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Role of Goosecoid, Xnot and Wnt antagonists in the maintenance of the notochord genetic programme in *Xenopus* gastrulae

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SUMMARY

The *Xenopus* trunk organiser recruits neighbouring tissues into secondary trunk axial and paraxial structures and itself differentiates into notochord. The inductive properties of the trunk organiser are thought to be mediated by the secretion of bone morphogenetic protein (BMP) antagonists. Ectopic repression of BMP signals on the ventral side is sufficient to mimic the inductive properties of the trunk organiser. Resultant secondary trunks contain somite and neural tube, but no notochord.

We show that inhibition of BMP signalling is sufficient for the initiation of the trunk organiser genetic programme at the onset of gastrulation. During late gastrulation, however, this programme is lost, due to an invasion of secreted Wnts from neighbouring tissues. Maintenance of this programme requires co-repression of BMP and Wnt signalling within the presumptive notochord region. To shed light on the molecular cascade that leads to the repression of the Wnt pathway, we looked for individual organiser genes whose overexpression could complement the inhibition of BMP signalling to promote notochord formation in the secondary trunks. Two genes, *gsc* and *Xnot*, were thus identified and shown to act in different ways. Xnot acts as a transcriptional repressor within the mesodermal region. Gsc acts in deeper vegetal cells, where it regulates *Frzb* expression to maintain *Xnot* expression in the neighbouring notochord territory.

These results suggest that, during gastrulation, the necessary repression of Wnt/β-catenin signalling in notochord precursors is achieved by the action of secreted inhibitors, such as Frzb, emitted by *gsc*-expressing dorsal vegetal cells.

Key words: Goosecoid, Xnot, Frzb, Transcription, Repressor, Notochord, Xwnt-8, BMP, *Xenopus*, Embryo

INTRODUCTION

Axial development in vertebrates is initiated and co-ordinated during gastrulation by a dorsal territory called the organiser. In amphibia, on the basis of molecular and embryological data (reviewed by Lemaire and Kodjabachian, 1996; Gerhart, 2001), Spemann's organiser has been subdivided into a trunk organiser and a head organiser. The trunk organiser, located in the upper dorsal marginal zone at the early gastrula stage (Zoltevitcz and Gerhart, 1997), acts as an inductive centre for two major embryonic events: neural induction in dorsal ectoderm and dorsalisation of equatorial mesoderm. These events are mediated by a careful balancing between the activity of BMPs, expressed in the ventrolateral mesodermal and ectodermal regions, and secreted BMP antagonists such as Chordin and Noggin, emitted by the trunk organiser (reviewed by Dale and Jones, 1999). Excess BMP signalling on the dorsal side results in an expansion of epidermis and ventrolateral mesoderm. Conversely, inhibition of BMP signalling on the ventral side of embryos leads to the formation of a secondary trunk that includes a neural tube and segmented somites (Suzuki et al., 1994). The head organiser derived from the vegetal edge of the dorsal marginal zone (Zoltewicz and Gerhart, 1997) induces anterior fates in the overlying presumptive neuroectoderm. It has been proposed that head formation requires co-inhibition of BMP and Wnt signalling, while trunk formation results from the secretion of BMP antagonists (Glinka et al., 1997). However, inhibition of Nodal signals is also implicated in head formation (Piccolo et al., 1999).

Studies on the organiser have so far mainly focused on its ability to organise surrounding tissues, while its intrinsic property to differentiate into axial mesendodermal structures has been left relatively unexplored. The head organiser forms head tissues such as the prechordal plate, which is involved in the separation of the eye fields, and the anterior endoderm. The trunk organiser mainly gives rise to the notochord, which acts as an important signalling centre for patterning neighbouring tissues (reviewed by Placzek, 1995; Currie and Ingham, 1996; Kim et al., 1997). In vertebrates, notochord formation is likely to involve the concerted action of several transcription factors. *no tail* (*ntl*) and *floating head* (*flh*) have been identified through genetic approaches in zebrafish (Halpern et al., 1993; Talbot et al., 1995). *ntl* belongs to a gene family of *Brachyury* (*T*)-type

transcription factor (Schulte-Merker et al., 1994). *flh* encodes a homeodomain transcription factor homologous to *Not* genes in *Xenopus* (*Xnot*) and chick (*Gnot* or *CNot*; von Dassow et al., 1993; Knezevic et al., 1995; Stein et al., 1996). In *ntl* mutants, it appears that the axial mesoderm is specified as floor plate (Halpern et al., 1997). By contrast, *flh* acts primarily by promoting notochord fates and repressing somitic fates, as muscle forms in place of notochord in the mutant embryos (Halpern et al., 1995; Amacher and Kimmel, 1998). At the onset of gastrulation, the expression domain of *flh* corresponds to the presumptive notochord region (Melby et al., 1996). In *Xenopus*, the restriction of *Xnot* domain to the presumptive notochord region occurs during gastrulation (von Dassow et al., 1993). Thus, *Xnot/flh* is the earliest gene that specifically marks the presumptive notochord region and is indeed required for its formation.

How is the expression domain of *Xnot/flh* established? The presumptive notochord region (*flh/Xnot* domain) is flanked laterally with the presumptive somitic mesoderm in the dorsal marginal zone. Inhibition of BMP signals is required for the specification of dorsal mesoderm, as ectopic activation of this signalling pathway on the dorsal side of embryos blocks formation of its derivatives, notochord and somitic mesoderm (reviewed by Dale and Jones, 1998). *Xwnt-8* is expressed in the ventrolateral part of the marginal zone (Chrisitian and Moon, 1993) and is implicated in partitioning of the most dorsal sector from the dorsolateral sector by positively regulating expression of myogenic genes and negatively regulating *Xnot* expression (Hoppler et al, 1996; Hoppler and Moon, 1998; Marom et al, 1999). These findings indicate that establishment of presumptive notochord region is based on repression between these two signals. The results presented here provide direct evidence that the co-repression of BMP and Wnt/β-catenin signals in mesoderm is sufficient to specify the presumptive notochord region. They also show that two homeobox-containing transcriptional repressors, Goosecoid and Xnot, are involved in this process in distinct manners.

MATERIALS AND METHODS

Embryos

Adult pigmented *Xenopus laevis* were purchased from Nasco (WI, USA) and CNRS (Rennes, France). Embryos were reared as previously described (Lemaire et al., 1998).

Construction of expression constructs for enRXnotHD and VP16XnotHD

Activating (VP16-XnotHD) and repressing (enR-XnotHD) forms of Xnot were constructed as follows: the region encoding the homeodomain of Xnot flanked by seven or six amino acids on either side and followed by a stop codon was PCR amplified with the two oligonucleotides XnotHD-F (5′-ccggagaTCTAGGACAGGACCCT-GCAAGTTAAAG-3′; *Bgl*II site underlined; *Xnot* sequences in capitals) and XnotHD-R (5′-agcaccgcg**gct**AGGCCTTCTTCTGCTC-CAGACTCTG-3′; *Sac*II site underlined; *Xnot* sequences in capitals, introduced stop codon in bold). The fragment was cloned into *Bam*HI-*Sac*II sites, replacing a fragment of *Mix.1* sequences in the constructs (pBSRN3-VP16Mix.1 and pBSRN3-enRMix.1) reported by Lemaire et al. (Lemaire et al., 1998).

