

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

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We present a loss-of-function study using antisense morpholino (MO) reagents for the organizer-specific gene *Gooseoid* (*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.

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Introduction

The isolation of the homeobox gene *Gooseoid* (*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 migra-

tion 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 *Gooseoids* 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*^{-/-}; *HNF3β*/*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 Jaekle, 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*

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(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 rhombo-

mere 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 brevicornis*, *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,

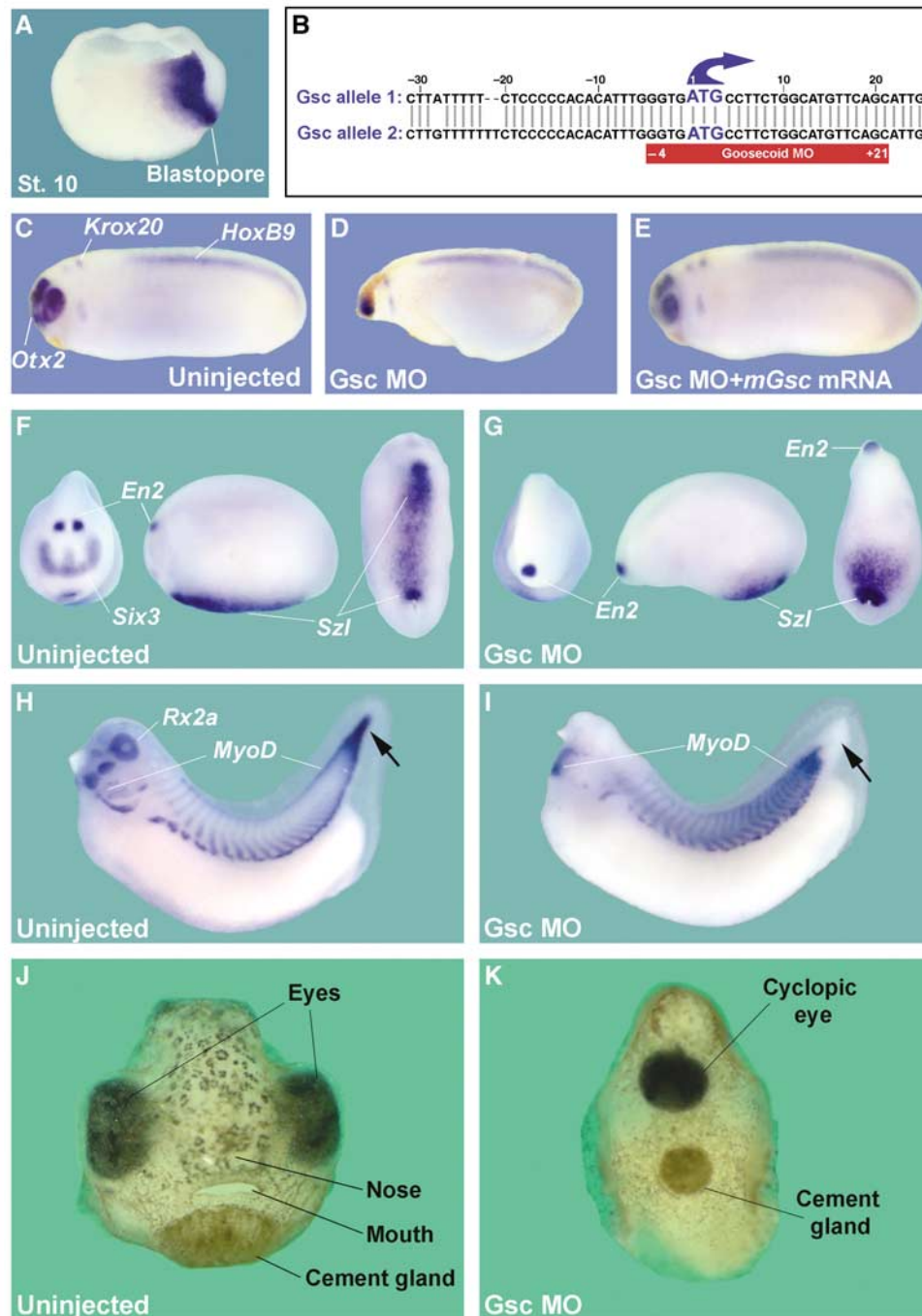


Figure 1 *Gsc* knockdown in *Xenopus* embryos causes loss of head structures and affects patterning of the AP and DV axes. (A) *Gsc* marks Spemann organizer endomesoderm at early gastrula. (B) *Gsc* MO targets both pseudoalleles of the *X. laevis* *Gsc* gene. (C–I) *Gsc* MO injection (136 ng total) causes loss of head structures, marked by *Otx2* (forebrain), *Six3* and *Rx2a* (forebrain and eyes), and *En2* (midbrain/hindbrain border) ($n = 106$; Supplementary Table 1). 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,

