Chd. (**A–E**) Gsc MO reduces *Chd* expression at gastrula 2.5-fold, whereas overexpression of *mGsc* mRNA greatly expands *Chd* expression. (**F–H**) Injection of 50 pg *mGsc* mRNA into one ventral blastomere at the four-cell stage leads to a range of dorsalized phenotypes, of which 50% develop secondary axes (38% partial; 12% complete with eyes, notochords, and cement glands). (**I, J**) Co-injection of Chd MO (34 ng) prevents second axis induction and dorsalization by *mGsc* mRNA in 97% of the embryos. (**K, L**) *mGsc* mRNA microinjection (200 pg total) induces *Chd* expression in UV-ventralized embryos at gastrula. (**M–O**) The rescue of head (*Otx2*) and pan-neural marker (*Sox2*) in UV embryos by *mGsc* overexpression ($n = 54$) has a complete requirement for Chd (co-injection of 136 ng Chd MO; $n = 59$).

treated with 2 ng/ml recombinant human Activin until stage 22, and analyzed by *in situ* hybridization and quantitative RT–PCR (Figure 3A). Elongation of animal caps by Activin was not blocked in the absence of Gsc, but Gsc-depleted caps failed to undergo neural differentiation, as shown by the lack of *Otx2* expression (Figure 3B–E) or that of *Sox2* (data not shown). This anti-neural effect in animal caps was stronger than in whole embryos, in which residual neural gene expression was always observed (Figure 3B and D, insets). These results suggest that Gsc is required for neural induction in Activin-treated animal caps, but not for their elongation (which is caused by the differentiation of dorsal mesoderm such as somites). This is in contrast with Chd, which is required for both elongation and neural differentiation of animal caps by Activin (Figure 3F and G). Thus, the dorsalizing effects of Activin have a complete requirement for Chd (Oelgeschläger *et al*, 2003), but only a partial one for Gsc.

To gain a better insight into the histotypic differentiation of Gsc-depleted caps, we next performed quantitative RT–PCRs (Figure 3H). The anterior neural markers *Otx2* and *Rx2a* and the organizer gene *Admp* were downregulated upon Gsc depletion, suggesting that in the wild-type embryo Gsc promotes the expression of these genes. *Gsc* expression itself—measured in samples from whole embryos at gastrula—was upregulated in Gsc-depleted embryos, in line with the previously described auto-inhibitory regulation of Gsc (Danilov *et al*, 1998). The somite markers *MyoD* and *α-Actin*, as well as the ventral mesoderm markers *Vent1*, *Vent2*, and *Evx1*, a proposed downstream target of Vent (Onichtchouk *et al*, 1998), were upregulated upon depletion of Gsc (Figure 3H). In addition, the ventrally expressed signaling factor *Wnt8* was upregulated in Gsc morphants. Yao and Kessler (2001) described that *Wnt8* is directly repressed by Gsc and, underscoring the importance of this interaction, we now found that Wnt8 MO suppresses the phenotype of Gsc morphants (Supplementary Figure 3). Taken together, the results suggest that the wild-type function of Gsc is to repress genes of paraxial and ventral mesoderm, while inducing neural markers and genes expressed in dorsal-most axial mesoderm. Gsc depletion caused a remarkably strong upregulation of the homeobox transcription factor *Vent1* (up to 25-fold), which prompted us to investigate more deeply the interplay between *Gsc* and the *Vent* genes.

Loss of Vent leads to dorsalization of the embryo

The Vent transcription factors consist of two genes in *Xenopus*: *Vent1* and *Vent2* (Gawantka *et al*, 1995;