Embryo injections

Injected mRNAs were synthesised *in vitro* with mMESSAGE mMACHINE kits (Ambion) as follows: activated form of *Xlim-1/3m* (Taira et al., 1994); *pintallavis* (Ruiz i Altaba and Jessell, 1992); *goosecoid* (a full-length cDNA of *goosecoid* was subcloned into pSP64T); *Xnot* (Gont et al., 1996); *Lef1ΔHMG* (Behrens et al., 1996); truncated form of type I BMP receptor (*tBR*) (Suzuki et al., 1994); *XSmad6* (a full-length cDNA of *XSmad6* was subcloned into pBSRN3). Morpholino oligos complementary to *β-catenin* (β-catenin-MO) were as described previously (Heasman et al., 2000).

Immunostaining and histology

Whole-mount immunostaining with MZ15 (notochord) antibody and histological analyses were carried out as described (Darras et al., 1997). Sections were stained with Haematoxylin/Eosin or only with Eosin after whole-mount X-gal staining.

In situ hybridisation and β-galactosidase staining

The following plasmid templates were linearised, and digoxigenin-substituted antisense RNA probes were transcribed with T3, T7 or SP6 RNA polymerase: *chordin* (Sasai et al., 1994); *goosecoid* (a full-length cDNA of *goosecoid* subcloned into pBluescript SK-); *XMyoD* (Hopwood et al., 1989); *Xnot* (Gont et al., 1993); *pintallavis* (a PCR-amplified coding region of *pintallavis* was subcloned into pGEM-T); *Xlim-1* (Taira et al., 1992); *Xwnt-8* (Lemaire and Gurdon, 1994); *XVent-1* (Gawantka et al., 1995); *XVent-2* (a coding region of *XVent-2* was subcloned into pBluescript KS+); *Xbra* (Smith et al., 1991); *Frzb* (Leyns et al., 1997). Embryos for whole-mount in situ hybridisation were processed as described (Gawanka et al., 1995). Whole-mount β-galactosidase staining was as described (Sanes et al., 1986). β-galactosidase activity was revealed with either salmon-Gal (red staining) or X-Gal (blue staining).

RESULTS

Secondary trunks induced by blocking BMP signals do not contain notochord

Inhibition of BMP signalling was originally reported to lead to the formation of a complete ectopic trunk, including a notochord, and in some cases even heads (Sasai et al., 1994). Other works, however, have suggested that inhibition of BMP is not sufficient for head or notochord formation (Suzuki et al., 1994; Glinka et al., 1997). This difference may have at least two origins. First, if ventral injections are not precisely targeted and a proportion of the injected embryos is injected laterally, the injected region may lie close to dorsal vegetal territories expressing secreted head organiser molecules such as Cerberus and Dickkopf (Bouwmeester et al., 1996; Glinka et al., 1998), which may complement the action of BMP antagonists. Second, as BMP antagonists such as Chordin are secreted proteins, their ventral overexpression may lead to the diffusion of the protein to lateral territories and again to a synergy with secreted head organiser genes.

To overcome these experimental limitations, we overexpressed in ventral blastomeres of four-cell embryos, molecules that cell autonomously block BMP signalling. In some experiments, a lineage tracer was co-injected together with anti-BMP molecules in order to verify that we precisely targeted a ventral position at the early gastrula stage (for examples, see Fig. 3B,F,J,N,R,V). Two molecules were tested in these experiments: a truncated form of the type 1 BMP receptor (tBR), and Smad6, an inhibitory Smad that is specific for the BMP pathway (Suzuki et al., 1994; Hata et al., 1998; H. Y. and P. L., unpublished). When *tBR* RNA (400 pg) was

expressed on the ventral side of embryos, the secondary axes observed always lacked anterior head structures (*n*=67) (Table 1 and Fig. 1D,F). Their trunks contained a neural tube and somitic muscle fused beneath the neural tube, but lack a notochord (Fig. 1E). Increasing the amount of injected *tBR* RNA (1000 pg) led to the same result, except that both primary and secondary trunks were shortened. Injection of 200 or 800 pg of *XSmad6* RNA had a qualitatively similar effect, the ectopic axes observed lacking both head and notochord (see Table 1). This result confirmed that the local ventral inhibition of BMP signals is not sufficient for the formation of 'complete' trunk, including a notochord. Interestingly, the ectopic structures induced by the local inhibition of BMP signalling, muscle and neural tube, correspond to the structures induced by grafting the trunk organiser (Spemann, 1931).

These results are consistent with the proposition that the trunk organiser acts by inhibiting BMP signals in neighbouring cells. They also establish that the inhibition of BMP signals alone is not sufficient for notochord formation.

Presence of inhibitory signals opposing notochord formation in the ventral marginal zone

Xwnt-8 is expressed in ventrolateral territories, its inhibition leads to enlarged notochord formation (Hoppler et al., 1996), while dorsal overexpression of *Xwnt-8* from the late blastula stage suppresses notochord as well as head development (Christian and Moon, 1993). These studies indicate that Xwnt-8 has a notochord-repressing activity. Therefore, the lack of notochord in the secondary trunk induced by inhibition of BMP signals might be due to the activity of Xwnt-8.

Expression of *Xwnt-8* gene is known to be regulated by BMP signals (Hoppler and Moon, 1998; Marom et al., 1999). We confirmed this observation in *tBR*-injected embryos. Expression of *Xwnt-8* was repressed during gastrulation (stages 10, 10.5 and 11) in the area where *tBR* RNA was injected (data not shown). Therefore, if Xwnt-8 is responsible for the lack of notochord in tBR-induced secondary trunks, the effect should originate from neighbouring cells. If this is the case, isolation of *tBR*-injected ventral marginal zone (VMZ) should result in formation of notochord. In order to test this hypothesis, the VMZs of embryos injected with 250 pg of *tBR* RNA were isolated with an arc of about 60° at the early gastrula stage, cultured in isolation until the early tailbud stage (stage 23) and monitored immunohistochemically for the formation of notochord (Fig. 1G-I). A large part of such explants differentiated into notochord (67%; *n*=15), while control VMZ explants never expressed the notochord-specific antigen (0%; *n*=34). By contrast, when tBR-VMZs were isolated with an arc of 120°, they did not differentiate into notochord (data not shown).

These results suggest the existence of a notochord-repressing signal(s) emitted by cells of the ventrolateral domains of gastrula embryos and that Xwnt-8 is likely to be a part of the signals.

Co-repression of BMP and Wnt/β-catenin signalling pathways is sufficient for notochord formation

We next tested whether the notochord-repressing signal(s) act via the Wnt pathway. To do this, *tBR* RNA was co-injected in the ventral marginal zone of four-cell embryos together with RNA for a truncated form of Lef1 (*Lef1ΔHMG*), which should repress the canonical Wnt pathway (Wnt/β-catenin pathway) in a cell autonomous fashion (Behrens et al., 1996). Co-injection of *tBR* (400 pg) and *Lef1ΔHMG* (800 pg) RNAs resulted in the formation of secondary trunks, containing notochord (82%; *n*=22) (Table 1 and Fig. 2A,B). This result shows that co-repression of BMP and Wnt/β-catenin signalling pathways is sufficient for the formation of notochord. Formation of notochord in secondary trunks of the Lef1ΔHMG plus tBR embryos was not associated with formation of head structures, presumably owing to the cell-autonomous action of the reagents used.

