Development 127, 4981-4992 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV2570

The role of *Xenopus dickkopf1* in prechordal plate specification and neural patterning

Olga Kazanskaya, Andrei Glinka and Christof Niehrs*

Division of Molecular Embryology, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany
*Author for correspondence

Accepted 5 September; published on WWW 24 October 2000

SUMMARY

***Dickkopf1* (*dkk1*) encodes a secreted WNT inhibitor expressed in Spemann's organizer, which has been implicated in head induction in *Xenopus*. Here we have analyzed the role of *dkk1* in endomesoderm specification and neural patterning by gain- and loss-of-function approaches. We find that *dkk1*, unlike other WNT inhibitors, is able to induce functional prechordal plate, which explains its ability to induce secondary heads with bilateral eyes. This may be due to differential WNT inhibition since *dkk1*, unlike *frzb*, inhibits Wnt3a signalling. Injection of inhibitory antiDkk1 antibodies reveals that *dkk1* is not only sufficient but also required for prechordal plate formation but not for notochord formation. In the neural plate *dkk1* is required for anteroposterior and dorsoventral patterning between mes- and telencephalon, where *dkk1* promotes anterior and ventral fates. Both the requirement of anterior explants for *dkk1* function and their ability to respond to *dkk1* terminate at late gastrula stage. *Xenopus* embryos posteriorized with bFGF, *BMP4* and *Smads* are rescued by *dkk1*. *dkk1* does not interfere with the ability of bFGF to induce its immediate early target gene *Xbra*, indicating that its effect is indirect. In contrast, there is cross-talk between BMP and WNT signalling, since induction of BMP target genes is sensitive to WNT inhibitors until the early gastrula stage. Embryos treated with retinoic acid (RA) are not rescued by *dkk1* and RA affects the central nervous system (CNS) more posterior than *dkk1*, suggesting that WNTs and retinoids may act to pattern anterior and posterior CNS, respectively, during gastrulation.**

Key words: *Blimp1*, *Hex*, *Shh*, Retinoic acid, Prechordal plate, *dkk1*, Organizer, WNT, BMP, FGF, *frzb*, *Xenopus*

INTRODUCTION

During early amphibian development neural induction and anteroposterior (a-p) as well as dorsoventral (d-v) patterning of the neuroectoderm are regulated by inducers released from Spemann's organiser and its derivatives. Many of the molecules secreted by the *Xenopus* organiser act by inhibiting signalling molecules of the BMP, WNT and Nodal-related families (Harland and Gerhart, 1997; Moon et al., 1997; Hsu et al., 1998; Piccolo et al., 1999). BMP inhibitors, like Chordin (Piccolo et al., 1996), Noggin (Zimmerman et al., 1996) and Follistatin (Hemmati-Brivanlou et al., 1994; Iemura et al., 1998), can induce ectopic trunks containing neural tube. WNT inhibitors like Frzb (Leyns et al., 1997; Wang et al., 1997a), Dkk1 (Glinka et al., 1998), and Wif1 (Hsieh et al., 1999) can cooperate with BMP inhibitors in inducing secondary heads containing forebrain and eyes. Inhibition of Nodal signalling, e.g. by Cerberus (Hsu et al., 1998; Piccolo et al., 1999) or Antivin (Thisse et al., 2000) is sufficient to induce forebrain and eyes in the absence of mesoderm. While these studies suggest an important role for various TGF-β and WNT inhibitors in neural induction and patterning, a full understanding of their physiological relevance requires loss-of-function studies.

Dickkopf1 (*dkk1*) encodes a secreted WNT inhibitor (Dkk1), which acts upstream of the WNT pathway component *dishevelled* but whose mechanism of action is unknown (Glinka et al., 1998). Like *Xenopus dkk1*, mouse (Glinka et al., 1998), human (Fedi et al., 1999; Krupnik et al., 1999) and zebrafish *dkk1* (Hashimoto et al., 2000) inhibit WNT/β-catenin signalling. *dkk1* is expressed in the leading edge of involuting endomesoderm as well as in the prechordal plate during *Xenopus* gastrulation. Together with BMP inhibitors it induces secondary heads. Overexpression of *dkk1* in *Xenopus* (Glinka et al., 1998) and zebrafish (Hashimoto et al., 2000) anteriorizes embryos, leading to shortened trunk and enlarged heads. Inhibition of Dkk1 in *Xenopus* using inhibitory antibodies leads to microcephalic embryos, typically exhibiting cyclopia (Glinka et al., 1998). While these studies suggest an important role for *dkk1* in specification of rostral structures they raised a number of questions.

Why does *dkk1* induce two eyes and other WNT inhibitors only one eye?

Coinjection of *dkk1* mRNA with BMP antagonists routinely induces complete heads containing two well-formed eyes. In contrast, both *frzb* and dominant negative *Xwnt8* together with BMP inhibitors induce only one ectopic eye (Glinka et al.,

1997), as does the head inducer *cerberus* (Bouwmeester et al., 1996). This is irrespective of the doses employed and appears to be a qualitative difference between these WNT antagonists and *dkk1*.

Is *dkk1* required for prechordal plate fate specification?

dkk1 overexpression anteriorizes embryos but it is unclear if this corresponds to an effect of *dkk1* on prechordal plate, or neuroectoderm, or both. In *Xenopus*, *dkk1* superinduces anterior neural markers together with BMP inhibitors in animal cap ectoderm (Glinka et al., 1998) and in zebrafish overexpression of *dkk1* promotes anterior neuroectoderm in antivin mRNA-injected embryos, which are devoid of most mesoderm (Hashimoto et al., 2000), indicating a direct effect on neuroectoderm. On the other hand, *Xenopus dkk1* superinduces prechordal plate markers together with BMP inhibitors in ventral mesoderm (Glinka et al., 1998) and overexpression of *dkk1* rescues mesendoderm formation in zebrafish mutant for *bozozok* (*boz*), a homeobox gene implicated in a pre-MBT WNT signalling pathway required for axis formation (Hashimoto et al., 2000). These latter results indicate an effect of *dkk1* on mesendoderm.

Does *dkk1* affect AP or DV neural patterning or both?

AntiDkk1 antibodies induce cyclopic embryos, indicating a requirement for *dkk1* in anterior neural patterning. However, cyclopia can be caused by defects in ventral midline signalling, i.e. DV patterning, or by defects in AP patterning, or both (Durston et al., 1989; Chiang et al., 1996; Schier et al., 1996; Sampath et al., 1998). Our previous experiments did not distinguish between these possibilities.

When is *dkk1* required for head induction?

Neural induction in vertebrates starts as early as the beginning of gastrulation (Gawantka et al., 1998; Grinblat et al., 1998) but patterning occurs still during neurulation (Sasai and De Robertis, 1997). *dkk1* expression starts in the early gastrula organizer but is maintained until late neurula in prechordal plate. It would be important to narrow down the critical period at which *dkk1* is required and to infer the stages when WNT signals relevant to *dkk1* action are active.

Which pathways does *dkk1* interact with?

Regarding upstream regulation, zebrafish *dkk1* expression is reduced in *boz*, *one eyed pinhead* (*oep*) and *squint* (*sqt*) mutants, suggesting that *dkk1* is a target of pre-MBT (midblastula transition) WNT signalling as well as Nodal signalling (Hashimoto et al., 2000). Regarding downstream components, *dkk1* functions as an inhibitory component of the posteriorizing WNT pathway, active after MBT. However, there are other secreted factors that also posteriorize embryos, including BMPs, FGFs and retinoic acid, and it is unclear if their pathways interact with *dkk1*.

Here we have addressed these questions, taking advantage of the availability of specific inhibitory Dkk1 antibodies that allowed us to test embryos for their requirement of *dkk1* during early axis formation.

MATERIALS AND METHODS

Embryo culture and dissections

In vitro fertilization, embryo culture and staging were carried out as described previously (Gawantka et al., 1995). Operations on embryos were performed using a microknife and fused glass capillary on plastic dishes coated with 1% agarose in 0.5× Barth solution (Peng, 1991). Explants were cultured in the presence of antibodies in 96-well plates pretreated with BSA. Brains of 4-day embryos were excized by forceps in 1× Barth solution.

Antibodies

Antibodies for blastocoel injections were purified as described previously (Glinka et al., 1998). Embryos from stages 8-11 were injected with 150 ng antibody 14 or 15 into the blastocoel and allowed to develop for 3 days for phenotype analysis or fixed at stages 13, 15 or 30 for in situ hybridization. For incubation of explants, antibodies were dialysed overnight against 0.5× Barth solution, and utilized at concentrations of 3.2 mg/ml.

RNA and DNA injections

Synthetic capped RNAs for microinjection were obtained by in vitro transcription using the Megascript kit (Ambion). DNA templates were linearised, transcribed and mRNAs injected per blastomere as follows: pCSFrzbI, *Not*I, SP6; 0.2 ng; pSP64tBR, *Eco*RI, SP6, 0.25 ng; pRNdkk1 *Not*I, SP6, 0.025ng; pT7TS-Xbhh *Bam*HI, T7, 0.5 ng; pBMP4, *Xho*I, T3, 0.13ng; pCSXSmad1, *Sfi*I, SP6, 0.5 ng. bFGF (Promega) was injected as protein into the blastocoel (25 pg/embryo). pCSmWnt3a DNA (Roelink and Nusse, 1991) was injected animally at the four-cell stage at 0.05 ng in all blastomeres.