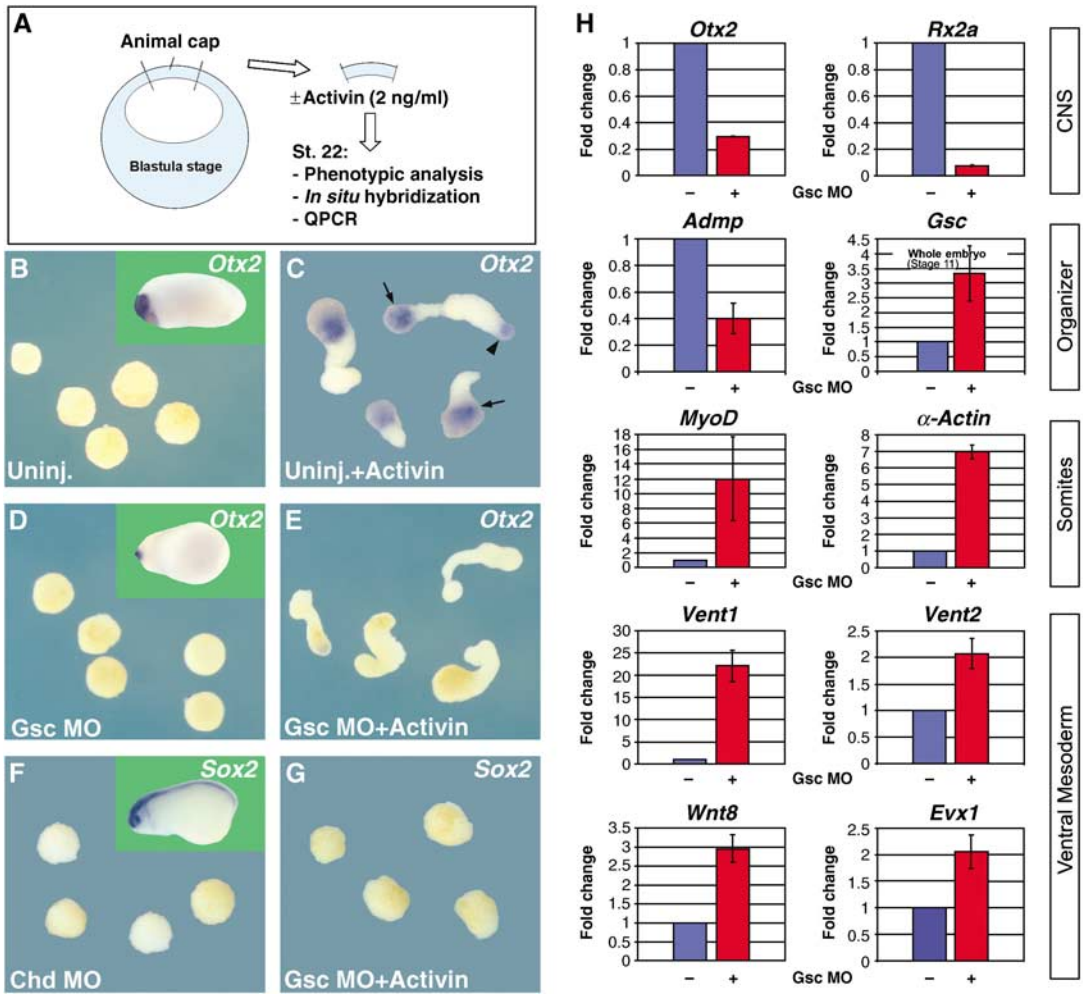

Figure 3 Gsc is required for secondary neural induction and mesoderm patterning in Activin-treated animal cap explants. (**A**) Experimental design ($n = 15$ or more per experimental set) (**B, C**) Untreated animal caps develop into atypical epidermis, whereas Activin treatment leads to elongation and brain formation, visualized by *Otx2* at the anterior pole (arrows). In addition, *Otx2* expression in anterior endoderm can be seen in one of the explants (arrowhead). (**D, E**) Gsc-depleted caps elongate after treatment with Activin, but lack *Otx2* neural staining. (**F, G**) Chd-depleted caps treated with Activin are unable to elongate, confirming the requirement of Chd for dorsal mesoderm and neural induction by Activin (Oelgeschläger *et al*, 2003). Insets show whole sibling embryos. (**H**) Quantitative RT–PCRs showing genes affected by depletion of Gsc include markers of anterior CNS, organizer, somites, and ventral mesoderm. Note that *Vent1* expression is increased more than 20-fold by Gsc knockdown.

Onichtchouk *et al*, 1996). To study their roles in the early embryo, MOs against both genes were designed. Radial injection of Vent1 MO into 2- to 4-cell stage embryos lead to a modest increase of *Sox2* expression at neurula stage, but seemed to have no effect on tadpole stage embryos (Figure 4B). Vent2 depletion broadened the neural plate and also moderately dorsalized the embryo at later stages (Figure 4C; Supplementary Figure 4). Strikingly, dorsalization was greatly increased when MOs for Vent1 and Vent2 were co-injected, causing the development of enlarged neural plates and head structures. At the tadpole stage, double Vent1/2-depleted embryos consisted of head structures with no tails and very short trunks (Figure 4D). Thus, Vent1 and Vent2 are partially redundant, as had been reported previously in zebrafish (Imai *et al*, 2001). Co-injection of mRNAs for *Vega1* and *Vega2*, the zebrafish homologues of *Vent1* and *Vent2* (Kawahara *et al*, 2000a, b; Melby *et al*, 2000), rescued the dorsalized phenotype of Vent1/2 knockdowns, indicating that the effect of these MOs was specific (Supplementary Figure 5).

Gsc and Vent1 and Vent2 have been proposed to repress each other in a cross-regulatory loop (Gawantka *et al*, 1995; Onichtchouk *et al*, 1996). Accordingly, *Gsc* expression would be expected to be upregulated upon Vent1/2 depletion. This was indeed the case, as shown in hemi-sectioned gastrula stage embryos (Figure 4E and F) and quantitative RT–PCRs in animal caps (Figure 4I). In addition, the expression of other dorsal genes, namely *Chd* (see insets in Figure 4E and F) and *Admp*, was increased upon Vent1/2 depletion (Figure 4I). The opposite result, upregulation of *Vent1* and *Vent2* expression in Gsc-depleted embryos, was also observed (Figure 4G and H). We conclude that Vent1 and Vent2 play an important role in repressing dorsal gene expression, since their depletion leads to a severe dorsalization of the embryo and a striking upregulation of *Gsc*.

Triple depletion of Goosecoid and Vent1/2 restores normal DV and AP pattern

What would be the result if transcription factors under opposite regulation were knocked down simultaneously? To

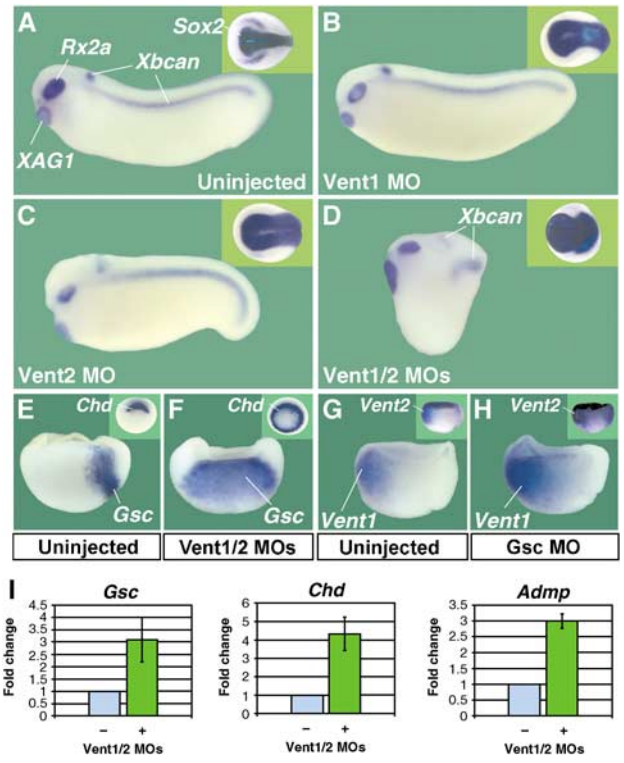

Figure 4 Double depletion of Vent1 and Vent2 causes severe dorsalization of the embryo. (**A–D**) Injection of either Vent1 or Vent2 MO expands the neural plate at neurula stage (insets), but only the combination of both MOs strongly dorsalizes tailbud stage embryos, with shortened body axes and large heads and cement glands ($n=122$; Supplementary Table I). (**E, F**) Vent1/2 depletion leads to transcriptional upregulation of *Gsc* (hemisections at stage 10; $n=15$) and *Chd* (insets in panels E and F; whole embryos, vegetal view; $n=18$). (**G, H**) Loss of Gsc increases *Vent1* and *Vent2* expression (hemisections at stage 10; $n=21$ and 15) (**I**) Quantitative RT–PCR analyses showing 3- to 4-fold upregulation of the organizer genes *Gsc*, *Chd*, and *Admp* in animal caps at gastrula stage after Vent1/2 depletion.

answer this question, we co-injected the MOs for Gsc, Vent1, and Vent2 radially at the four-cell stage. Surprisingly, 80% of triple-depleted embryos were rescued to an almost normal pattern, with well-formed axial structures such as somites (marked by *MyoD*), spinal cord (*HoxB9*), notochord (not shown), brain (*Six3*, *Krox20*), and heart (*Nkx2.5*) (Figure 5A–F). At the neurula stage, the expression domains of *Sox2* (neural plate), *En2* (midbrain/hindbrain border), and *XAG1* (cement gland) that were strongly expanded in Vent1/2 morphants, were rescued to normal in triple-depleted embryos (see insets in Figure 5A–C and G–I). To show that the doses of MOs used in the triple knockdown experiments effectively depleted the activities of the three genes, we injected Gsc MO, Vent1/2 MOs and Vent1/2/Gsc MOs in various concentrations; even the lowest doses caused identical phenotypes, indicating that the loss-of-function in the triple morphant embryos was complete (Supplementary Figure 6).