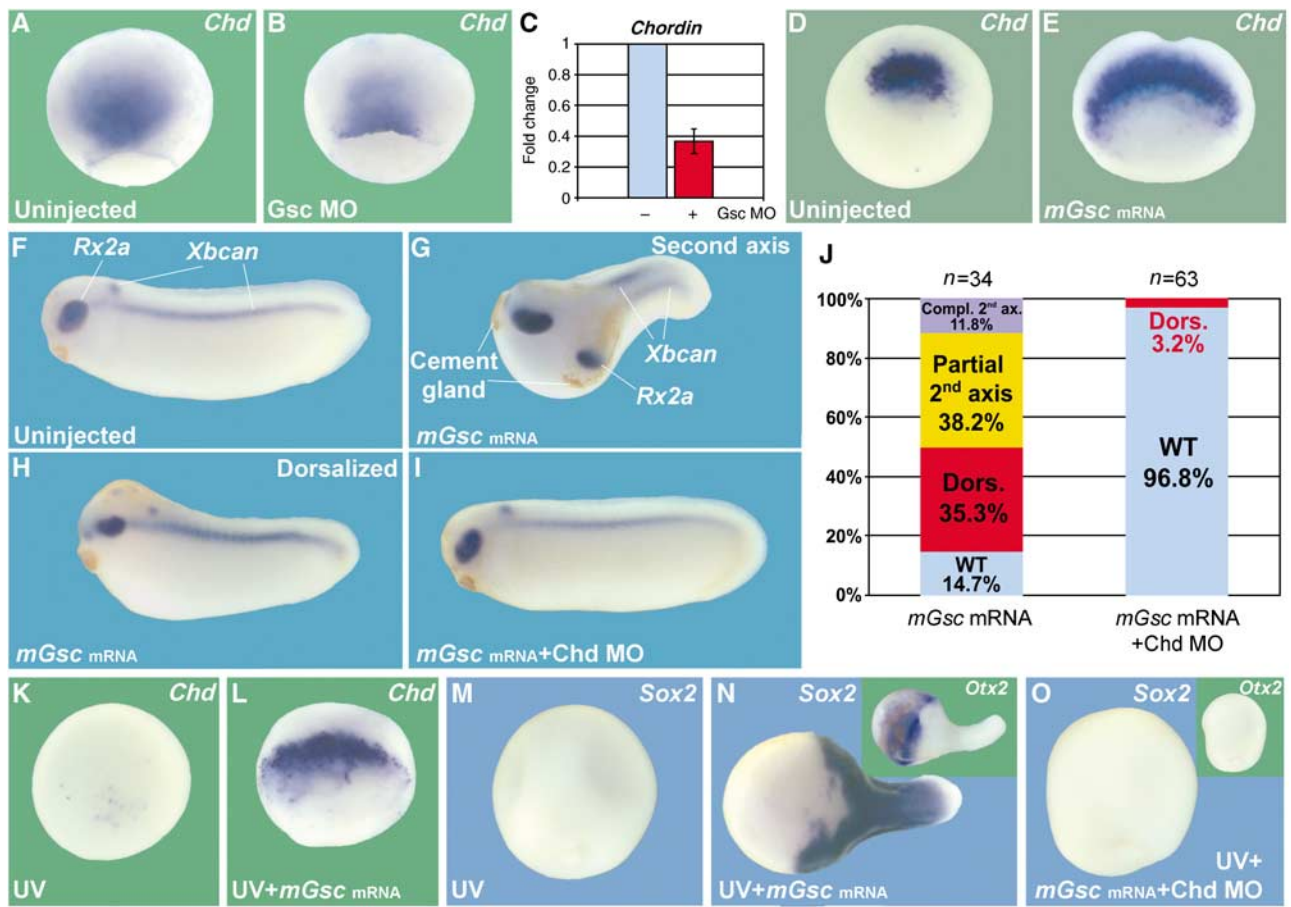


Figure 2 The dorsalizing effects of *mGsc* mRNA injection require *Chd*. (A–E) *Gsc* MO reduces *Chd* expression at gastrula 2.5-fold, whereas overexpression of *mGsc* mRNA greatly expands *Chd* expression. (F–H) Injection of 50 pg *mGsc* mRNA into one ventral blastomere at the four-cell stage leads 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-neuronal 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;