Whole-mount in situ hybridization, histological sections and TUNEL staining

Whole-mount in situ hybridization was performed as described by Harland (1991) with modifications (Hollemann et al., 1998). Histological 50 µm sections of albumin-gelatine embedded embryos were cut using a vibratome. TUNEL staining was carried out as described (Hensey and Gautier, 1998).

Luciferase assays and RT-PCR

For luciferase assays the BMP-responsive luciferase reporter construct pVent2-Luc (BRE) (Candia et al., 1997) and assays were carried out as described (Glinka et al., 1996). Embryos were injected with 25 pg pVent2-Luc and the following mRNAs: preprolactin (0.5 ng, control), *BMP4* (0.25 ng), *dkk1* (0.075 ng), *frzb* (0.5ng) or *noggin* (0.05 ng). RT-PCR assays were carried out as described (Gawantka et al., 1995). Gene-specific primers were as follows: *H4*, *Xvent1* (Gawantka et al., 1995), *Xbra* (Glinka et al., 1996), *Xvent2* (Onichtchouk et al., 1996), *szl* (Salic et al., 1997).

RESULTS

dkk1 specifies the prechordal plate and is required for bilateral eye formation

Ventral coinjection of dominant-negative Bmp-2/4 receptor (*tBR*) mRNA (Suzuki et al., 1994) with *dkk1* mRNA induces heads containing two eyes, while coinjected *frzb* (Leyns et al., 1997; Wang et al., 1997a) and *tBR* mRNAs induce heads with only one eye (Fig. 1A-C). To further test if this reflects a non-equivalence between these two WNT inhibitors we asked if *frzb* could rescue the phenotype resulting from the loss of Dkk1 function, the microcephaly induced by injected antiDkk1 Ab. This polyclonal antibody (anti15 Ab) was raised against a peptide epitope which is not conserved in *dkk2-4* and not even

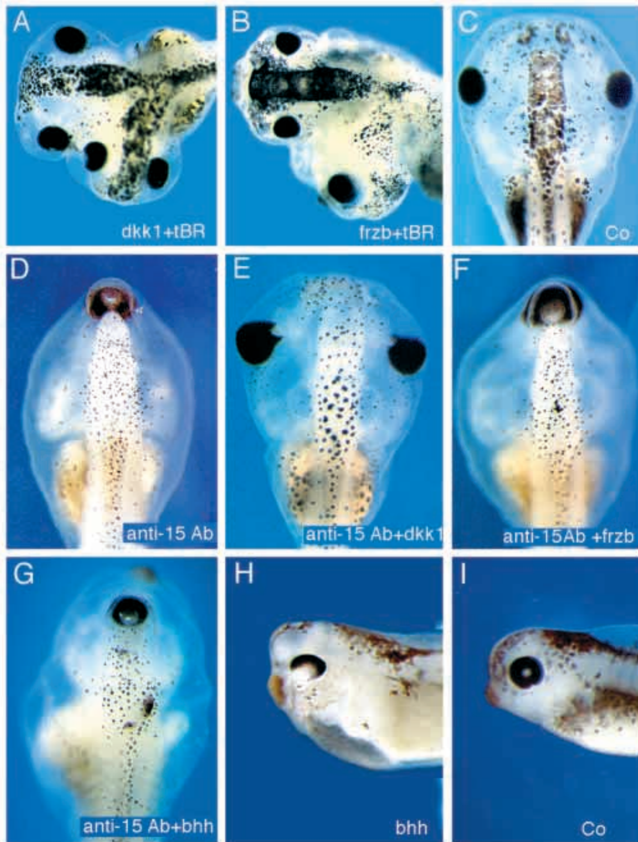

Fig. 1. *dkk1* but not *frzb* induces complete secondary heads. Coinjections of *tBR* mRNAs with *dkk1* (A) or *frzb* (B) into ventral blastomeres of four-cell embryos results in the development of secondary heads with two eyes or one eye, respectively. Embryos shown are 4 days postfertilisation (pf). (C-G) *dkk1* rescues the cyclopic phenotype elicited by anti15 Ab, while *frzb* and *bhh* are unable to rescue cyclopia. Embryos are day 6 pf, shown from the dorsal side, anterior up. (C) Uninjected control embryo. (D) Embryo injected with anti15 Ab into the blastocoel. (E) Embryo injected radially with *dkk1* mRNA and anti15 Ab. (F) Embryo injected radially with *frzb* mRNA and anti15 Ab. (G) Embryo injected radially with *bhh* mRNA and anti15 Ab. (H) Embryo injected radially with *bhh* mRNA. (I) Uninjected control embryo.

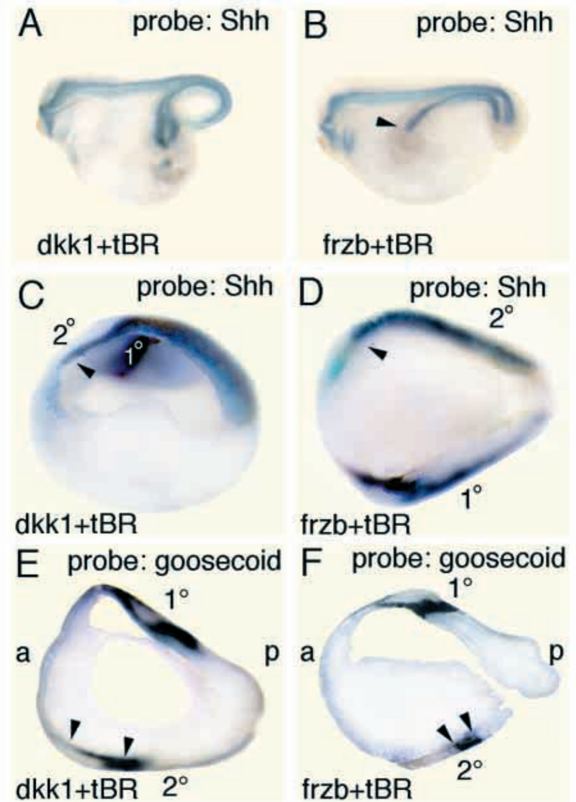

Fig. 2. *dkk1* but not *frzb* induces complete prechordal plates. (A,B) In situ hybridization with *sonic hedgehog* (*Shh*) in embryos coinjected at the four-cell stage ventrally with *tBR* and either *dkk1* (A) or *frzb* (B). Stage-30 embryos are shown from the anterior. Arrowhead indicates the anterior limit of *Shh* expression. (C-D) in situ hybridization for *Shh* in embryos injected at the four-cell stage ventrally with nuclear *lacZ* lineage tracer, *tBR* and either *dkk1* (C) or *frzb* (D). Stage-16 embryos were cut sagittally; secondary axes are marked by light blue β-gal staining. Arrowheads indicate the anterior limit of secondary *Shh* expression. Note that the expression in the prechordal plate in (D) is lacking. (E,F) In situ hybridization for *goosecoid* (*gsc*) in embryos injected as in A and B. Sagittal sections of stage-14 embryos are shown. Arrowheads indicate the mesodermal zone of *gsc* expression in the anterior mesoderm of the induced secondary axis (2°). Note that mesodermal *gsc* expression extends anterior to the neuroectodermal in the primary axis (1°) and secondary axis induced by *dkk1*, but is in register with the neural expression in *frzb*-injected embryos (F). a, anterior; p, posterior.

in mouse *dkk1*. It recognizes the extracellular form of Dkk1 and its effects can be completely blocked by preincubation with specific epitope-peptide as well as by extra Dkk1, attesting to its specificity (Glinka et al., 1998). Microinjection of anti15 Ab induces predominantly cyclopic embryos (Fig. 1D) while control Ab injection has no effect (not shown). However, while *dkk1* rescues head formation as shown previously, *frzb* injection only leads to a slight expansion of the cyclopic eye and forebrain but does not rescue bilateral eyes in anti15 Ab treated embryos (Fig. 1D-F). We conclude that *frzb* cannot substitute for the loss of *dkk1*, despite their common function as WNT antagonists.

Hedgehog emanating from the prechordal plate is thought be be a ventral midline signal necessary for splitting the eye field (Roelink et al., 1994; Ekker et al., 1995a,b; Macdonald et al., 1995; Chiang et al., 1996; Li et al., 1997). We therefore analyzed whether forced Hedgehog expression could rescue bilateral eye formation in antiDkk1 Ab-injected embryos. We used *banded hedgehog* (*bhh*), which has similar activities to *Shh* but is more potent (Ekker et al., 1995a). However, injected *bhh* mRNA does not rescue bilateral eye formation, although it elicits a potent *hedgehog* overexpression phenotype, most notably ventral eye defects (Fig. 1H), and it expands ventral midline tissue (not shown). It cannot be ruled out that *bhh* fails to rescue cyclopia because in order to do so its expression, unlike that of *dkk1,* needs to be localized.