The experiment described above, however, did not address whether co-inhibition of BMP and Wnt signalling is necessary within the presumptive notochord cells or whether it is required for the formation of a notochord-inducing centre in more vegetal territories. Block or downregulation of BMP activity is a prerequisite for the specification of the dorsal mesoderm,

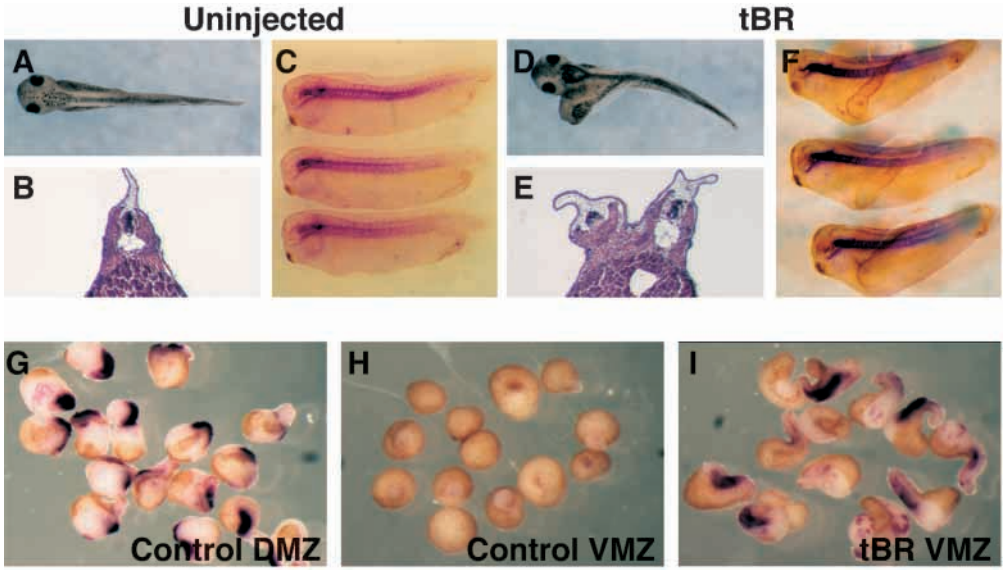

Fig. 1. Effect of ventral tBR injection on whole embryos and explanted ventral marginal zones. (A-C) Uninjected embryos. (D-F) Embryos injected ventrally with tBR RNA. (A,D) Dorsal view of tadpoles. (B,E) Transverse sections through tadpoles. (C,F) Immunostaining of the notochord with the monoclonal antibody MZ15 (the otic vesicle is also labelled with MZ15). (G-I) Ventral marginal zone explants differentiate into notochord in the absence of BMP signals. Dorsal (G) or ventral (H,I) marginal zones were isolated at early gastrula stage from uninjected embryos (G,H) or embryos injected ventrally with *tBR* RNA (I). Explants were cultured until stage 23, and immunostained with MZ15.

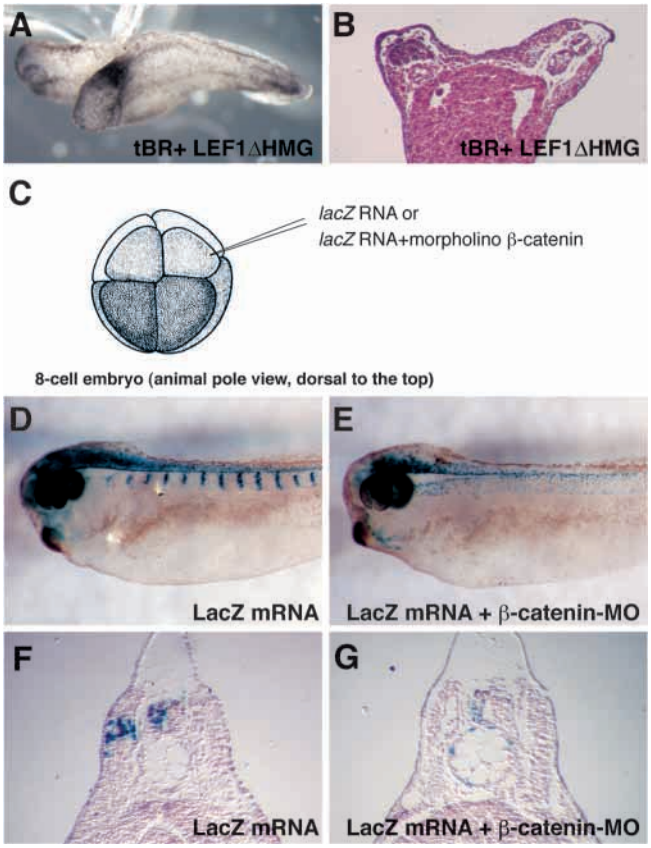

Fig. 2. Local repression of BMP and canonical Wnt signalling leads to notochord formation. (A) Lateral view of an embryo injected ventrally with *tBR* and *Lef1ΔHMG* RNA. (B) Cross-section through the embryo shown in A. (C) Position of injection for D-G. (D,E) Lateral view of cleared embryos injected with the indicated reagents. (F,G) Cross-sections through embryos injected with the indicated reagents. β-galactosidase activity was revealed in blue at stage 32.

which can be subdivided into somitic mesoderm and notochord (reviewed by Dale and Jones, 1999). If the co-repression of BMP and Wnt signals within the dorsal mesoderm is essential for the differentiation of notochord, ectopic inhibition of the Wnt signal in the presumptive somitic region, where BMP activity is also blocked or low (Dosch et al., 1997), should result in its transformation into notochord. In order to test this hypothesis, we wanted to block Wnt signals cell autonomously within dorsolateral mesoderm. However, as the Wnt/β-catenin signalling pathway is required during early blastula stages for dorsal specification, it was not possible to inject *Lef1ΔHMG* RNA. Therefore, we took advantage of a novel antisense technique using morpholino oligonucleotides complementary to *β-catenin* mRNA (β-catenin-MO) (Heasman et al., 2000). β-catenin-MO inhibits the accumulation of endogenous β-catenin protein, disrupting dorsal tissue formation when injected at the two- or four-cell stage (Heasman et al., 2000). However, injection at the eight-cell stage does not interrupt the formation of dorsal axis (Heasman et al., 2000). Therefore, injection of β-catenin-MO at the eighth-cell stage should block only the late functions of β-catenin. We injected β-catenin-MO into the lateral-marginal part of one of the dorso-animal blastomeres of the eight-cell embryo (Fig. 2C). This part of the embryo is adjacent to the presumptive notochord region and fated to form part of the presumptive somitic mesoderm and nervous system (Dale and Slack, 1987). Consistently, embryos injected with *lacZ* RNA showed β-galactosidase activity in part of the somitic mesoderm as well as in the nervous system, but rarely in the notochord (Fig. 2D,F). When β-catenin-MO (5 ng) and *lacZ* RNA were co-injected into the same position, resultant embryos appeared to be morphologically normal (Fig. 2E). However, β-galactosidase activity was no longer observed in the somitic mesoderm, but was instead detected in notochord (Fig. 2E,G), indicating a fate-transformation of injected cells from somitic mesoderm into notochord. It should be noted that no staining was seen in the prechordal plate, indicating the lack of a fate transformation into a notochord-inducing centre. This result suggests that cell-autonomous inhibition of Wnt/β-catenin signals in the dorsal mesoderm cells is sufficient to