This extraordinary rescue in embryos in which Vent1/2 and Gsc were knocked down shows that the dorsalizing effect of the Vent1/2 depletion is mediated by the upregulation of Gsc and vice versa. It is startling that the loss of three transcription factors—shown to have important effects in single loss- and gain-of-function situations—is without much consequence on the overall pattern formation of the embryo. The only defect we observed in triple depleted embryos was a marked reduction in blood tissue, marked by *Scl* (Figure 5G–I). We also observed that the triple MO embryos usually did not survive beyond stage 30. Thus, the loss of Gsc, Vent1 and Vent2 can be compensated to a remarkable extent during early development but not later on.

Quantitative RT–PCRs of animal cap explants at gastrula stage supported these findings. For example, *Chd* expression was increased four-fold by Vent1/2 knockdown, but was restored to normal levels in triple-depleted cap samples (Figure 5J). *Szl*, which is expressed in the ventral center as member of the *Bmp4* synexpression group, was downregulated upon Vent1/2 removal, indicating it is a downstream target of Vent. Vent1/2/Gsc knockdown, however, restored expression levels of *Szl* to normal (Figure 5K). Similar effects were observed for *BAMBI* (data not shown). Depletion of Chd had stronger effects than Gsc, for it was epistatic to Vent1/2 (Supplementary Figure 7). The data indicate that the dorsalization effects of Vent1/2 loss-of-function are mediated by the upregulation of Gsc and that, reciprocally, the effects of Gsc depletion are mediated by the upregulation of Vent1/2.

Self-regulation in half-embryos

Bisection of wild-type embryos at blastula stage along the DV axis (Reversade and De Robertis, 2005) leads to the formation of ventral half belly-pieces (called Bauchstücke by Hans Spemann) that consist of ventral tissues, whereas dorsal halves self-regulate to form well-proportioned half-sized embryos (Figure 6A–D). *HoxB9*, which is a spinal cord and ventral mesoderm marker (Wright *et al*, 1990), marks only ventral mesoderm in ventral halves, which are devoid of neural tissue marked by *Sox2* (Figure 6D, inset). Knockdown of Gsc did not affect the ventral half and, as expected, reduced head development in the dorsal halves (Figure 6E and F). In contrast, Vent1/2 depletion not only dorsalized the dorsal halves, resulting in large head structures, but also caused dorsalization of the ventral half-embryos. These ventral halves formed elongated axial neural structures expressing *Sox2*, *HoxB9* and *Krox20* (as well as mesodermal *MyoD* in somites and *Xbcan* in the notochord, data not shown), which in most cases lacked the forebrain marker *Six3* (Figure 6G and H). To investigate whether Gsc was involved in the dorsalization of ventral half-embryos caused by Vent1/2 depletion, we analyzed triple Vent1/2/Gsc morphants. It was found that the depletion of Gsc reversed the phenotypes of Vent1/2 knockdown to the wild-type pattern causing the differentiation of belly-pieces (Figure 6I and J). These results suggest several conclusions. First, because Vent1/2-depleted dorsal halves were only partially dorsalized, we believe additional ventralizing signals must exist on the dorsal side. Second, the development of Vent1/2-depleted ventral halves into embryos with dorsal mesodermal and neural structures is mediated exclusively by Gsc, since in triple knockdowns belly-pieces lacking all dorsal tissues are formed. Finally, it seems that *Gsc* and the *Vent1/2* genes are predominantly required in their normal side of expression, while after removal of all three they seem to be dispensable for embryonic pattern formation.

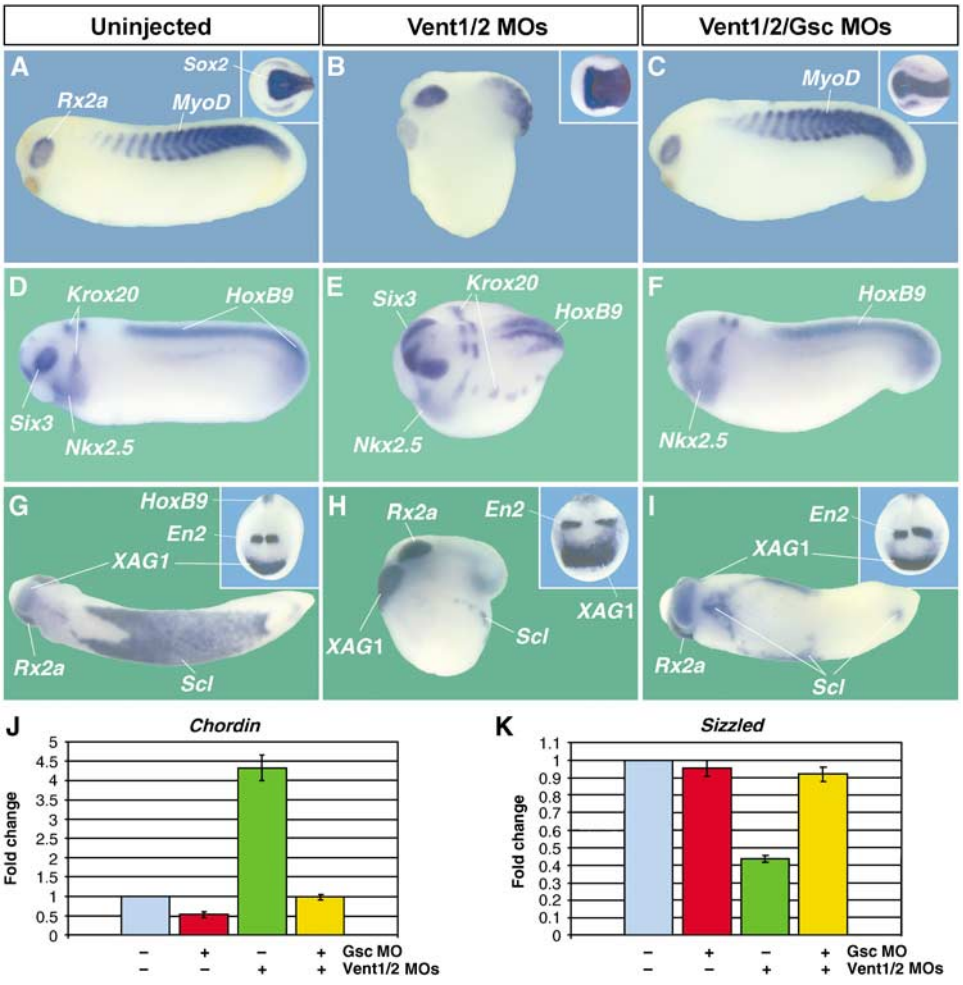

Figure 5 Gsc is required for the dorsalization caused by Vent1/2 knockdown. (**A–I**) Co-injection of Gsc MO restores normal pattern in Vent1/2-depleted whole embryos ($n = 53$; Supplementary Table I). At the neurula stage, knockdown of Vent1/2/Gsc reduces the neural plate (*Sox2*) back to normal size (insets in panels A–C). In addition, the expansion of the cement gland and midbrain in Vent1/2 morphants is rescued in triple knockdown embyros (insets in panels G–I). Note that blood formation (*Scl*) is not rescued in the triple depletions (I). All MOs were injected at the same dose (45 ng each). (**J**) The upregulation of *Chd* expression by Vent1/2 MO is restored to control levels in Vent1/2/Gsc-depleted animal caps at gastrula stage. (**K**) Expression of *Szl* is downregulated by Vent1/2 MO, but restored to normal levels when Gsc is also depleted.