We next tested the effect of *dkk1* and *frzb* on ectopic *sonic hedgehog* (*Shh*) (Ekker et al., 1995a) expression in the prechordal plate. In situ hybridization shows that secondary heads induced by *dkk1* expressed *Shh* in the ventral forebrain (n=32; Fig. 2A), as well as in the axial mesoderm rostral to

the notochord, the position normally occupied by prechordal plate (*n*=26; Fig. 2C). This is unlike the secondary axes induced by *frzb* (*n*=22), where *Shh* expression did not reach up to forebrain level and remained in the outer tissue layer (Fig. 2B,D). Coinjected *lacZ* marker shows that frzb/tBR expressing cells are capable of reaching a position ahead of the *Shh* expression limit, though, suggesting that specification, and not migration, is compromised. We examined the expression of another prechordal plate marker, *goosecoid* (*gsc*) (Cho et al., 1991) in early neurulae (stage 13). In *dkk1*-injected embryos all of the examined secondary heads (*n*=37) showed a zone of prechordal plate expression underlining and extending anterior to the neuroectodermal *gsc* expression (Fig. 2E, arrowheads). In contrast, in *frzb*-injected embryos the mesodermal and neuroectodermal *gsc* expression domains were always in register (Fig. 2F). We conclude that in secondary heads induced by *frzb*, unlike *dkk1*, prechordal plate cells, i.e. those expressing *gsc* and *Shh* simultaneously, are reduced and do not reach their proper position. This difference in formation of a proper secondary prechordal plate may explain the ability of *dkk1* to induce bilateral eyes. However, Hedgehog signalling alone is not sufficient to rescue cyclopia induced by loss of *dkk1*, suggesting that additional ventral midline signals are affected in these embryos.

The above results indicated that *dkk1* is sufficient to promote prechordal plate fate in conjunction with BMP inhibitors. We next asked whether *dkk1* is also necessary for specification of this tissue. We analyzed the prechordal plate markers *XHex* (Newman et al., 1997), *XBlimp1* (de Souza et al., 1999) and *gsc*, as well as the notochord marker *XNot2* (Gont et al., 1993), in control neurulae, neurulae injected with antiDkk1 Ab, or neurulae overexpressing *dkk1*. Fig. 3 shows that all prechordal plate markers were reduced in Ab-injected neurulae and expanded in *dkk1* mRNA-injected embryos. The expression domain of *Xhex*, which extends anterior of the prechordal plate into endoderm, was more sensitive to the Ab and to *dkk1* overexpression than the posterior (black arrow in Fig. 3B,C). *XNot2* expression showed that notochord was shortened and broadened by *dkk1* overexpression (Fig. 3L). While the notochord marker *XNot2* expanded following *dkk1* injection it was hardly affected in Ab-injected embryos, suggesting that *dkk1* is not required for notochord formation. We conclude that *dkk1* is both necessary and sufficient for promoting prechordal plate specification.

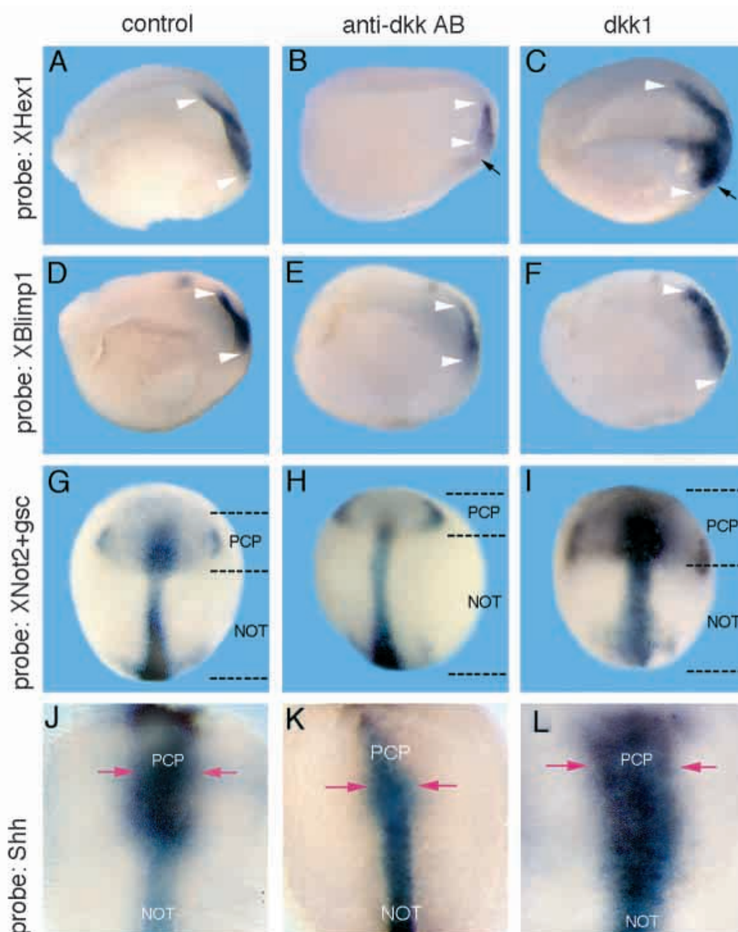

Fig. 3. *dkk1* is required for prechordal plate formation. (A,D,G) Uninjected controls, (B,E,H) Embryos injected with anti15 Ab into the blastocoel at blastula stage and (C,F,I) embryos injected radially at the four-cell stage with *dkk1* mRNA. In situ hybridization with probes to *XHex1* (A-C), *XBlimp1* (D-F) and *XNot2* + *gsc* (G-I). (A-F) Embryos from early neurula stage (stage 13) were cut sagittally; dorsal side up, anterior to the right. White arrowheads indicate the anterior and posterior limits of expression. (G-I) embryos from stage 13 are shown from the dorsal side, anterior side up. (J-L) The prechordal plates of control (J), anti15 Ab-injected (K), or *dkk1* mRNA-injected embryos (L) are shown from gastrocoel, anterior side is up. Red arrows indicate the lateral limit of *Shh* expression. PCP, prechordal plate, NOT, notochord.

dkk1 is required for a-p and d-v patterning of anterior neuroectoderm

dkk1 and AP patterning

To analyze the role of *dkk1* in neural patterning we compared the expression of neural markers in embryos injected with antiDkk1 Ab, or injected with *dkk1* mRNA or treated with retinoic acid (RA). RA is a posteriorizing agent, which like antiDkk1 Ab injection, can induce cyclopia and microcephaly (e.g. Durston et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991a,b; Sharpe, 1991; reviewed in Sasai and De Robertis, 1997), and it was of interest to know whether both treatments act in a similar fashion.

We first analyzed the effect of *dkk1* on AP patterning (Fig. 4). *dkk1* RNA or DNA injection induces an expanded head region, including larger eyes and telencephalon. Expression of the telencephalic marker *BF1* (Bourguignon et al., 1998), the anterior neural plate marker *Xanf1* (Zaraisky et al., 1992) as well as *XOtx2*, whose anterior and posterior expression domains mark the cement gland and midbrain, respectively (Blitz and Cho, 1995; Pannese et al., 1995), was greatly expanded posteriorly and laterally following *dkk1* injection (Fig. 4B,F,J). This can also be seen in dissected brains from tadpole embryos (Fig. 4O,S). In contrast, mesencephalon as well as hindbrain and otic placodes were reduced, depending on the injected dose, as indicated by reduced or absent *En2* (Fig. 4B) (Hemmati-Brivanlou et al., 1991) and *Krox20* (Bradley et al., 1993) expression (marking rhombomeres 3 and

5, not shown). Likewise, the posterior domain of *XOtx2* expression, which marks future mesencephalon and diencephalon caudally to the zona limitans, was significantly reduced (Fig. 4J). We conclude that *dkk1* is able to anteriorize the neural plate.