Table 1. Formation of trunk and presence of notochord in the secondary trunk

Experiment	RNA injected into embryos (pg per embryo)	Number of embryos	Secondary trunk (%)	Notochord in the secondary trunk (%)
I-1	*Xsmad6* (200)	74	96	0
I-2	*Xsmad6* (800)	12	100	0
I-3	*tBR* (400)	75	89	0
I-4	*tBR* (1000)	21	95	0
II-1	*lef1ΔHMG* (800)	10	0	0
II-2	*lef1ΔHMG* (800) + *tBR* (400)	22	100	82
III-1	*gsc* (50)	48	19	0
III-2	*gsc* (50) + *tBR* (400)	84	87	52
III-3	*Xnot* (100)	45	0	–*
III-4	*Xnot* (100) + *tBR* (400)	82	95	85
III-5	*3m* (250)	22	59	0
III-6	*3m* (250) + *tBR* (400)	35	100	0
III-7	*pintallavis* (200)	30	0	–
III-8	*pintallavis* (200) + *tBR* (400)	30	60	–

The indicated mRNA amount was injected into the marginal zone of two ventral blastomeres of 4-cell embryos. The embryos were scored at the tailbud stage for the formation of secondary trunks as well as for the presence of notochord in the secondary trunks.

*In the embryos injected ventrally with *Xnot* RNA (100 pg), contrary to a previous report (Gont et al., 1996), we did not observe duplication or enlargement of the primary notochord.

convert them into notochord. Therefore, co-repression of BMP and Wnt/β-catenin signals is likely to be involved in the specification of presumptive notochord region.

Organiser genes are induced but not maintained in the ventral mesoderm in the absence of BMP signals

As the presumptive notochord region resides within the organiser, we first asked whether organiser genes are ectopically activated by local inhibition of ventral BMP activity. We looked at the expression of the organiser genes *chordin*, *goosecoid* (*gsc*), *pintallavis*, *Xlim-1*, *Xnot* and *frzb* in *tBR*-injected embryos at the mid- (stage 10.5-11) and late (stage 13) gastrula stages (Sasai et al., 1994; Cho et al., 1991; Ruiz i Altaba and Jessell, 1992; Taira et al., 1992; von Dassow et al., 1993; Leyns et al., 1997; Wang et al., 1997a).

All organiser genes tested were ectopically expressed on the ventral side of injected mid gastrulae (Fig. 3B,F,J,N,R,V), appearing slightly less intense than on the dorsal side. By the late gastrula stage, however, expression of *gsc*, *pintallavis*, *Xnot*, *Xlim-1* and *frzb* was lost in the midline structures of the forming secondary trunks (Fig. 3H,L,P,T,X). Expression of *chordin* was maintained in the involuting mesoderm of the late gastrula embryos (Fig. 3D), suggesting that BMP signals remain blocked during gastrulation in the secondary axes, as in the primary axes. These results suggest that inhibition of BMP signals is sufficient to initiate the molecular programme of the organiser, but not to maintain it, which could explain the lack of notochord in tBR-induced secondary trunks.

Gsc and Xnot complement the inhibition of BMP signalling in notochord formation

If the organiser genes whose expression was not maintained in the *tBR*-injected VMZ are involved in notochord formation, their overexpression together with tBR might complement the action of tBR to cause ectopic notochord formation. To test this possibility, we carried out the following experiments. Synthetic mRNAs for individual organiser genes were injected alone or in combination with *tBR* RNA on the ventral side of four-cell embryos. The presence of notochord in the induced secondary axes was monitored both morphologically and immunohistochemically at the tailbud stage.

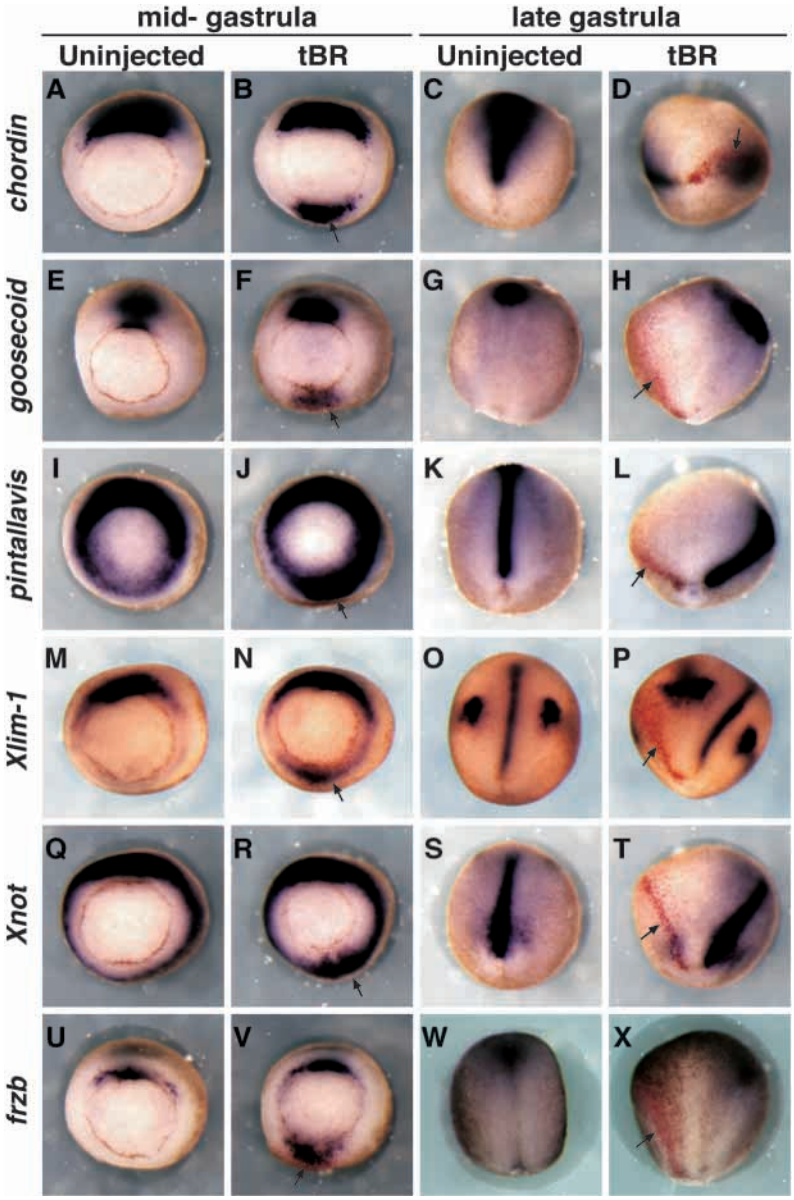

Fig. 3. Expression of organiser genes is initiated on the ventral side when BMP signals are ectopically blocked, but is not maintained by the late gastrula stage. (A,E,I,M,Q,U) Vegetal view of uninjected mid-gastrulae (stage 11). (B,F,J,N,R,V) Vegetal view of mid-gastrulae injected ventrally with *tBR* RNA and *lacZ* RNA as lineage tracer. (C,G,K,O,S,W) Dorsal-vegetal view of uninjected late gastrulae (stage 13). (D,H,L,P,T,X) Dorsal-vegetal view of late gastrulae injected ventrally with tBR RNA. The identity of the probes used is indicated on the left of each row of panels. Note that the expression of all the genes tested is lost at the late gastrula stage, with the exception of *chordin* (D). β-galactosidase activity is shown in red, gene expression in purple.