Discussion

The results presented in this loss-of-function study by MO knockdown strengthen the view that *Gsc* and *Vent* homeobox genes have mutually opposing roles in patterning the mesoderm of the *Xenopus* embryo. First, single Gsc knockdown produced head truncations and increased ventral tissue in whole embryos. Second, all the effects of *Gsc* mRNA overexpression could be blocked by Chd MO. Third, Vent1 and Vent2 MOs strongly synergized with each other, causing severe dorsalizations accompanied by the massive upregulation of *Gsc* expression over the entire endomesodermal region. Fourth, triple Vent1/2/Gsc knockdown embryos developed with an almost completely normal pattern, without either the ventralized phenotype of the Gsc MO, or the dorsalizing influence of Vent1/2 MOs. This lead to the surprising conclusion that the basic DV and AP patterning can be achieved in the absence of these three important transcription factors. Thus, it appears that these homeobox genes are engaged principally in cross-regulatory interactions with each other to ensure robust development.

Goosecoid is required for early patterning in Xenopus embryos

The discovery of Gsc was a very exiting moment, because it provided a marker for the Spemann organizer that, when overexpressed, could induce secondary axes and other aspects of the inducing activities of organizer tissue (reviewed in De Robertis, 2006). *Gsc* homologues have been found in all animals that have been studied, ranging from flatworms to humans (Blum *et al*, 1992; De Robertis, 2004). Therefore, the lack of a gastrulation phenotype of Gsc knockouts in the mouse (Rivera-Pérez *et al*, 1995; Yamada *et al*, 1995; Belo *et al*, 1998) and in *Drosophila* (Goriely *et al*, 1996; Hahn and Jaeckle, 1996) was very puzzling. In zebrafish, however, Seiliez *et al* (2005) recently reported cyclopia and anterior truncations in Gsc morphants. In *Xenopus*, work using antimorphic Goosecoids, VP16 fusions, or antisense RNA, all had indicated a role for Gsc in patterning the early mesoderm (Steinbeisser *et al*, 1995; Ferreiro *et al*, 1998; Latinkic and Smith, 1999; Yao and Kessler, 2001).

Using a Gsc MO, we have now confirmed that Gsc is required for early patterning in *Xenopus*. The Gsc knockdown

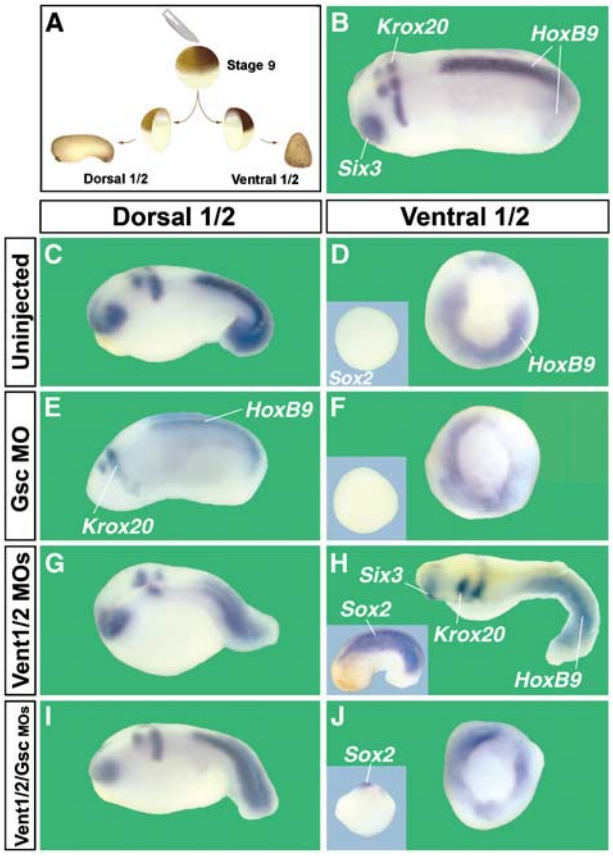

Figure 6 Knockdown of Gsc and Vent1/2 restores normal development of dorsal and ventral half-embryos ($n = 52$ or more per experimental set). (**A**) Embryos were bisected into dorsal and ventral halves at blastula stage. (**B**) Control sibling at the same magnification as the other panels. (**C, D**) Bisectioned control embryos form smaller but well-proportioned dorsal half-embryos, whereas ventral halves differentiate into belly-pieces that express *HoxB9* in the ventral mesoderm (Wright *et al*, 1990) but are devoid of neural tissue, as indicated by the lack of *Sox2* expression (inset). (**E, F**) Gsc depletion (136 ng MO) causes a reduction of the head in dorsal halves, whereas ventral halves are not affected. (**G, H**) Dorsal halves of Vent1- and Vent2-depleted embryos (45 ng each) are dorsalized, but retain overall DV patterning. The corresponding ventral halves are strongly dorsalized, including expression of spinal cord (*HoxB9*), brain (*Krox20, Six3*), and pan-neural *Sox2* marker (inset). (**I, J**) Remarkably, both halves of triple knockdown embryos (45 ng each) develop as the uninjected control half-embryos.

phenotypes included truncations and fusions of the forebrain and eyes, and dose-responsive ventralization of dorsal mesoderm. Quantitative RT–PCR studies in Activin-treated animal cap explants confirmed that Gsc MO causes ventralization, inhibiting the organizer gene *Admp* and the brain markers *Otx2* and *Rx2a*, and inducing expression of ventral markers, such as *Wnt8*, *Vent2*, and *Evx1* (Figure 3H). Gsc depletion caused a particularly strong induction of the homeobox transcription factor *Vent1* (over 20-fold), providing the initial impetus to investigate the relationship between Gsc and Vent. In addition to the head phenotype, somite formation was affected by the loss of Gsc, and *MyoD* expression was lost in the tip of the tail (Figure 1I, arrow), a region in which *Vent2* is expressed (Onichtchouk *et al*, 1996). Onichtchouk *et al* (1998) have described Vent2 as a repressor of muscle formation in this region; perhaps loss of Gsc causes an upregulation of *Vent2*, which in turn may lead to increased repression of *MyoD*.