Consistent with these gain-of-function results, we found that loss of *dkk1* posteriorizes the neural plate. Injection into the blastocoel of anti14 control antibody, which recognizes intracellular Dkk1, had no detectable phenotypic effect (*n*=45), whereas microinjection of anti15 Ab led reproducibly to microcephaly (100%) and cyclopia (74%) (*n*=120). 5-22% of the embryos showed complete deletion of eyes and forebrain. Such embryos were lacking expression of *BF1* and had reduced *Xanf1* expression (Fig. 4C,G). *XOtx2* expression shifted to the very anterior part of the brain and its posterior domain was expanded (Fig. 4K). While *En2* expression at the mid-hindbrain boundary was expanded (Fig. 4C), *Krox20* expression in the hindbrain was unaffected (data not shown). This phenotype differs significantly from that induced following RA treatment, where brain structures are reduced up to hindbrain, as is highlighted by reduction of all markers, including *En2* expression (compare Fig. 4C,D, P,Q, T,U). The loss of anterior neural structures in antiDkk1 Ab-injected embryos is not due to increased cell death, as monitored by TUNEL staining of neurula embryos (data not shown), and hence is due to repatterning. We conclude that *dkk1* is required for specification of neural structures anterior of the mid-hindbrain boundary.

dkk1 and DV patterning

The role of *dkk1* in prechordal plate formation makes an effect on DV patterning of the anterior neural plate very likely, since it is known that genetic or mechanic ablation of the prechordal plate affects ventral forebrain structures (Adelmann, 1936a,b). Indeed, analysis of dissected brains indicated that *dkk1* RNA injection leads to an expansion and antiDkk1 Ab injection to a massive loss of ventral tel-and diencephalon (Fig. 4S,T). To further study DV patterning of the anterior neural plate we analyzed *XNot2*, which is also a marker of dorsal neuroectoderm, expressed bilaterally in the dorsal diencephalon, as well as of *Shh*, a marker for the ventral midline. *dkk1* RNA injection leads to expansion of *Shh* and a reduction of *XNot2* expression, and conversely antiDkk1 Ab injection leads to expansion of *XNot2* and reduction of *Shh* expression (Fig. 5). Thus, *dkk1* is necessary and sufficient for ventralization of anterior neural plate. Similar to antiDkk1 Ab treatment, RA reduces ventroanterior structures, leading to a collapse and fusion of

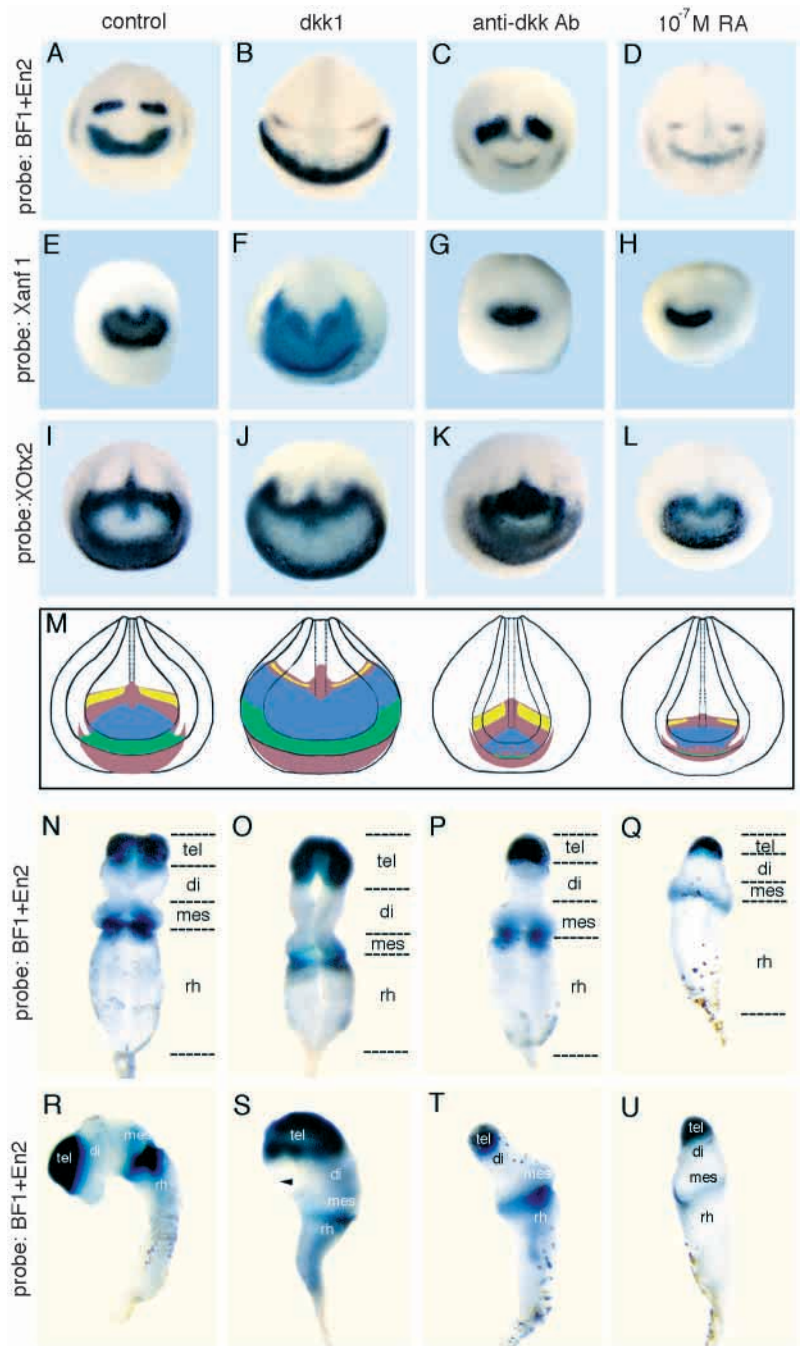

Fig. 4. *dkk1* regulates anteroposterior patterning of neurectoderm. (A,E,I) control embryos, (B,F,J) embryos injected radially at the four-cell stage with *dkk1* (C,G,K) embryos injected with anti15 Ab into the blastocoel and (D,H,L) embryos treated with 10^{-7} M retinoic acid (RA). In situ hybridization for *BF1* and *En2* (A-D), *Xanf1* (E-H), and *XOtx2* (I-L). Midgastrula-stage embryos (stage 15) are shown from anterior, dorsal side up. (M) Schematic diagram of the *BF1* (dark green), *Xanf1* (blue), *XOtx2* (violet) and *En2* (yellow) expression under the treatments indicated above. Note the changes in size of the head anlage and change in the proportions between its anterior and posterior regions. (N-U) *dkk1* affects forebrain at the expense of midbrain, whereas RA affects both regions. (N-U) In situ hybridization for *BF1* and *En2* under the treatments indicated above. Brains of stained embryos were excised from 4-day embryos and are shown from the dorsal (N-Q) and lateral (R-U) sides, anterior side up. Note the expansion of tel- and diencephalon and reduction of mesencephalon in O and S and the opposite changes in P and T. di, diencephalon; mes, mesencephalon; rh, rhomencephalon; tel, telencephalon.

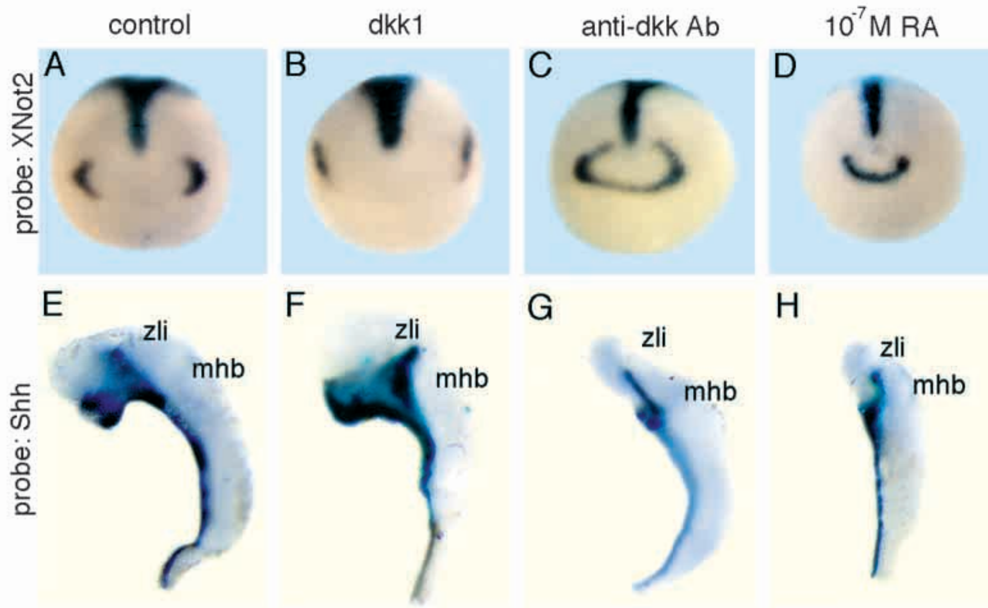

Fig. 5. *dkk1* regulates dorsoventral patterning of neuroectoderm. (A,E) Control embryo, (B,F) embryos radially injected at the four-cell stage with *dkk1*, (C,G) injected with anti15 Ab into the blastocoel and (D,H) treated with 10^{-7} M RA. In situ hybridisation for *XNot2* (A-D) and *Shh* (E-H). (A-D) Embryos are shown from the anterior, dorsal side up, (E-H) brains excised from day 4 pf. Brains are shown from the lateral side. zli, zona limitans intrathalamica, mhb, midbrain-hindbrain boundary.

XNot2 expression on the midline and to a reduction of *Shh* expression (Fig. 5D,H).