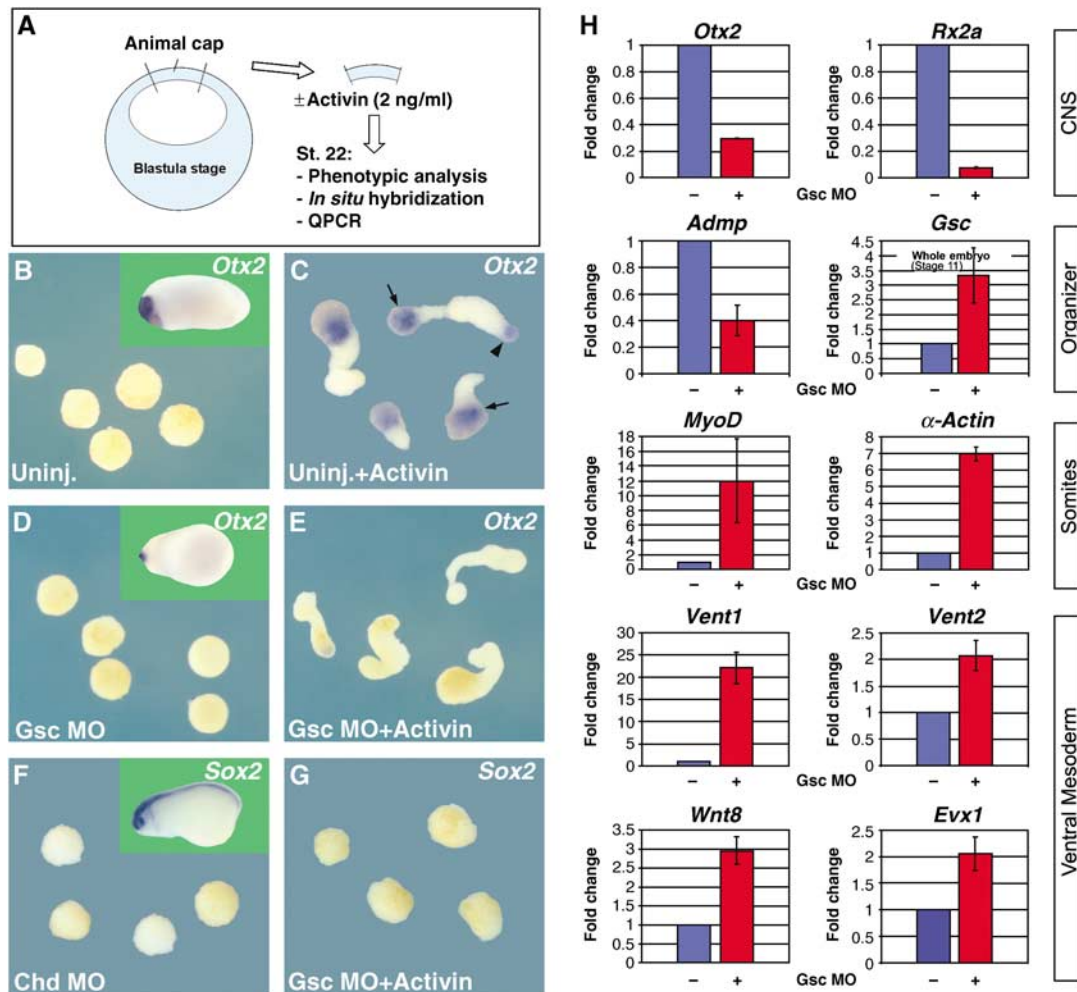


Figure 3 *Gsc* is required for secondary neural induction and mesoderm patterning in Activin-treated animal cap explants. (A) Experimental design ($n = 15$ or more per experimental set) (B, C) Untreated animal caps develop into atypical epidermis, whereas Activin treatment leads to elongation and brain 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* knock-downs, 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