Experiments using Chd MO showed that the dorsalizing effects of injecting *mGsc* mRNA into wild-type or UV-treated embryos were mediated by the upregulation of Chd. This was a particularly satisfying result, since Chd was initially isolated as a downstream target gene of Gsc in the Spemann organizer (Sasai *et al*, 1994). The fact that Gsc is a transcriptional repressor makes a direct induction of *Chd* transcription unlikely. Chd activation may be mediated in part by a double repression mechanism, whereby Gsc represses Vents which in turn repress *Chd* expression (Melby *et al*, 1999). In addition, *Chd* transcription is also activated by Nodal/Activin signaling.

Goosecoid and genetic redundancy

Embryos must have highly redundant regulatory systems to ensure that a perfectly proportionate animal is produced time after time. However, our understanding of how genetic redundancy works is very rudimentary. It has been argued that perhaps a second mouse *Gsc* gene might explain the lack of gastrulation phenotype in the mouse (Belo *et al*, 1998). However, the present results suggest that an alternative explanation may be possible. In *Xenopus*, loss of Gsc is devoid of effect in the absence of Vent1 and Vent2. Therefore, it could be that in the mouse the Vent regulatory system might be less prominent than in the more rapidly developing *Xenopus* embryo. In this respect, it is interesting to note that clear *Vent* homologues have neither been found in the mouse nor in the *Drosophila* genome. The genes most closely related to *Vent* in the mouse are *BarX1* and *BarH1*, members of the *Bar* family of homeobox genes, that are defined by *Drosophila BarH1*, which share with Vents a rare amino-acid substitution at position 47 of the third helix of the homeodomain (Thr instead of Val or Ile) (Kappen *et al*, 1993). Interestingly, in addition to the Niehrs group (Onichtchouk *et al*, 1996), *Vent2* was isolated independently by Papalopulu and Kintner (1996), who named it *Xenopus Bar-related* (*Xbr-1*), as well as by Ladher *et al* (1996), who named it *Xom*, for its similarity to *Om(1D)*, the *Drosophila annasae* homologue of *D. melanogaster BarH1*. Both groups based the relationship of the newly discovered *Xbr-1/Xom* and *Drosophila BarH1/Om(1D)* on the sequence similarity (approximately 55%) in the homeodomain, as well as on the similarities in the expression pattern of the genes in the eye. As has been proposed for *Vents* (Onichtchouk *et al*, 1998), *Bar* genes also function as antineural agents. They achieve this by inhibiting the transcription of bHLH transcription factors, such as *Drosophila Atonal* or vertebrate *Neurogenin* (Saito *et al*, 1998; Lim and Choi, 2003). Mouse BarX1 and BarH1 are also involved in other processes, such as tooth development and stomach organogenesis (Tissier-Seta *et al*, 1995; Reig *et al*, 2006).

In humans, a *Vent*-like homeobox gene, called *VENTX*, has been described (Moretti *et al*, 2001). Although *VENTX* is only distantly related to the *Vent* genes, it shares the Thr substitution of the *Vent*- and *Bar*-subclass of homeobox genes. Two observations also indicate that human *VENTX* might indeed be a true homologue of the *Xenopus* and zebrafish *Vent/Vega* genes: first, microinjected *VENTX* mRNA ventralizes zebrafish embryos and, second, VENTX protein was detected in immature bone marrow and erythroleukemia cells.

From these results, Moretti and co-workers concluded that VENTX—like Vent and Vega—may be involved in mesoderm patterning and maintenance of hematopoietic stem cells in the adult. It appears that the *Vent* genes have adopted a prominent functional role in *Xenopus* and zebrafish, while in the mouse and *Drosophila* the *Bar* genes might carry out some of the functions of the missing *Vents*.

Gsc homologues, however, are found throughout the animal kingdom, from flatworms to vertebrates, and it is therefore unlikely that this endomesodermal gene is not an important player in embryogenesis, despite the genetic redundant mechanisms that are at play in some animals. In the case of the mouse embryo, the simplest interpretation would be that Gsc lacks a gastrulation phenotype because mice lost the *Vent* genes. We have analyzed the syntenic region of the mouse genome, and failed to find a *VENTX* murine homologue (V Sander, unpublished observations). Searching for a true murine *Vent* homologue seems important, and perhaps such a gene might be found by screening for BMP-inducible genes, since *Vent2* is a primary response gene to BMP4 (Rastegar *et al*, 1999; Trindade *et al*, 1999; Karaulanov *et al*, 2004).

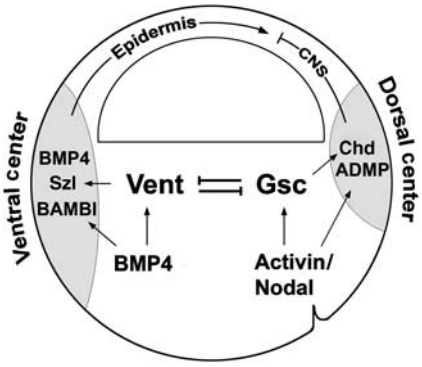

Figure 7 Model of regulatory mechanisms for pattern formation at gastrula. In the dorsal center, Activin/Nodal signals phosphorylate Smad2/3 to activate *Gsc* expression. The expansion of Chd and ADMP can also be achieved by Gsc-independent pathways. In the ventral center, BMP4/7 signals phosphorylate Smad1/5/8 and lead to the expression of *Vent1/2*. BMP4 is also able to activate ventral center secreted proteins by Vent-independent mechanisms. The function of Gsc and Vent is to regulate each other, providing an intracellular compensatory mechanism that works in concert with the extracellular networks of growth factors and their antagonists.

Self-regulation of the DV mesodermal field

The interaction between Vent1, Vent2, and Gsc had never been tested in a triple loss-of-function situation, which proved an interesting experiment. The triple knockdown of Gsc and Vent1/2 led to the surprising result of the restoration of normal embryonic patterning, not only in whole embryos but also in bisected dorsal and ventral half-embryos. First, this result argues that the dorsalization caused by Vent1/2 depletion is entirely mediated by Gsc. The Gsc MO, which had only a moderate effect on the dorsal half of the embryo when injected alone, had a very strong effect on Vent1/2-depleted ventral halves, reversing all dorsal cell differentiations to ventral mesoderm (Figure 6H and J). Second, it raises the question of how the embryo can compensate for the simultaneous loss of three genes, when single and double knockdowns are strongly affected. Two opposing homeodomain repressors are transcriptionally upregulated when one signaling center or the other is depleted. Removing both transcription factors negates the phenotype. It should be pointed out that this regulation might not be exclusively transcriptional. Dawid and co-workers have reported that in zebrafish Vega1 and Vega2 can directly bind to Gsc protein in immunoprecipitation experiments (Kawahara *et al*, 2000b). If binding of Gsc inhibited the activity of Vent/Vega, this could provide a simple mechanism for reinforcing the transcriptional regulation at the protein level. This is an aspect of the DV patterning system that deserves more study in the future.

