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Authors
Sander, Veronika
Reversade, Bruno
De Robertis, EM

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

Veronika Sander, Bruno Reversade and EM De Robertis*

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 dorsaled the embryo. Unexpectedly, simultaneous depletions 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 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-Perez 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 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 Pv1) 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...
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). Radical 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-

meree 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 (Oegelshläger 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,
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,
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;...
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

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**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.
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). Scl, 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 Scl 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 dorsoalized 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 dorsoalized, 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 4 Double depletion of Vent1 and Vent2 causes severe dorsoalization 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 dorsoalizes 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.
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 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 Szi is downregulated by Vent1/2 MO, but restored to normal levels when Gsc is also depleted.
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 Eox1 (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 Vent 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 zebraloph 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), 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.

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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 Goosecoid-1 mouse embryos, 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′-GCAGGAAGATCCACGCAATA-3′; Vent1 MO 5′-GTA CATAGAGATCCTGGGACCT-3′; and Vent2 MO 5′-GTATCATCTCTTACGAGTCT-3′. X. tropicalis Vent1 and Vent2 MOs had been reported previously (Pollil and Amaya, 2002), but were not useful for the pseudohaploidy species X. laevis. The Chordin MO was as indicated otherwise, injected four times vegetally (136 ng total). For in situ whole-mount in situ hybridization, samples were extracted using the Absolutely RNA MicroPrep kit (Stratagene).

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