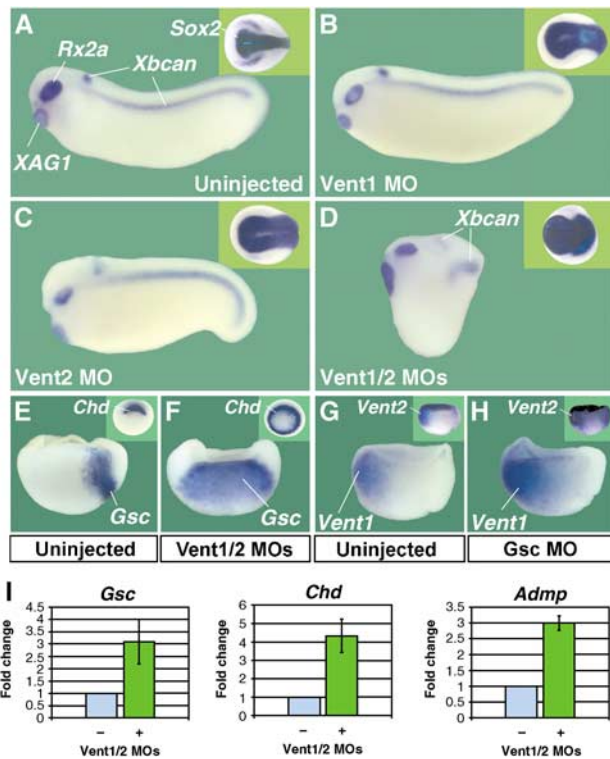


Figure 4 Double depletion of *Vent1* and *Vent2* causes severe dorsalization of the embryo. (A–D) Injection of either *Vent1* or *Vent2* MO expands the neural plate at neurula stage (insets), but only the combination of both MOs strongly dorsalizes tailbud stage embryos, with shortened body axes and 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 *Xbcanc* 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.

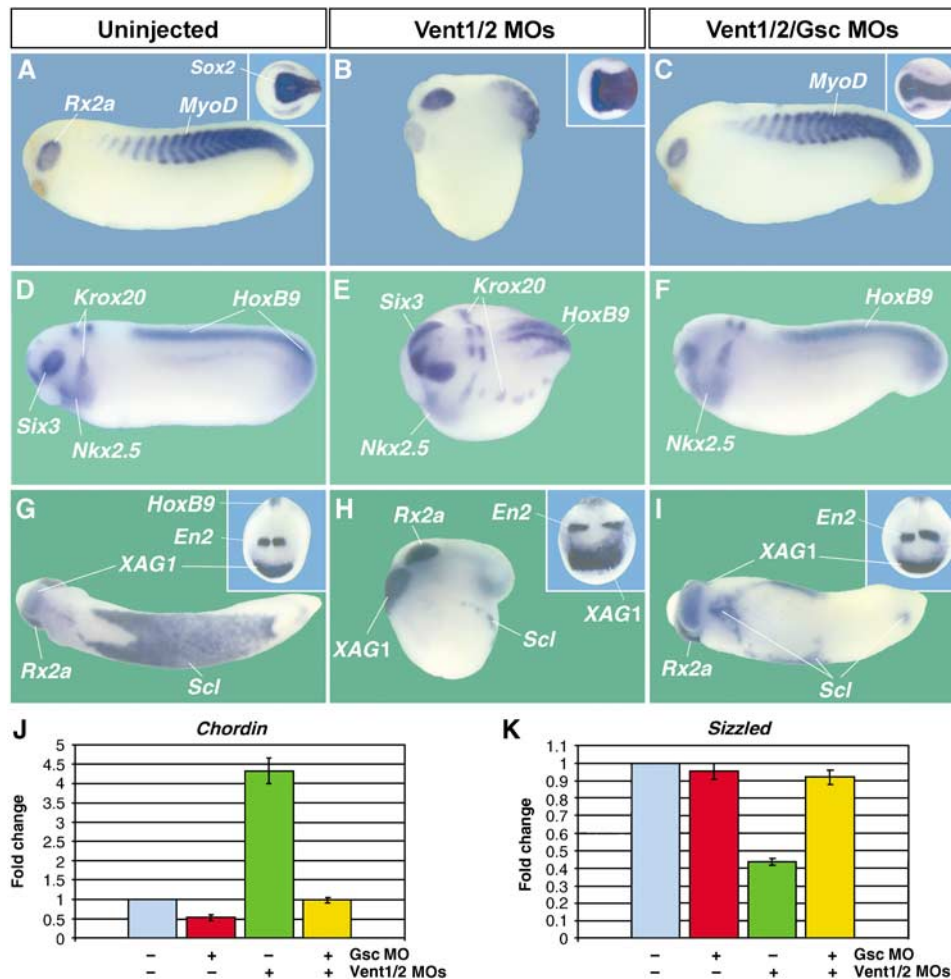


Figure 5 *Gsc* is required for the dorsalization caused by *Vent1/2* knockdown. (A–I) Co-injection of *Gsc* MO restores normal pattern in *Vent1/2*-depleted whole embryos ($n = 53$; Supplementary Table I). At the neurula stage, knockdown of *Vent1/2/Gsc* reduces the neural plate (*Sox2*) back to normal 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 embryos (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.