Time window of *dkk1* action

To understand when *dkk1* acts in the specification of anterior neural tissue, we performed two types of experiment. First, we determined up to which stage antiDkk1 Ab is able to affect head structures. We dissected the head anlage during mid-late gastrula and cultured it in isolation. Such explants contain all three germ layers (anterior neuroectoderm, anterior endomesoderm) and they will develop into well-patterned heads (Fig. 6A-E). We dissected such explants at various stages and cultured them in the presence of anti15 or control Ab. While explants treated with control Ab developed into normal patterned heads (85%, *n*=44; Fig. 6D,E), antiDkk1 treated explants were cyclopic when cultured from late gastrula onwards (stage 12.5) (83%, *n*=57; Fig. 6B). However, when added from early neurula (stage 13) onwards, the Ab no longer had an effect and explants formed again well-patterned heads (87%, *n*=56; Fig. 6C). Second, we determined the stage at which head anlagen lose competence to respond to *dkk1*. We grafted animal caps of albino

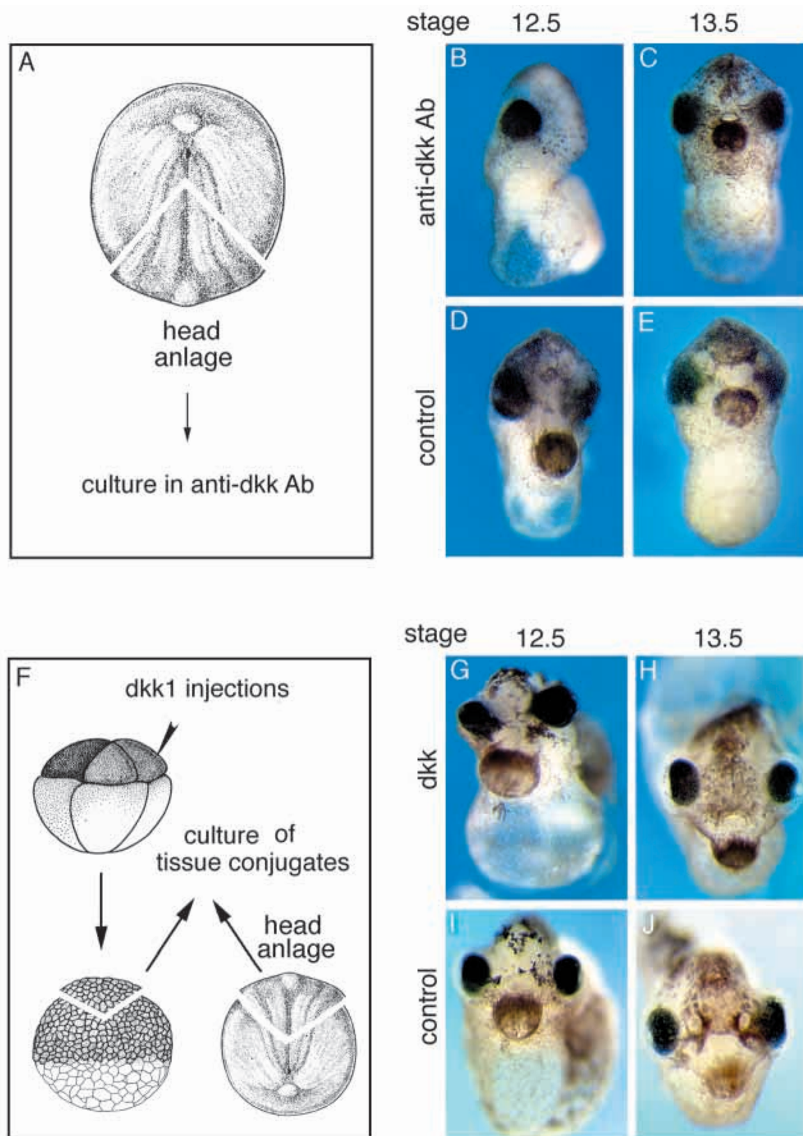

Fig. 6. Timing of *dkk1* action. (A,F) Schematic drawings of experiments. The anterior halve of the gastrocoel roof containing the head anlage was excised from stage 12.5 or 13.5 embryos and cultured in the presence of anti14 (control, D,E) or anti15 Ab (B,C) until stage 20, then transferred to 0.5× Barth solution and cultured for an additional 24 hours. Note cyclopia in (B). (F-J) Anterior halves of gastrocoel roof excized from stage-12.5 or -13.5 embryos were conjugated with animal caps, injected with *dkk1* mRNA (G,H) or control animal caps (I,J) and cultured for 2 days. Note that the head in G is strongly anteriorised.

embryos injected with *dkk1* RNA to head anlagen from pigmented embryos of three stages, late gastrula (stage 12.5), early (stage 13.5) and mid neurula (stage 14.5). Only stage 12.5 explants (76%; n=21) had enlarged forebrain and big eyes compared to the control (Fig. 6G); the later stages were normal (100%, n=17; Fig. 6H). Thus, following both overexpression as well as inhibition, we find that *dkk1* acts until late gastrula stage.

dkk1 interacts with BMP and FGF signalling pathways

dkk1 functions as a WNT inhibitor in a variety of assays, in line with the view that its role in head formation pertains to its antagonizing the posteriorizing action of WNTs after MBT (reviewed in Niehrs, 1999). However, FGF, BMP and RA have been implicated in neural posteriorization in addition to WNTs (reviewed in Slack, 1994; Harland and Gerhart, 1997; Sasai and De Robertis, 1997). These factors may either act as posteriorzing signals independent of WNTs or they may act by ultimately activating a posteriorizing WNT pathway. In the latter case, their effects should be rescued by *dkk1*.

To analyze these relationships, embryos were posteriorized by incubation with RA (Fig. 7A), blastomere injection of *BMP4* (Fig. 7D) (Dale et al., 1992; Jones et al., 1992), *Smad1* (Fig. 7G) (Meersman et al., 1997), *Smad5* (Table 1) (Suzuki et al., 1997) or mouse *Wnt3a* (Fig. 7M) (Roelink and Nusse, 1991) mRNAs or by injection of bFGF protein into the blastocoel (Fig. 7J). As summarized in Table 1, *dkk1* mRNA rescued the posteriorization by all of these reagents, with the exception of RA (Fig. 7B). The WNT inhibitor *frzb* also efficiently rescued *BMP4*-, *XSmad-1*- and bFGF-treated embryos but, unlike *dkk1*, it failed to revert posteriorisation induced by *Wnt3a* (Fig. 7I), previously shown to be unaffected by *frzb* (Wang et al., 1997b). These results indicate that the posteriorizing effects of forced BMP and FGF expression can be compensated by a concomitant downregulation of WNT signalling and that the RA pathway acts independently of posteriorizing WNTs.

Table 1. *dkk1* is able to rescue *BMP4*-, *Xsmads*-, *mWnt3A*- and bFGF-, but not RA-induced, posteriorization

Injected reagent	Posteriorized embryos (%)	Normal or anteriorised embryos (%)	n
10^{-6} M RA	100	0	36
10^{-7} M RA	81	19	40
10^{-6} M RA/*dkk1*	100	0	29
10^{-7} M RA/*dkk1*	84	16	29
BMP4	71	29	49
BMP4/dkk1	0	100	51
BMP4/frzb	8	92	51
XSmad1	90	10	31
XSmad1/dkk1	0	100	23
XSmad1/frzb	4	96	26
XSmad5	87	13	39
XSmad5/dkk1	6	94	35
bFGF	91	9	44
bFGF/*dkk1*	11	89	45
bFGF/*frzb*	5	95	21
mWnt3A	95	5	44
mWnt3A/dkk1	8	92	26
mWnt3A/frzb	95	5	19

n, number of embryos injected.

The fact that *dkk1* and *frzb* rescue BMP- and FGF-treated embryos raised the question of whether signalling of these growth factors may directly require WNT signalling. To test this we carried out animal cap assays and studied the induction of FGF and BMP target genes between blastula and gastrula stages by RT-PCR. Induction of the FGF target gene *Xbra* is known to be a direct response, which is not blocked by cycloheximide (Smith et al., 1991). Neither *dkk1* nor *frzb* interfere with *Xbra* induction by bFGF (Fig. 8A). This indicates that direct FGF signalling is unaffected by these