One of the properties of the vertebrate embryo that has intrigued researchers since the beginnings of experimental embryology, is its ability to self-regulate pattern after experimental perturbation (reviewed by De Robertis, 2006). Recent work has suggested that molecules of similar biochemical activities but under opposite transcriptional control expressed on the dorsal or ventral side of the embryo might explain the formation of a self-regulating morphogenetic field. So far, this proposition has been tested for secreted proteins produced in the Spemann organizer, such as Chd and ADMP, and proteins expressed ventrally as part of the *Bmp4* synexpression group (also called the ventral center), such as BMP4/7, the Xolloid-related Chordinase (Xlr) and its competitive inhibitor Sizzled (Reversade and De Robertis, 2005; Lee *et al*, 2006). Both Gsc and Vent are homeodomain proteins that function as transcription repressors. However, they are under the opposite transcriptional control by Smad1/5/8 (which are activated by BMP), and Smad2/3 (which are activated by Activin and Nodal) and might be considered part of the intracellular mechanism that maintains the morphogenetic field in the mesoderm.

In the triple knockdown situation, a safety net of extracellular dorsal and ventral center molecules might still be able to adjust and mediate self-regulation. We have tested this assumption by removing BMP4 or ADMP in addition to Vent1/2/Gsc. As shown in Supplementary Figure 8, both quadruple knockdowns resulted in strongly dorsalized embryos. This indicates that removing additional components of the regulatory safety network disrupts the self-regulation that can be still achieved in Vent1/2/Gsc triple morphants.

Figure 7 describes a model in which Gsc and Vent are considered central players in the gastrula embryo. The dorsal center is induced by Activin/Nodal signals, and Chordin and ADMP are induced by Gsc-dependent and independent pathways. On the ventral side, BMP4/Smad1 and Vent positively cross-regulate each other (Onichtchouk *et al*, 1996; Schuler-Metz *et al*, 2000; Henningfeld *et al*, 2002), inducing other components of the *Bmp4* synexpression group, such as BAMBI and Sizzled. In the dorsal center, *Gsc* is a primary response gene to Activin/Nodal signaling (Cho *et al*, 1991), and in the ventral center *Vent2* is a primary response gene to BMP4 (Rastegar *et al*, 1999; Trindade *et al*, 1999; Karaulanov *et al*, 2004). The transcriptional repressors Gsc and Vent strongly oppose each other, in order to establish and maintain a balance between dorsal and ventral pattern formation. DV patterning is a crucial process in early development, and our results suggests that the embryo has enough redundancy to provide a remarkable double assurance mechanism, such that when Gsc and Vent1/2 are removed, they can still be compensated by an extracellular mechanism involving the BMP4 and ADMP morphogens.

This situation, in which the removal of a very important developmental gene results in normal development in certain mutant backgrounds, is reminiscent of the case of Nanos in the early embryonic patterning of *Drosophila.* The posterior morphogen Nanos clears ubiquitously distributed maternal *hunchback* transcripts from the posterior half of the embryo. Lack of the maternal determinant Nanos leads to severe abdomen defects. However, flies double mutant for maternal *nanos* and *hunchback* develop completely normally (Struhl, 1989). Thus, the compensation mediated by the simultaneous removal of counteracting factors might help provide an understanding of the molecular mechanisms of genetic redundancy that goes beyond gene duplication hypotheses.

Goosecoid and cancer

Gsc is an old, intensely studied gene that might still yield more surprises, such as the one found in the present study for the mutual requirement of Gsc and Vent to self-regulate pattern. One particularly interesting recent development is the discovery by Weinberg and co-workers that Gsc is a major mediator of epithelial–mesenchymal transition in mammary tumor metastases (Hartwell *et al*, 2006). Thus, a gene that promotes cell migration in the prechordal plate in the *Xenopus* embryo (Niehrs *et al*, 1993), can be co-opted by cancer cells to promote invasiveness. This leaves us with the question of whether in these metastatic tumors the opposing interactions between Gsc and Vents, so important during *Xenopus* embryogenesis, might also come into play.

Materials and methods

Morpholino oligos

Antisense MOs for *X. laevis Gsc*, *Vent1*, and *Vent2* were obtained from Gene Tools LLC and consisted of the following sequences: Gsc MO, 5′-GCTGAACATGCCAGAAGGCATCACC-3′; Vent1 MO 5′-GTCA ATAGAGAATCCCTGTTGAACC-3′; and Vent2 MO 5′-GTCATCTTG TCTGTATTAGTCCT-3′. *X. tropicalis* Vent1 and Vent2 MOs had been reported previously (Polli and Amaya, 2002), but were not useful for the pseudotetraploid species *X. laevis*. The Chordin MO was as described (Oelgeschläger *et al*, 2003). MOs were resuspended in sterile water to a concentration of 1 mM. Prior to microinjections, the MOs were heated at 95°C for 30 s, placed on ice, and, unless indicated otherwise, injected four times vegetally (136 ng total). For the double Vent1/2 depletions, the two MOs were mixed at a ratio of 1:1:1 with water, the triple Vent1/2/Gsc MO mix was prepared at a ratio of 1:1:1, and a total dose of 45 ng per MO was injected in each case.

Embryological methods

mRNA for *mGsc* was transcribed from a pBluescriptII KS(−) construct (Blum *et al*, 1992). Procedures for mRNA synthesis and whole-mount *in situ* hybridization are available at www.hhmi.ucla.edu/derobertis/index.html. For animal cap explant studies, 2- to 4-cell embryos were injected four times into the animal pole with either Gsc MO, Vent1/2 MO, or all three MOs. Animal explants were isolated at stage 8, treated with 2 ng/ml recombinant human/mouse/rat Activin A (R&D Systems), and cultured until stage 22 (Sive *et al*, 2000). UV irradiation was performed as described (Steinbeisser *et al*, 1995). DV bisections were prepared from embryos with strong DV polarity (Klein, 1987) at stage 9 in 0.3 × Barth's solution as described (Reversade and De Robertis, 2005).