Injection of mRNA for either an activated form of *Xlim-1* (*Xlim-1/3m*) (Taira et al., 1994) (250 pg) or *gsc* (50 pg) in two ventral blastomeres at the four-cell stage lead to formation of secondary trunks that did not contain notochord (Table 1). Injection of *pintallavis* (200 pg) or *Xnot* (100 pg) RNA alone did not result in any detectable phenotype (Table 1). When RNA for *tBR* (400 pg) was co-injected together with *Xlim-1/3m* (250 pg) or *pintallavis* RNA (200 pg), the secondary trunks did not contain notochord (Table 1). When an increased amount of *Xlim-1/3m* (500 pg) or *pintallavis* RNA (400 pg) was injected together with *tBR* RNA, embryos showed severe gastrulation defects and did not form secondary trunks. Some embryos injected with *Xlim-1/3m* (500 pg) plus *tBR* RNA displayed secondary trunks, which did not contain notochord (data not shown). By contrast, in secondary trunks induced by co-injection of mRNA for *gsc* (50 pg) or *Xnot* (100 pg) with *tBR* RNA (400 pg), notochord formation was observed at a

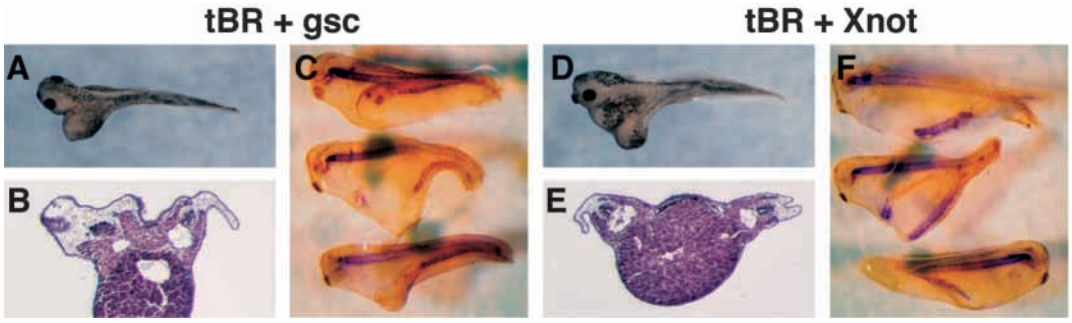

Fig. 4. *gsc* and *Xnot* complement inhibition of BMP signals to promote notochord formation. (A-C) Tadpoles injected ventrally with *tBR* and *gsc* RNA. (D-F) Embryos injected ventrally with *tBR* and *Xnot* RNA. (A,D) External views of injected tadpole. (B,E) Transverse sections through tadpole larvae. (C,F) Lateral views of cleared injected embryos after immunostaining with MZ15.

high frequency (52% and 85%, respectively; Table 1; Fig. 4). Notochords induced by *tBR* plus *gsc* were located more posteriorly in secondary trunks and were thinner than those in the *tBR*/*Xnot*-induced secondary trunks (Fig. 4B,C,E,F). It should be noted that the secondary axes induced in this manner never contained anterior head structures (Fig. 4A,D).

Thus, this result shows that both *gsc* and *Xnot* are able to complement the inhibition of BMP signals to promote notochord formation.

Xnot acts in notochord formation as a transcription repressor

Gsc is a homeobox-containing transcription factor that is likely to act as a repressor (Danilov et al., 1998; Ferreiro et al., 1998). *Xnot* also encodes a homeodomain protein, but it is not known whether it acts as a transcription activator or repressor. To address this issue, we constructed repressor- and activator-forms of this molecule by fusing its homeobox domain with either the Engrailed-repressor domain (enR) or the VP16-activator domain (Fig. 5A). We then co-expressed each molecule together with tBR on the ventral side of four-cell embryos. When enR-XnotHD was expressed with tBR, notochord formation was observed in the secondary trunks, while co-expression of VP16-XnotHD and tBR did not result in notochord formation (Fig. 5B,C). Hence, Xnot appears to act as a transcriptional repressor in notochord formation. Consistent with this interpretation, careful analysis of the primary sequence of Xnot reveals the presence of a conserved motif (called eh1) that has been shown to be involved in active transcriptional repression by the Engrailed protein (Fig. 5D) (Smith and Jaynes, 1996).

We then made use of the VP16-Xnot construct to antagonise the function of the endogenous Xnot protein in the dorsal mesoderm. Overexpression of VP16-XnotHD in dorsal marginal zone (DMZ) explants suppressed the formation of endogenous notochord formation (Fig. 5F). This effect was rescued by co-injection of wild type *Xnot* (Fig. 5G).

Altogether, these results indicate that Xnot acts as a transcription repressor required for notochord formation.

Gsc is able to act in vegetal blastomeres, while Xnot acts exclusively in mesoderm

During gastrulation, the endogenous expression domains of *Xnot* and *gsc* appear complementary. *Xnot* marks the presumptive notochord region (von Dassow et al., 1993; Fig. 6C,D), while *gsc* is expressed in the prechordal plate (Steinbeisser and DeRobertis, 1993; Fig. 6G,H). As early as late blastula (stage 9.5) or early gastrula (stage 10) stage, they are already expressed in largely non-overlapping domains