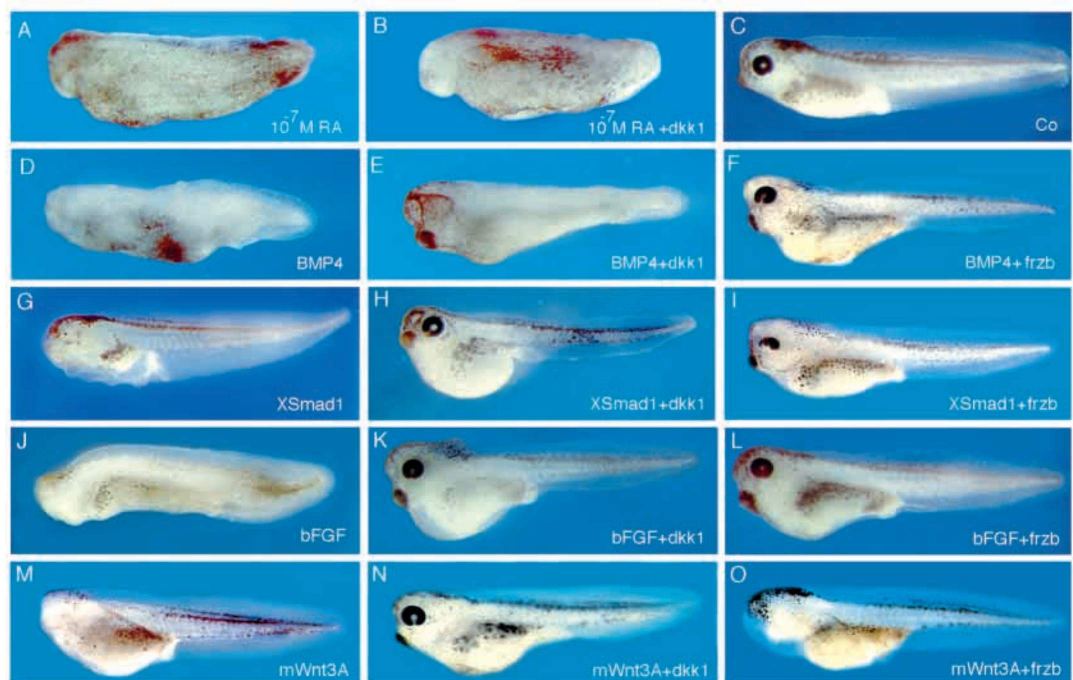

Fig. 7. *dkk1* rescues embryos posteriorized by bFGF, *BMP4*, *XSmad1* and *Wnt3A*, but not by RA. Where indicated, embryos were injected radially at the four-cell stage with *dkk1* or *frzb* mRNA. (A,B) Embryos treated from stages 8-13 with 10^{-7}M retinoic acid (RA). (D-F) Embryos were injected radially at the four-cell stage with *BMP4* or (G-I) *XSmad1* mRNA. (J-L) Embryos were injected radially at the four-cell stage with *dkk1* or *frzb* mRNA and at the blastula stage with bFGF protein into the blastocoel. (M-O) Embryos were injected animally at the four-cell stage with *mWnt3A* plasmid DNA. (C) Uninjected control.

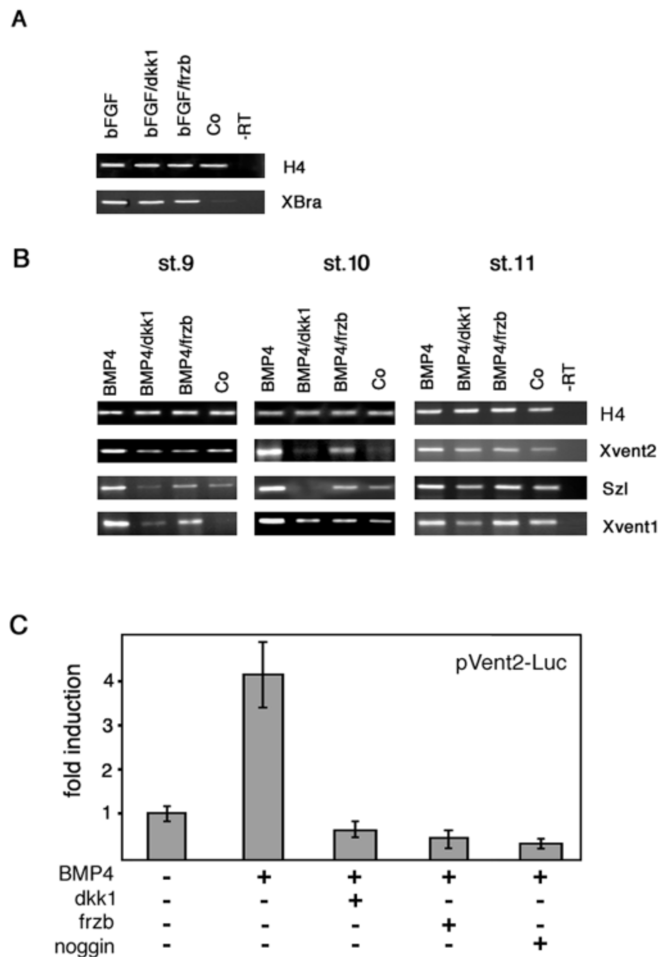

Fig. 8. Signalling of BMP but not FGF is sensitive to WNT inhibitors. (A) *Xbra* induction by bFGF is insensitive to WNT inhibitors. RT-PCR analysis of *Xbra* expression in animal caps. Embryos were uninjected (Co) or injected animally at the four-cell stage with *dkk1* or *frzb* mRNA. Animal caps were cut at stage 8, cultivated with or without bFGF and analysed for induction of *Xbra* at stage 10.5. (B) Induction of BMP target genes shows a transient requirement for WNT signalling. RT-PCR analysis of *Xvent2*, *szl* and *Xvent1* expression in animal caps. Embryos were injected animally at the four-cell stage with *BMP4* mRNA or *BMP4* plus *dkk1* or *frzb* mRNAs as indicated. Animal caps were cut at stage 8 and analysed for induction of mesodermal markers at stages 9, 10, and 11. –RT, minus reverse transcription control samples; H4, histone H4 for normalisation. (C) Induction of the *Xvent2* promoter by *BMP4* requires WNT signalling. Embryos were coinjected animally at the four-cell stage with the BMP-responsive reporter plasmid pVent2-Luc and either *preprolactin* mRNA (control, first column), or *BMP4*, *dkk1*, *frzb* or *noggin* mRNAs as indicated. At stage 10, luciferase assays were carried out with extracts of whole embryos in triplicate samples.

WNT inhibitors and that WNT-dependent posteriorisation by FGF occurs indirectly, e.g. by secondary induction of WNTs.

Surprisingly, in the analogous experiment with *BMP4*-injected animal caps, induction of all tested target genes, *Xvent2* (Onichtchouk et al., 1996), *sizzled* (Salic et al., 1997) and *Xvent1* (Gawantka et al., 1995) is sensitive to *dkk1* and *frzb* (Fig. 8B). *Xvent2* is an immediate early response gene to BMP signalling (Ladher et al., 1996; Rastegar et al., 1999) and its promoter contains a well-characterized BMP response element (Hata et al., 2000; Henningfeld et al., 2000). *BMP4*-induced activation of a luciferase reporter construct containing this promoter fragment (Candia et al., 1997) is also sensitive to *dkk1* and *frzb* in stage-10 embryos (Fig. 8C). However, while *dkk1* and *frzb* inhibit induction of BMP targets at blastula and early gastrula stage, the requirement for WNT signalling fades by midgastrula (stage 11; Fig. 8B). The results indicate that BMP, unlike FGF signalling, requires endogenous WNT signalling between the blastula and early gastrula stages.

DISCUSSION

dkk1 is able to induce entire heads when coexpressed with BMP inhibitors and injection of inhibitory antibodies leads to microcephaly in *Xenopus* embryos. These results, together with the expression in the anterior endomesoderm, indicate that *dkk1* plays an important role in head induction. However, these findings raise questions regarding the role of *dkk1* in different germ layers, the timing of its action and the pathways with which it interacts. Here we present a detailed analysis of the requirement for *dkk1* during axial patterning. Our study provides compelling evidence for a physiological role of WNT signalling in neural and mesendodermal regionalization and characterizes the epistatic interactions of WNT with respect to BMP and FGF signalling during this process.

dkk1 and anterior endomesoderm formation

Our results indicate that *dkk1* is necessary and sufficient to promote formation of the anterior endomesoderm. Specifically, we observe that prechordal plate markers such as *XBlimp1*, *gsc*, and *XHex* are reduced following antiDkk1 Ab injection. In addition, the anterior expression of *XHex*, labeling the anterior endoderm, is also reduced. These genes are superinduced and expanded following *dkk1* overexpression but they are not induced ectopically, e.g. in ventral mesoderm or posterior chordamesoderm. This indicates that other factors in addition to WNT inhibitors are required for prechordal plate formation, most likely BMP inhibitors, since coexpression of *dkk1* with BMP inhibitors can induce prechordal plate markers ectopically in ventral mesoderm (Glinka et al., 1998). Notochord formation does not appear to require *dkk1*, even though *dkk1* overexpression expands notochord territory (this study) and rescues notochord in *boz* mutants (Hashimoto et al., 2000). While previous studies suggested that WNTs are able to ventroposteriorize dorsal mesoderm (Christian and Moon, 1993; Hoppler et al., 1996; Fredieu et al., 1997) and that simultaneous BMP and WNT inhibition is sufficient to convert mesoderm into prechordal plate (Glinka et al., 1997, 1998; Piccolo et al., 1999), our data provide the first evidence that WNT inhibition (via *dkk1*) is indeed required for this process in vivo.

The induction of bilateral eyes by *dkk1* but not by *frzb* can be explained by the ability of the former to promote proper prechordal plate formation. While *frzb* also induces prechordal plate markers such as *gsc*, this induced tissue never reaches an anteroventral position. We also note that *frzb*, unlike *dkk1*, is unable to induce ectopic *XHex* expression when coinjected with tBR (data not shown). This difference in prechordal plate specification suggests that endomesoderm is antagonized by

more than one WNT and that *dkk1* and *frzb* do not inhibit the same set of WNTs. This is supported by the inability of *frzb* to rescue antiDkk1 Ab-injected embryos. Indeed, we show that Wnt3a, which is required for posterior development in mice (Takada et al., 1994), is inhibited by *dkk1* but not by *frzb* (Wang et al., 1997b). Interestingly, *bhh* mRNA injection was also insufficient to rescue cyclopic embryos following antiDkk1 Ab injection. This indicates that there are other ventral midline signals in addition to Hedgehog acting downstream of *dkk1* that are required for splitting of the eye field.