Quantitative RT–PCR

Total RNA of either three whole embryos or 10 animal caps per sample was extracted using the Absolutely RNA Microprep kit (Stratagene), and cDNA synthesis was carried out using random hexamer priming and the StrataScript Reverse Transcriptase. Quantitative RT–PCR was performed on the Mx3000P (Stratagene) using the Brilliant SYBR Green QPCR Master Mix (Stratagene). Measurements were performed in quadruplicates and normalized to the expression levels of *ODC* (*Ornithine decarboxylase*). Fold change values (x) were calculated using the following formula: $x = 2^{-\Delta\Delta Ct}$. Bars indicate standard deviations. The primer sequences were: α-Actin, fwd: TCCCTGTACGCTTCTGGTCGTA, rev: TCTCAAA GTCCAAAGCCACATA; Admp, fwd: GATGATGGAAGGAGAGGA, rev: TCATGTTCTGACCCAAAG; Chd, fwd: GTTGTACATTTGGTGGGAA, rev: ACTCAGATAAGAGCGATCA; Gsc, fwd: GCTGAT-TCCACCAGT GCCTCACCAG, rev: GGTCCTGTGCCTCCTCTTCCTCCTG; MyoD, fwd: AGGTCCAACTGCTCCGACGGCATGAA, rev: AGGAGAGAATCC AGTTGATGGAAACA; ODC, fwd: CAGCTAGCTGTGGTGTGG, rev: CAACATGGAAACTCACACC; Otx2, fwd: GGATGGATTTGTTACAT CCGTC, rev: CACTCTCCGAGCTCACTTCCC; Rx2a, fwd: AGACTGGT GGCTATGGAG, rev: ATACCTGCACCCTGACTT; Szl, fwd: GTCTTCC TGC-TCCTCTGC, rev: AACAGGGAGCACAGGAAG; Vent1, fwd: TTCCCTTCAGCATGGT-TCAAC, rev: GCATCTCCTTGGCATATTTGG; Wnt8, fwd: TATCTGGAAGTTGCAGCA-TACA, rev: GCAGGCACTCT CGTCCCTCTGT. The PCR cycling conditions for 40 cycles were: denaturation at 95°C for 30 s, annealing at 55°C for 60 s, and extension at 72°C for 30 s.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We are indebted to Drs I Dawid, D Kimelman, and C Niehrs for providing DNA constructs. We also thank C Metzinger, D Geissert, and T Boe for technical assistance. This work was supported by the NIH (R37 HD21502-21). EMDR is an investigator at the Howard Hughes Medical Institute.

References

Ault KT, Dirksen M-L, Jamrich M (1996) A novel homeobox gene *PV.1* mediates induction of ventral mesoderm in *Xenopus* embryos. *Proc Natl Acad Sci USA* **93:** 6415–6420

Belo JA, Leyns L, Yamada G, De Robertis EM (1998) The prechordal midline of the chondrocranium is defective in *Goosecoid-1* mouse mutants. *Mech Dev* **72:** 15–25

Blum M, Gaunt SJ, Cho KWY, Steinbeisser H, Blumberg B, Bittner D, De Robertis EM (1992) Gastrulation in the mouse: the role of the homeobox gene *goosecoid*. *Cell* **69:** 1097–1106

Cho KWY, Blumberg B, Steinbeisser H, De Robertis EM (1991) Molecular nature of Spemann's organizer: the role of the *Xenopus* homeobox gene *goosecoid*. *Cell* **67:** 1111–1120

Danilov V, Blum M, Schweickert A, Campione M, Steinbeisser H (1998) Negative autoregulation of the organizer-specific homeobox gene *goosecoid*. *J Biol Chem* **273:** 627–635

De Robertis EM (2004) Goosecoid. In: *Gastrulation*, Stern CD (ed). pp 581–589. New York: Cold Spring Harbor Laboratory Press

De Robertis EM (2006) Spemann's organizer and self-regulation in amphibian embryos. *Nat Rev Mol Cell Biol* **4:** 296–302

Fainsod A, Steinbeisser H, De Robertis EM (1994) On the function of BMP-4 in patterning the marginal zone of the *Xenopus* embryo. *EMBO J* **13:** 5015–5025

Ferreiro B, Artinger M, Cho KWY, Niehrs C (1998) Antimorphic *goosecoids*. *Development* **125:** 1347–1359

Filosa S, Rivera-Perez JA, Perea Gomez A, Gansmuller A, Sasaki H, Behringer RR, Ang S-L (1997) *Goosecoid* and *HNF-3*β genetically interact to regulate neural tube patterning during mouse embryogenesis. *Development* **124:** 2843–2854

Gawantka V, Delius H, Hirschfeld K, Blumenstock C, Niehrs C (1995) Antagonizing the Spemann organizer: the role of the homeobox gene *Xvent-1*. *EMBO J* **14:** 6268–6279

Goriely A, Stella M, Coffinier C, Kessler D, Mailhos C, Dessain S, Desplan C (1996) A functional homologue of *goosecoid* in *Drosophila*. *Development* **122:** 1641–1650

Hahn M, Jaeckle H (1996) *Drosophila goosecoid* participates in neural development but not in body axis formation. *EMBO J* **15:** 3077–3084

Hartwell KA, Muir B, Reinhardt F, Carpenter AE, Sgroi DC, Weinberg RA (2006) The Spemann organizer gene, *Goosecoid*, promotes tumor metastasis. *Proc Natl Acad Sci USA* **103:** 18969–18974

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

Henningfeld KA, Friedle H, Rastegar S, Knöchel W (2002) Autoregulation of *Xvent-2B*; direct interaction and functional cooperation of Xvent-2 and Smad1. *J Biol Chem* **277:** 2097–2103

Imai Y, Gates MA, Melby AE, Kimelman D, Schier AF, Talbot WS (2001) The homeobox genes *vox* and *vent* are redundant repressors of dorsal fates in zebrafish. *Development* **128:** 2407–2420

Kappen C, Schughart K, Ruddle FH (1993) Early evolutionary origin of major homeodomain sequence classes. *Genomics* **18:** 54–70

Karaulanov E, Knoechel W, Niehrs C (2004) Transcriptional regulation of BMP4 synexpression in transgenic *Xenopus*. *EMBO J* **23:** 844–856

Kawahara A, Wilm T, Solnica-Krezel L, Dawid IB (2000a) Antagonistic role of *vega1* and *bozozok/dharma* homeobox genes in organizer formation. *Proc Natl Acad Sci USA* **97:** 12121–12126

Kawahara A, Wilm T, Solnica-Krezel L, Dawid IB (2000b) Functional interaction of *vega2* and *goosecoid* homeobox genes in zebrafish. *Genesis* **28:** 58–67

Klein SL (1987) The first cleavage furrow demarcates the dorsal–ventral axis in *Xenopus* embryos. *Dev Biol* **120:** 299–304

Ladher R, Mohun TJ, Smith JC, Snape AM (1996) *Xom*: a *Xenopus* homeobox gene that mediates the early effects of BMP-4. *Development* **122:** 2385–2394

Latinkic BV, Smith JC (1999) *Goosecoid* and *mix.1* represses *Brachyury* expression and are required for head formation in *Xenopus*. *Development* **125:** 1769–1779

Lee HX, Ambrosio AL, Reversade B, De Robertis EM (2006) Embryonic dorsal–ventral signaling: secreted frizzled-related proteins as inhibitors of tolloid proteinases. *Cell* **124:** 147–159