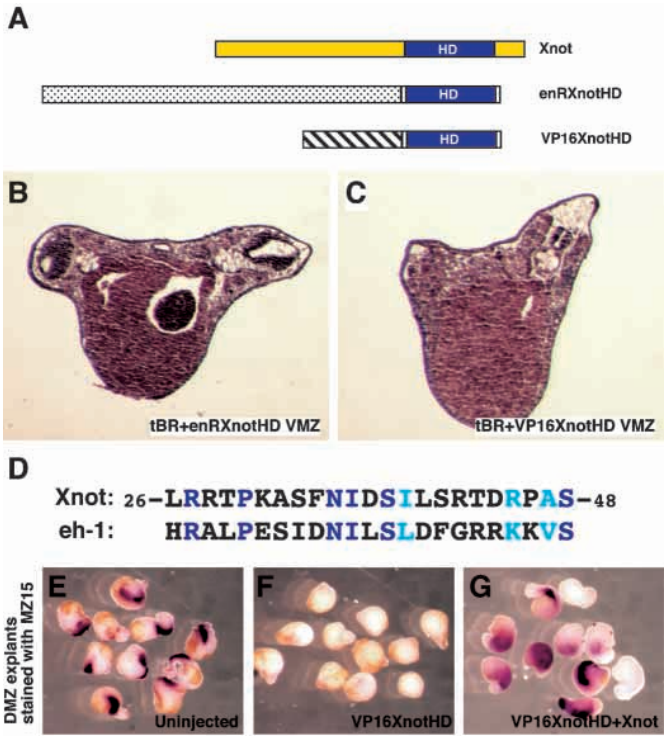

Fig. 5. Xnot acts as a transcriptional repressor. (A) Schematic of constructs. The homeodomain is shown in blue. The repressor domain (enR) of *engrailed* is indicated by a dotted box. The VP16 activator domain is represented by a hatched box. (B,C) Cross-sections through stage 32 embryos, which were co-injected ventrally at the four-cell stage with RNA for *tBR* and either (B) *enRXnotHD* (25pg) or (C) *VP16XnotHD* (100pg). (D) Sequence comparison of Xnot (amino acids 26-48) and the active repressing domain (eh-1) of engrailed. Identical amino acids are in dark blue. Conserved amino acids are in light blue. (E-G) Immunostaining with MZ15 of DMZ explants from (E) uninjected embryos, or from (F) embryos injected with *VP16XnotHD* RNA (200pg) or (G) *VP16XnotHD* (200pg) plus wild-type *Xnot* (100pg) RNA.

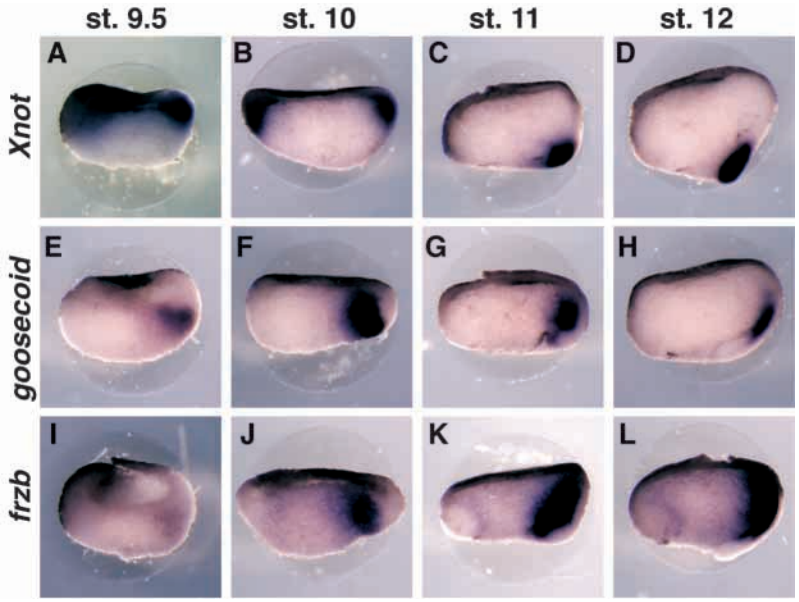

Fig. 6. Comparative analysis of the expression of *Xnot*, *gsc* and *frzb* during the course of gastrulation. Embryos at the indicated stages were bisected along the dorsoventral axis, and processed for whole-mount *in situ* hybridisation analyses with the indicated probes. Dorsal is towards the right.

along the animal-vegetal axis (Zoltewicz and Gerhart, 1997; Fig. 6A,B,E,F). These observations suggest that *Xnot* and *gsc* are involved in notochord formation in distinct manners.

We first tested the spatial competence of cells to respond to *gsc* and *Xnot* by forming notochord. *tBR* RNA was first injected in the VMZ of four-cell embryos to antagonise BMP signalling in a broad domain. Then, at the 32-cell stage, a mixture of *lacZ* and *gsc* or *Xnot* RNAs was injected into either B4, C4 or D4 blastomeres (Fig. 7A). At the tailbud stage, we monitored injected embryos for the position of the injected cells and for the presence of ectopic notochord. B4 normally gives rise mainly to ectoderm, C4 to both mesoderm and endoderm, and D4 to endoderm (Dale and Slack, 1987). Injection of *Xnot* or *gsc* RNA in B4 was not able to trigger notochord development. Injection of either *gsc* and *Xnot* in C4 led to notochord development in 55% (n=11) and 100% (n=8) of cases, respectively, with the injected cells contributing mainly to the ectopic trunk mesoderm (Fig. 7C,F). Injection into D4 blastomeres of mRNA for *gsc*, but not for *Xnot*, also led to notochord development (20%, n=10), the stained cells being found anterior to the notochord in what could be the pharyngeal endoderm (Fig. 7D,G).

This suggests that Xnot is active only in the mesoderm precursors, while Gsc is able to act in more vegetal cells. This result is therefore consistent with their endogenous expression domains during gastrulation, where *Xnot* is expressed in posterior trunk mesoderm while *gsc* is expressed in anterior endomesoderm (Fig. 6).

gsc acts upstream of *Xnot* in notochord formation

We then addressed the relationship between *gsc* and *Xnot* in notochord formation. We overexpressed *gsc* or *Xnot* together with *tBR* on the ventral side of embryos and analysed their ability to maintain each other's expression during gastrulation. When Gsc and tBR were co-expressed on the ventral side of embryos, expression of *Xnot* was maintained in the axial mesoderm of forming secondary trunks of late gastrula

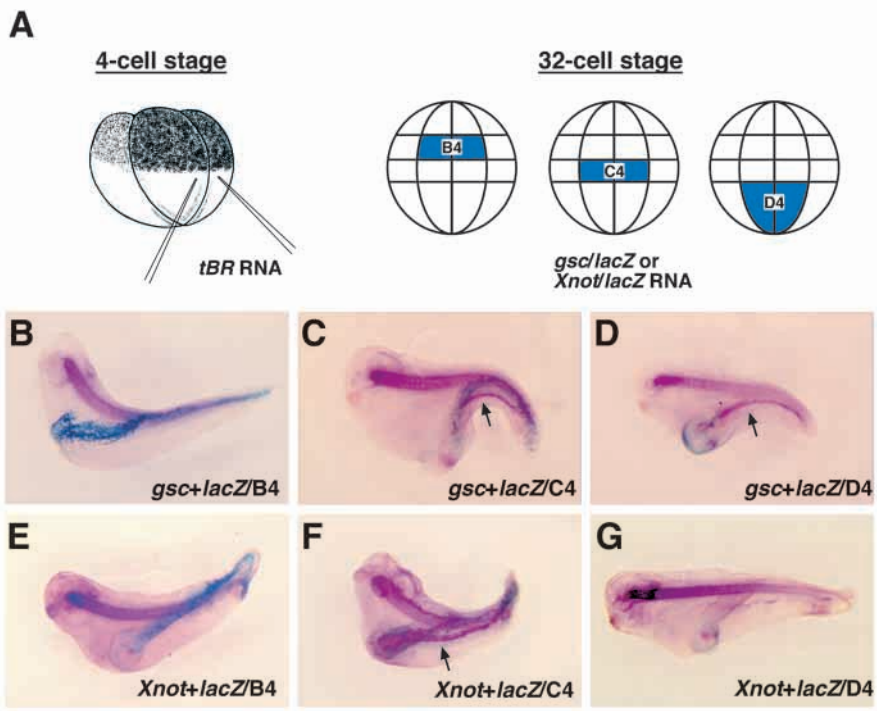

Fig. 7. *gsc* is able to act in vegetal blastomeres, while *Xnot* acts exclusively in the marginal zone. (A) Experimental scheme. *tBR* RNA was injected into ventral blastomeres of four-cell embryos. When embryos reached the 32-cell stage, *gsc* or *Xnot* RNA was injected into B4, C4 or D4 blastomeres together with *lacZ* RNA. (B-G) Cleared tailbud-stage embryos, injected with the indicated combination of factors/blastomeres, and showing the position of the X-gal staining (blue) and of the immunoreactivity for MZ15 (red).