Xenopus dkk1 is expressed in the deep endomesoderm of the gastrula, including both the prospective prechordal plate and anterior endoderm, and becomes confined to the prechordal plate in neurulae. In zebrafish and mouse, *dkk1* is initially expressed in extraembryonic endoderm, the yolk syncytial layer and the anterior visceral endoderm (AVE), respectively, and later becomes confined to the prechordal plate (Glinka et al., 1998; Pearce et al., 1999; Hashimoto et al., 2000). It will be interesting to dissect the respective roles of *dkk1* in these two tissues.

dkk1 and anterior neural patterning

Our data indicate that *dkk1* is required for patterning of the entire anterior neural plate since both a-p as well as d-v markers are affected in Ab-injected embryos. *dkk1* acts by anteriorizing the neural plate from midbrain onwards and by ventralizing it. This is consistent with the observation that WNTs not only posteriorize but also dorsalize neuroectoderm, promoting neural crest formation (Saint-Jeannet et al., 1997; Chang and Hemmati-Brivanlou, 1998; Dorsky et al., 1998; LaBonne and Bronner-Fraser, 1998). Thus, WNTs and their antagonists such as Dkk1 integrate the patterning of both neural axes. An important question is whether *dkk1* patterns neuroectoderm only indirectly via its promoting endomesoderm formation or whether neuroectodermal cells directly respond to *dkk1*. Hashimoto et al. (2000) carried out an elegant experiment where they prevented anterior endomesoderm formation by *antivin* mRNA injection and asked whether *dkk1* could still anteriorize neural plate. Indeed, in these embryos *dkk1* anteriorized the neuroectoderm but could not rescue endomesoderm, indicating that ectodermal cells can respond directly to *dkk1*. Further evidence for a direct action of *dkk1* on neuroectoderm is the observation that *Xenopus* animal caps, which are of purely ectodermal origin, express anterior neural markers in response to *dkk1* mRNA injection (Glinka et al., 1998). Thus, *dkk1* directly affects both neuroectoderm and endomesoderm.

The requirement for *dkk1* both in prechordal plate and AP neural patterning argues against the notion that the prechordal plate is only involved in DV and not AP neural patterning. This was inferred from studying *oep* mutant embryos, which are cyclopic but contain forebrain structures, and where a mature prechordal plate does not form (Schier et al., 1997). The suggestion that prechordal plate only promotes ventral midline signalling and not anteriorization also conflicts with the findings that extirpation of anterior endoderm only affects heart induction while extirpating prospective prechordal plate deletes head structures (Schneider and Mercola, 1999). Furthermore, the prechordal plate is a potent neural-anteriorizing tissue (Mangold, 1933; Bradley et al., 1996; Foley et al., 1997; Pera and Kessel, 1997; Zoltewicz and Gerhart, 1997). Close inspection of *oep* mutant embryos reveals a reduction of forebrain markers (Grinblat et al., 1998) and furthermore, prechordal plate markers are still expressed in *oep* mutant embryos during early gastrulation (Strähle et al., 1997).

The requirement for *dkk1* in neural patterning supports the two-inhibitor model, which proposes that head formation involves simultaneous inhibition of BMP and WNT signals (Glinka et al., 1997; Niehrs, 1999). A requirement for BMP inhibition in head formation has recently been demonstrated by the finding that *noggin*/*chordin* double-homozygous mutant mice show severe forebrain defects and cyclopia (Bachiller et al., 2000). This is consistent with the notion that a BMP-dependent gradient of positional information is present across the entire gastrula ectoderm (Knecht et al., 1995; Wilson et al., 1997; Barth et al., 1999).

WNTs as posteriorizing signals

Classical experiments by Nieuwkoop, Saxen and Toivonen predicted that posteriorizing signals act during gastrulation to repattern an initially anterior neural induced state (Gilbert and Saxen, 1993; Nieuwkoop, 1997). A number of candidates for such signals have since been proposed, including WNTs (reviewed in Niehrs, 1999), FGFs (reviewed in Slack, 1994) BMPs (Dale et al., 1992; Jones et al., 1992) and RA (e.g. Durston et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991a,b; Sharpe, 1991; reviewed in Sasai and De Robertis, 1997). The requirement for *dkk1* in anterior neural patterning provides compelling evidence for a physiological role of WNTs in posteriorization. Our data indicate that WNTs that antagonize head formation and interact with *dkk1* are active until late gastrula. That neural patterning may mostly occur prior to neurulation is consistent with previous studies showing patterned expression of neural markers during early gastrulation (Gawantka et al., 1998; Grinblat et al., 1998). Various WNTs are expressed in tissues relevant to neural patterning. *Xwnt8* is expressed in the lateroventral mesoderm and is a prime candidate for inhibiting notochord and prechordal plate fates (Smith and Harland, 1991). *Xwnt1*, *-3A* (Wolda et al., 1993) and *-7B* (Chang and Hemmati-Brivanlou, 1998) are predominantly expressed in gastrula ectoderm and/or neurula posterior neuroectoderm, suggesting that they are responsible for posteriorizing neural fates. They may act in either vertical (Nieuwkoop, 1997; Poznanski and Keller, 1997; Chen et al., 2000) and/or planar type signalling (Ruiz i Altaba, 1993, 1998) to inhibit rostral and induce more caudal gene expression.

The results show that the posteriorizing action of FGFs may be due to their acting through WNTs because they can be rescued by *dkk1* and *frzb*. Similarly, the requirement for WNT signaling during neural crest induction has been shown to be direct, whereas FGF-mediated neural crest induction may be indirect and mediated by WNT signals (LaBonne and Bronner-Fraser, 1998). Furthermore, the posteriorizing action of FGF in Noggin-treated animal caps is dependent on endogenous WNT signalling (McGrew et al., 1997). Finally, experiments with a dominant negative *FGF receptor1* argued against a posteriorizing role for FGF in early neural patterning (Kroll and Amaya, 1996). Thus, the active principle behind FGF-mediated posteriorization appears to be induced WNT signalling.

That BMPs antagonize anterior neural cell fates and head formation has long been known (Dale et al., 1992; Jones et al., 1992) and BMP inhibitors are indeed required for head formation since *Chordin/noggin* double homozygous mutant mice lose forebrain structures and are cyclopic (Bachiller et al., 2000). What is the relationship between early BMP- and WNT signalling? We show that *BMP4*-posteriorized embryos are rescued by *dkk1*. Furthermore, our results reveal that there is cross-talk between BMP and WNT signalling pathways until the early gastrula stage since induction of the immediate BMP target gene *Xvent2* is sensitive to WNT inhibitors. A similar cross-talk between WNT and TGF-β pathways during gastrula induction of *Xtwn* expression has been demonstrated to be due to direct binding of Smad4 to Lef1 (Nishita et al., 2000). However, the WNT/BMP cross-talk appears to be transient since at stage 10.5 (Marom et al., 1999) or 11 (this work), BMP-induced target gene induction becomes independent of WNT antagonists. Yet, the relationship between BMP and WNT signalling is further complicated by the positive regulation of *Xwnt8* expression by *BMP4* after MBT (Marom et al., 1999). Furthermore, before MBT the WNT/β-catenin signalling pathway of the Nieuwkoop center appears to repress *BMP4* expression (Baker et al., 1999). Thus, at least three interactions between BMP and WNT signals in early *Xenopus* can be distinguished. First, WNT signalling represses BMP4 in the early organiser (Baker et al., 1999). Second, in regions where BMP4 signalling is activated, it depends on concomitant WNT signalling between blastula and early gastrula stages. There is evidence for unidentified ventralizing WNTs that may be involved in this process (Itoh and Sokol, 1999; Kühl et al., 2000). Third, by midgastrula direct BMP4 signalling becomes independent of WNT signalling and is required for *Xwnt8* expression (Marom et al., 1999). With regard to the coinhibition of head induction by WNTs and BMPs, we favor a model where these pathways synergize during the latter phase to antagonize head formation. This synergy may either occur at the level of common or distinct target genes specifying rostral fates.

Unlike bFGF and *BMP4*, RA induces embryonic microcephaly in a fashion which cannot be rescued by *dkk1*. This indicates an independent posteriorizing pathway from that of WNTs. This is also supported by the way that RA and antiDkk1 Ab influence neural patterning. Both affect AP (e.g. Durston et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991a,b; Sharpe, 1991; reviewed in Sasai and De Robertis, 1997) and DV patterning (Franco et al., 1999; this work) and induce microcephaly, but in RA-treated embryos neural structures including the midbrain are reduced while in antiDkk1 Ab-injected embryos the midbrain is slightly increased. Furthermore, loss-of-function studies in *Xenopus* and mouse indicated that retinoid signalling is required for patterning neural tissue posterior of the hindbrain (Blumberg et al., 1997; Dupe et al., 1999; Niederreither et al., 1999; Sharpe and Goldstone, 2000). This raises the possibility that WNTs pattern the neural plate between forebrain and midbrain and RA between hindbrain and spinal cord.