Lewis SL, Khoo P-L, De Young RA, Bildsoe H, Wakamiya M, Behringer RR, Mukhopadhyay M, Westphal H, Tam PPL (2006) Genetic interaction of *Gsc* and *Dkk1* in head morphogenesis of the mouse. *Mech Dev* **124:** 157–165

Lim J, Choi K-W (2003) Bar homeodomain proteins are anti-proneural in the *Drosophila* eye: transcriptional repression of *atonal* by Bar prevents ectopic retinal neurogenesis. *Development* **130:** 5965–5974

Melby AE, Beach C, Mullins M, Kimelman D (2000) Patterning the early zebrafish by the opposing actions of *bozozok* and *vox/vent*. *Dev Biol* **224:** 275–285

Melby AE, Clements WK, Kimelman D (1999) Regulation of dorsal gene expression in *Xenopus* by the ventralizing homeodomain gene *Vox*. *Dev Biol* **211:** 293–305

Moretti PAB, Davidson AJ, Baker E, Lilley B, Zon LI, D'Andrea RJ (2001) Molecular cloning of a human *Vent*-like homeobox gene. *Genomics* **76:** 21–29

Niehrs C (2001) The Spemann organizer and embryonic head induction. *EMBO J* **20:** 631–637

Niehrs C, Pollet N (1999) Synexpression groups in eukaryotes. *Nature* **402:** 483–487

Niehrs C, Keller R, Cho KWY, De Robertis EM (1993) The homeobox gene *goosecoid* controls cell migration in *Xenopus* embryos. *Cell* **72:** 491–503

Niehrs C, Steinbeisser H, De Robertis EM (1994) Mesodermal patterning by a gradient of the vertebrate homeobox gene *goosecoid*. *Science* **263:** 817–820

Oelgeschläger M, Kuroda H, Reversade B, De Robertis EM (2003) Chordin is required for the Spemann organizer transplantation phenomenon in *Xenopus* embryos. *Dev Cell* **4:** 219–230

Onichtchouk D, Gawantka V, Dosch R, Delius H, Hirschfeld K, Blumenstock C, Niehrs C (1996) The *Xvent-2* homeobox gene is part of the *BMP-4* signalling pathway controlling dorsoventral patterning of *Xenopus* mesoderm. *Development* **122:** 3045–3053

Onichtchouk D, Glinka A, Niehrs C (1998) Requirement for *Xvent-1* and *Xvent-2* gene function in dorsoventral patterning of *Xenopus* mesoderm. *Development* **125:** 1447–1456

Papalopulu N, Kintner C (1996) A *Xenopus* gene, *Xbr-1*, defines a novel class of homeobox genes and is expressed in the dorsal ciliary margin of the eye. *Dev Biol* **174:** 104–114

Polli M, Amaya E (2002) A study of mesoderm patterning through the analysis of the regulation of *Xmyf-5* expression. *Development* **129:** 2917–2927

Rastegar S, Friedle H, Frommer G, Knoechel W (1999) Transcriptional regulation of *Xvent* homeobox genes. *Mech Dev* **81:** 139–149

Reig G, Cabrejos ME, Concha ML (2006) Functions of BarH transcription factors during embryonic development. *Dev Biol* **302:** 367–375

Reversade B, De Robertis EM (2005) Regulation of ADMP and BMP2/4/7 at opposite embryonic poles generates a self-regulating morphogenetic field. *Cell* **123:** 1147–1160

Rivera-Pérez JA, Mallo M, Gendron-Maguire M, Gridley T, Behringer RR (1995) *Goosecoid* is not an essential component of the mouse gastrula organizer but is required for craniofacial and rib development. *Development* **121:** 3005–3012

Saito T, Sawamoto K, Okano H, Anderson DJ, Mikoshiba K (1998) Mammalian BarH homologue is a potential regulator of neural bHLH genes. *Dev Biol* **199:** 216–225

Sasai Y, Lu B, Steinbeisser H, Geissert D, Gont LK, De Robertis EM (1994) *Xenopus chordin*: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* **79:** 779–790

Sander V, Mullegger J, Lepperdinger G (2001) *Xenopus* brevican is expressed in the notochord and the brain during early embryogenesis. *Mech Dev* **102:** 251–253

Schmidt JE, von Dassow G, Kimelman D (1996) Regulation of dorsal–ventral patterning: the ventralizing effects of the novel *Xenopus* homeobox gene *Vox*. *Development* **122:** 1711–1721

Schuler-Metz A, Knöchel S, Kaufmann E, Knöchel W (2000) The homeodomain transcription factor Xvent-2 mediates autocatalytic regulation of BMP-4 expression in *Xenopus* embryos. *J Biol Chem* **275:** 34365–34374

Seiliez I, Thisse B, Thisse C (2005) FoxA3 and goosecoid promote anterior neural fate through inhibtion of Wnt8a activity before the onset of gastrulation. *Dev Biol* **290:** 152–163

Sive HL, Grainger RM, Harland RM (2000) *Early Development of Xenopus laevis: A Laboratory Manual*. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press

Steinbeisser H, De Robertis EM, Ku M, Kessler D, Melton DA (1993) *Xenopus* axis formation: induction of *goosecoid* by injected *Xwnt-8* and activin mRNAs. *Development* **118:** 499–507

Steinbeisser H, Fainsod A, Niehrs C, Sasai Y, De Robertis EM (1995) The role of *gsc* and *BMP-4* in dorsal-ventral patterning of the marginal zone in *Xenopus*: a loss-of-function study using antisense RNA. *EMBO J* **14:** 5230–5243

Struhl G (1989) Differing strategies for organizing anterior and posterior body pattern in *Drosophila* embryos. *Nature* **338:** 741–744

Tissier-Seta J-P, Mucchielli M-L, Mark M, Mattei M-G, Goridis C, Brunet J-F (1995) *Barx1*, a new mouse homeodomain transcription factor expressed in cranio-facial ectomesenchyme and the stomach. *Mech Dev* **51:** 3–15

Trindade M, Tada M, Smith JC (1999) DNA-binding specificity and embryological function of Xom (Xvent-2). *Dev Biol* **216:** 442–456

Wright CV, Morita EA, Wilkin DJ, De Robertis EM (1990) The *Xenopus* XlHbox 6 homeo protein, a marker of posterior neural induction, is expressed in proliferating neurons. *Development* **109:** 225–234

Yamada G, Mansouri A, Torres M, Stuart ET, Blum M, Schultz M, De Robertis EM, Gruss P (1995) Targeted mutation of the murine *goosecoid* gene results in craniofacial defects and neonatal death. *Development* **121:** 2917–2922

Yao J, Kessler DS (2001) Goosecoid promotes head organizer activity by direct repression of Xwnt8 in Spemann's organizer. *Development* **128:** 2975–2987

Zhu L, Belo JA, De Robertis EM, Stern CD (1999) *Goosecoid* regulates the neural inducing strength of the mouse node. *Dev Biol* **216:** 276–281