Gooseoid is required for early patterning in *Xenopus* embryos

The discovery of *Gsc* was a very exciting 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 Jaekle, 1996) was very puzzling. In zebrafish, however, Seiliez *et al* (2005) recently reported cyclopia and anterior truncations in *Gsc* morphants. In *Xenopus*, work using anti-morphic *Gooseoids*, VP16 fusions, or antisense RNA, all had indicated a role for *Gsc* in patterning the early mesoderm (Steinbeisser *et al*, 1995; Ferreira *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

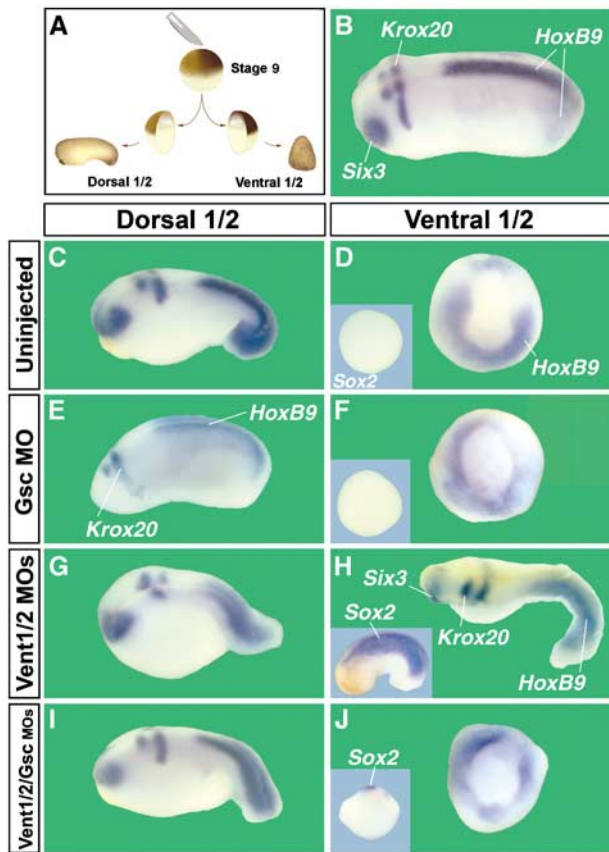


Figure 6 Knockdown of *Gsc* and *Vent1/2* restores normal development of dorsal and ventral half-embryos ($n = 52$ or more per experimental set). (A) Embryos were bisected into dorsal and ventral halves at blastula stage. (B) Control sibling at the same magnification as the other panels. (C, D) Bisectioned 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 knock-down 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 11, 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).

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),

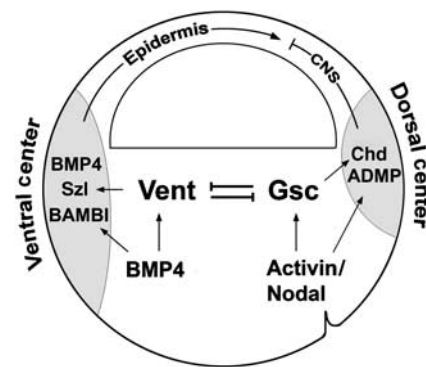


Figure 7 Model of regulatory mechanisms for pattern formation at gastrula. In the dorsal center, Activin/Nodal signals phosphorylate Smad2/3 to activate *Gsc* expression. The expansion of *Chd* and *ADMP* can also be achieved by *Gsc*-independent pathways. In the ventral center, BMP4/7 signals phosphorylate 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.

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'-GTCAATAGAGAATCCCTGTTGAACC-3'; and *Vent2* MO 5'-GTCATCTTG TCTGTATTAGTCT-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 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.

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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: TCCTGTACGCTTCTGGTCTGTA, rev: TCTCAA GTCCAAAGCCACATA; *Admp*, fwd: GATGATGGAAGGAGAGGA, rev: TCATGTTCTGACCCAAAG; *Chd*, fwd: GTTGATCATTTGGTGGAA, rev: ACTCAGATAAGAGCGATCA; *Gsc*, fwd: GCTGAT-TCCACCAGT GCCTCACCAG, rev: GGTCCTGTGCCTCTCTCTCTCTG; *MyoD*, fwd: AGGTCCAAGTCTCCGACGGCATGAA, rev: AGGAGAGAATCC AGTTGATGGAAACA; *ODC*, fwd: CAGCTAGCTGTGGTGTGG, rev: CAACATGGAAACTCACACC; *Otx2*, fwd: GGATGGATTTGTTACAT CCGTC, rev: CACTCTCCGAGCTCACTTCCC; *Rx2a*, fwd: AGACTGGT GGCTATGGAG, rev: ATACCTGCACCCTGACTT; *Szl*, fwd: GTCTTC 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>).

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