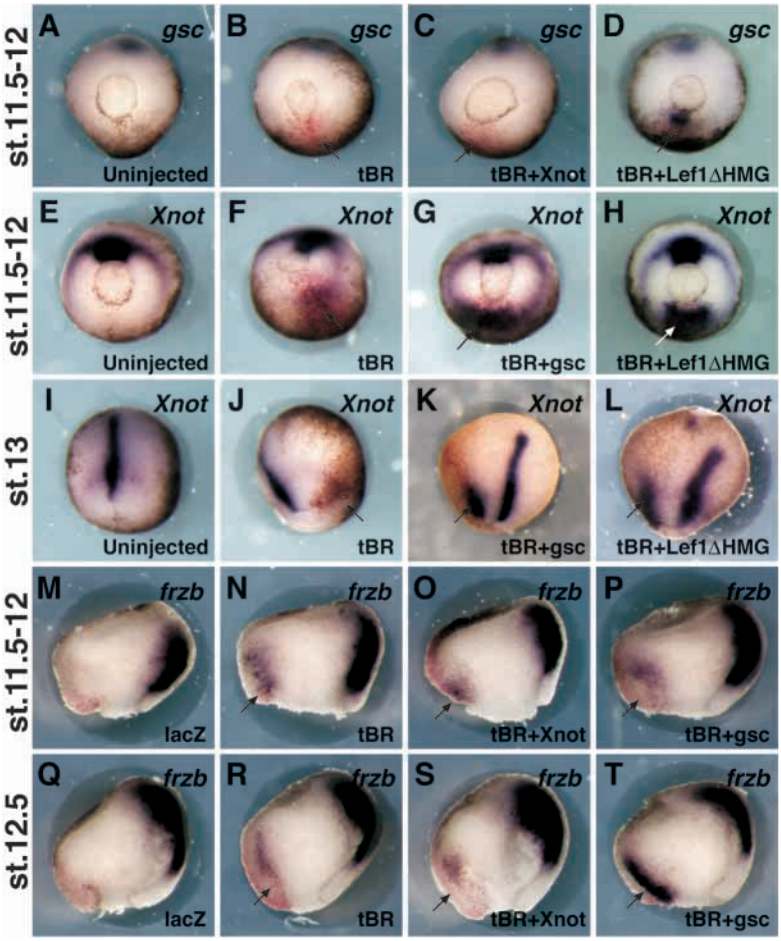

Fig. 8. Regulatory interactions between *Xnot*, *gsc* and *frzb* during notochord formation. Embryos at the indicated stages (left) were injected with the indicated combination of factors (lower right corner of each panel) and processed for in situ hybridisation with the indicated probes (upper right corner of each panel). (A-H) Vegetal views. (I-L) Dorsal or dorso-lateral views. (M-T) Embryos were bisected along the dorsoventral axis before being processed for whole-mount in situ hybridisation. Injected cells are revealed by their β-galactosidase activity (red). Gene expression is shown in purple.

embryos (compare Fig. 8G,K with *tBR*-injected embryos in Fig. 8F,J). By contrast, secondary trunks induced by Xnot and tBR had already lost the expression of *gsc* when analysed at the mid-late gastrula stage (stage 11.5-12, Fig. 8C). These results suggest that *gsc* acts upstream of *Xnot* to maintain the expression of this gene and promote notochord formation.

This and the previous section therefore suggest that during notochord formation, *gsc* acts in vegetal cells by maintaining the expression of *Xnot* in notochord precursors via the action of an extracellular molecule(s). We next addressed the identity of the extracellular molecule(s) regulated by *gsc*.

Frzb may be the notochord-promoting signal regulated by *gsc*

We showed above that Wnt/β-catenin signalling pathways, probably activated by Xwnt-8, should be blocked to promote notochord formation (Fig. 2). We reasoned that the non-cell-autonomous effect of *gsc* could be mediated by secreted Wnt antagonists. *frzb* encodes a molecule with homology to the extracellular domain of the putative Wnt receptor, Frizzled, and has been shown to specifically block embryonic responses to Xwnt-8 (Leyns et al., 1997; Wang et al., 1997a; Wang et al., 1997b). It also has been reported that radial injection of *gsc* RNA into *Xenopus* embryos results in an expansion of the endogenous expression domain of *frzb* (Leyns et al., 1997). These results indicate that Frzb is a good candidate for the extracellular molecule regulated by *gsc*.

As shown above, the tBR-injected ventral marginal zones initiate but do not maintain expression of *frzb* during gastrulation (Fig. 3V,X). We therefore asked whether the *frzb* expression is maintained when *tBR* and *gsc* RNAs are co-injected. At the late gastrula stage (stage 12.5), maintenance of ectopic *frzb* expression was barely observed in embryos injected ventrally with *tBR* RNA alone, or with *Xnot* and *tBR* RNAs (Fig. 8N,O,R,S). When *tBR* and *gsc* RNAs are co-injected ventrally, however, ectopic *frzb* expression was maintained (Fig. 8P,T).

This result suggests that Frzb could mediate the non-cell autonomous effect of *gsc* on the maintenance of *Xnot* expression in the presumptive notochord region by antagonising Wnt ligands emitted from neighbouring ventrolateral cells. Consistent with this view, when RNAs for *tBR* and *Lef1ΔHMG* were injected ventrally at the four-cell stage, resultant gastrula embryos maintained ectopic expression of *Xnot* (Fig. 8H,L). We also found evidence for a positive regulatory loop between *gsc* and Wnt inhibition. While expression of *frzb* was strongly maintained in embryos injected with *tBR* and *gsc*, we also detected maintenance of *gsc* expression in ventral marginal zones injected with *tBR* and *Lef1ΔHMG* (Fig. 8D).

DISCUSSION

Co-repression of BMP and Wnt signals and formation of organiser derivatives

It has been shown that simultaneous inhibition of BMP and Wnt signals is sufficient to promote head formation in the secondary axes (Glinka et al., 1997). Current models propose that Wnt inhibitors such as Dickkopf1 and Frzb, which are emitted by anterior endomesoderm, act by blocking the posteriorising effect of Wnts on neuroectoderm, thus allowing anterior neurectodem to develop (McGrew et al., 1997, Hashimoto et al., 2000; reviewed by Kiecker and Niehrs, 2000).

The repression of Wnt signalling in overlying anterior neural territories is not the sole function of the Wnt inhibitors secreted by the head organiser. For example, inhibition of Wnt signalling by Dkk1 is central to the formation of the prechordal plate, a head organiser derivative (Kazanskaya et al., 2000).

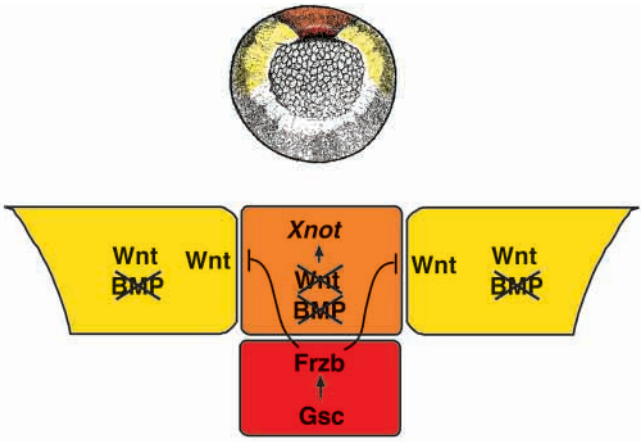

Fig. 9. A model for notochord formation. BMP signals are blocked in dorsal mesoderm in both somitic (yellow) and notochord (orange) territories. Wnt signals are also blocked in the notochord region, allowing maintenance of *Xnot* expression. This state is maintained by the antagonistic action of Frzb, secreted from the prechordal plate (red). *frzb* expression is positively regulated by Gsc.

Frzb, on the other hand, is not able to induce ectopic prechordal plate, illustrating that these molecules may antagonise different Wnts. Our results now show that the co-repression of BMP and Wnt signals is required for the formation of notochord. Dkk1, however, does not seem to be involved in this process (Kazanskaya et al., 2000), and our results suggest that Frzb may be the Wnt inhibitor involved.

Wnt signalling is transduced by at least two different pathways: one is mediated through β-catenin, the second involves the stimulation of protein kinase C (reviewed by Kuhl et al., 2000). We found that the Wnt/β-catenin pathway should be inhibited for notochord formation. We further addressed where the inhibition should take place to promote notochord formation and found that inhibition of Wnt/β-catenin signals in dorsolateral mesodermal cells is sufficient to convert their fate into notochord in a cell-autonomous manner. Therefore, the formation of both prechordal plate and notochord, the main organiser derivatives, requires co-repression of BMP and Wnt signals.

Mode of action of Xnot and Gsc in promotion of the notochord cell fate

In this study, we show that Xnot acts downstream of the co-inhibition of BMP and Wnt signals to promote notochord formation. A series of genetic and embryological studies with zebrafish embryos have revealed that *flh*, a zebrafish homologue of *Xnot*, plays an essential role in promotion of notochord differentiation by repressing muscle fate (Talbot et al., 1993; Melby et al., 1996: Amacher and Kimmel, 1998). *flh* is expressed exclusively in the presumptive notochord region (Melby et al., 1996). In the anterior notochord, *flh* seems to act solely to repress the function of a gene called *spadetail* (*spt*), which is required for the formation of trunk somitic muscle (Kimmel et al., 1989; Amacher and Kimmel, 1998; Griffin et al., 1998). Our demonstration that Xnot is a transcriptional repressor raises the interesting possibility that this gene may directly regulate the zygotic expression of the *Xenopus* homologue of *spadetail* (*antipodean/VegT*; Stennard et al., 1999).

In *Xenopus*, it has been shown that *Xwnt-8* positively regulates expression of myogenic genes such as *XMyoD* and *XMyf-5*, and negatively that of *Xnot* (Hoppler et al., 1996; Hoppler and Moon, 1998; Marom et al., 1999). Consistently, zygotic overexpression of *Xwnt-8* in the dorsal side of *Xenopus* embryos leads to transformation of notochord to somitic muscle (Christian and Moon, 1993). The apparent opposite effect of Xwnt-8 on the regulation of the myogenic genes and *Xnot* could occur either independently or linearly. In the latter case, one can propose that Xwnt-8 signal negatively regulates *Xnot* at the transcription level, while Xnot is repressing expression of the myogenic genes in notochord precursors. We showed that overexpression of *Xnot* resulted in the formation of notochord in the tBR-induced secondary trunks. Production of Xnot protein from injected RNA by-passes the postulated negative transcriptional influence of *Xwnt-8*, and thus promotes notochord formation.

In the endogenous situation, however, there must be mechanisms to protect the presumptive notochord region from the inhibitory effect of Xwnt-8 and allow the maintained expression of *Xnot* in this region (Fig. 9). We propose that *gsc* acts in this process by regulating the expression of a Wnt antagonist, *frzb*, which is known to bind and inhibit Xwnt-8 (Leyns et al., 1997; Wang et al., 1997a). Exclusion of Xwnt-8 from the most dorsal marginal zone is also mediated at the transcriptional level. Expression of *Xwnt-8* gene is initiated in the ventrolateral marginal zone, but never in the dorsal marginal zone. It has been suggested that *gsc* is responsible for the repression of *Xwnt-8* expression in the dorsal marginal zone (Christian and Moon, 1993). We also found that ventral injection of *gsc* (50 pg) RNA alone effectively represses the expression of *Xwnt-8* during gastrulation (stages 10, 10.5 and 11), while expression of *Xvent-1*, another ventrolateral gene, is repressed only at the onset of gastrulation (stage 10) (H. Y. and P. L., unpublished). As Gsc is a transcription repressor, the repression of *Xwnt-8* gene might be directly mediated by Gsc (Danilov et al., 1998; Ferreiro et al., 1998). Therefore, at least two mechanisms are acting to exclude Xwnt-8 from the most dorsal marginal zone: (1) repression of *Xwnt-8* by Gsc at the transcription level; and (2) inhibition by Frzb through a direct binding to Xwnt-8.

Wnt repression and generation of chordate characteristics?

All chordates, at some stage of their life cycle, possess a notochord and a dorsal hollow neural tube. The notochord, in particular, is a central characteristic that unites tunicates, amphioxus and vertebrates in the phylum Chordata. We show here that repression of Wnt signalling by secreted Wnt antagonists is required for the formation of the *Xenopus* notochord. Wnt ligands are present in the genomes of both deuterostomes and protostomes, and even in *Hydra* (Hobmayer et al., 2000). However, secreted Wnt inhibitors have so far only been indentified in chordate genomes, and are notably absent from the sequenced genomes of both *Drosophila melanogaster* and *Caenorhabditis elegans*. Interestingly, both Frzb- and Dkk-like molecules are present in the genome of the ascidian *Ciona intestinalis* (D. Caillol and P. L., unpublished), a tunicate. Hence, although the data are still incomplete and we cannot rule out the possibility that Wnt antagonists are present in basal deuterostomes such as hemichordates and echinoderms, they

suggest that the emergence of several types of secreted Wnt antagonists may have coincided with the emergence of the notochord.

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