We are greatful to A. Brändli, S. Ekker, P. Krieg, M. Pannese, N. Pollet, H. Steinbeisser and A. Zaraisky for reagents. We thank M. Brand and T. Bouwmeester for critical reading of the manuscript.

REFERENCES

Adelmann, H. B. (1936a). The problem of cyclopia. Pt. I. *Quart. Rev. Biol.* **11**, 161-182.

Adelmann, H. B. (1936b). The problem of cyclopia. Pt. II. *Quart. Rev. Biol.* **11**, 284-304.

Bachiller, D., Klingensmith, J., Kemp, C., Belo, J. A., Anderson, R. M., May, S. R., McMahon, J. A., McMahon, A. P., Harland, R. M., Rossant, J. and De Robertis, E. M. (2000). The organizer factors Chordin and Noggin are required for mouse forebrain development. *Nature* **403**, 658-661.

Baker, J. C., Beddington, R. S. and Harland, R. M. (1999). Wnt signaling in Xenopus embryos inhibits bmp4 expression and activates neural development. *Genes Dev.* **13**, 3149-3159.

Barth, K. A., Kishimoto, Y., Rohr, K. B., Seydler, C., Schulte-Merker, S. and Wilson, S. W. (1999). Bmp activity establishes a gradient of positional information throughout the entire neural plate. *Development* **126**, 4977-4987.

Blitz, I. L. and Cho, K. W. Y. (1995). Anterior neuroectoderm is progressively induced during gastrulation: the role of the Xenopus homeobox gene orthodenticle. *Development* **121**, 993-1004.

Blumberg, B., Bolado, J. J., Moreno, T. A., Kintner, C., Evans, R. M. and Papalopulu, N. (1997). An essential role for retinoid signaling in anteroposterior neural patterning. *Development* **124**, 373-379.

Bourguignon, C., Li, J. and Papalopulu, N. (1998). XBF-1, a winged helix transcription factor with dual activity, has a role in positioning neurogenesis in *Xenopus* competent ectoderm. *Development* **125**, 4889-4900.

Bouwmeester, T., Kim, S.-H., Sasai, Y., Lu, B. and De Robertis, E. M. (1996). Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature* **382**, 595-601.

Bradley, L., Wainstock, D. and Sive, H. (1996). Positive and negative signals modulate formation of the *Xenopus* cement gland. *Development* **122**, 2739-2750.

Bradley, L. C., Snape, A., Bhatt, S. and Wilkinson, D. G. (1993). The structure and expression of the Xenopus Krox-20 gene: conserved and divergent patterns of expression in rhombomeres and neural crest. *Mech. Dev.* **40**, 73-84.

Candia, A. F., Watabe, T., Hawley, H. B., Onichtchouk, D., Zhang, Y., Derynck, R., Niehrs, C. and Cho, K. W. Y. (1997). Cellular interpretation of multiple TGF-beta signals: intracellular antagonism between activin/BVg1 and BMP-2/4 signaling mediated by Smads. *Development* **124**, 4467-4480.

Chang, C. and Hemmati-Brivanlou, A. (1998). Neural crest induction by Xwnt7B in Xenopus. *Dev. Biol.* **194**, 129-134.

Chen, Y., Hollemann, T., Pieler, T. and Grunz, H. (2000). Planar signalling is not sufficient to generate a specific anterior/posterior neural pattern in pseudoexogastrula explants from Xenopus and Triturus. *Mech. Dev.* **90**, 53-63.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383**, 407-413.

Cho, K. W., Blumberg, B., Steinbeisser, H. and De Robertis, E. M. (1991). Molecular nature of Spemann's organizer: the role of the Xenopus homeobox gene goosecoid. *Cell* **67**, 1111-1120.

Christian, J. L. and Moon, R. T. (1993). Interactions between Xwnt-8 and Spemann organizer signaling pathways generate dorsoventral pattern in the embryonic mesoderm of Xenopus. *Genes Dev.* **7**, 13-28.

Dale, L., Howes, G., Price, B. M. and Smith, J. C. (1992). Bone morphogenetic protein 4: a ventralizing factor in early Xenopus development. *Development* **115**, 573-585.

de Souza, F. S. J., Gawantka, V., Gomez, A. P., Delius, H., Ang, S.-L. and Niehrs, C. (1999). The zinc finger gene Xblimp1 controls anterior endomesodermal cell fate in Spemann's organizer. *EMBO J.* **18**, 6062-6072.

Dorsky, R. I., Moon, R. T. and Raible, D. W. (1998). Control of neural crest cell fate by the Wnt signalling pathway. *Nature* **396**, 370-373.

Dupe, V., Ghyselinck, N. B., Wendling, O., Chambon, P. and Mark, M. (1999). Key roles of retinoic acid receptors alpha and beta in the patterning of the caudal hindbrain, pharyngeal arches and otocyst in the mouse. *Development* **126**, 5051-5059.

Durston, A. J., Timmermans, J. P., Hage, W. J., Hendriks, H. F., de Vries, N. J., Heideveld, M. and Nieuwkoop, P. D. (1989). Retinoic acid causes an anteroposterior transformation in the developing central nervous system. *Nature* **340**, 140-144.

Ekker, S. C., McGrew, L. L., Lai, C. J., Lee, J. J., von, K. D., Moon, R. T. and Beachy, P. A. (1995a). Distinct expression and shared activities of

members of the hedgehog gene family of *Xenopus laevis*. *Development* **121**, 2337-2347.

Ekker, S. C., Ungar, A. R., Greenstein, P., von, K. D., Porter, J. A., Moon, R. T. and Beachy, P. A. (1995b). Patterning activities of vertebrate hedgehog proteins in the developing eye and brain. *Curr. Biol.* **5**, 944-955.

Fedi, P., Bafico, A., Nieto Soria, A., Burgess, W. H., Miki, T., Bottaro, D. P., Kraus, M. H. and Aaronson, S. A. (1999). Isolation and biochemical characterization of the human Dkk-1 homologue, a novel inhibitor of mammalian Wnt signaling. *J. Biol. Chem.* **274**, 19465-19472.

Foley, A. C., Storey, K. G. and Stern, C. D. (1997). The prechordal region lacks neural inducing ability, but can confer anterior character to more posterior neuroepithelium. *Development* **124**, 298329-96.

Franco, P. G., Paganelli, A. R., Lopez, S. L. and Carrasco, A. E. (1999). Functional association of retinoic acid and hedgehog signaling in *Xenopus* primary neurogenesis. *Development* **126**, 4257-4265.

Fredieu, J. R., Cui, Y., Maier, D., Danilchik, M. V. and Christian, J. L. (1997). Xwnt-8 and lithium can act upon either dorsal mesodermal or neuroectodermal cells to cause a loss of forebrain in Xenopus embryos. *Dev. Biol.* **186**, 100-114.

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

Gawantka, V., Pollet, N., Delius, H., Pfister, R., Vingron, M., Nitsch, R., Blumenstock, C. and Niehrs, C. (1998). Gene expression screening in Xenopus identifies molecular pathways, predicts gene function and provides a global view of embryonic patterning. *Mech. Dev.* **77**, 95-141.

Gilbert, S. F. and Saxen, L. (1993). Spemann's organizer: models and molecules. *Mech. Dev.* **41**, 73-89.

Glinka, A., Delius, H., Blumenstock, C. and Niehrs, C. (1996). Combinatorial signalling by Xwnt-11 and Xnr3 in the organizer epithelium. *Mech. Dev.* **60**, 221-231.

Glinka, A., Wu, W., Delius, H., Monaghan, P. A., Blumenstock, C. and Niehrs, C. (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature* **391**, 357-362.

Glinka, A., Wu, W., Onichtchouk, D., Blumenstock, C. and Niehrs, C. (1997). Head induction by simultaneous repression of Bmp and wnt signalling in Xenopus. *Nature* **389**, 517-519.

Gont, L. K., Steinbeisser, H., Blumberg, B. and De Robertis, E. M. (1993). Tail formation as a continuation of gastrulation: the multiple cell populations of the Xenopus tailbud derive from the late blastopore lip. *Development* **119**, 991-1004.

Grinblat, Y., Gamse, J., Patel, M. and Sive, H. (1998). Determination of the zebrafish forebrain: induction and patterning. *Development* **125**, 4403-4416.

Harland, R. M. (1991). In situ hybridization: an improved whole-mount method for Xenopus embryos. *Methods Cell Biol.* **36**, 685-695.

Harland, R. M. and Gerhart, J. (1997). Formation and function of Spemann's organizer. *Annu. Rev. Dev. Biol.* **13**, 611-667.

Hashimoto, H., Itoh, M., Yamanaka, Y., Yamashita, S., Shimizu, T., Solnica-Krezel, L., Hibi, M. and Hirano, T. (2000). Zebrafish Dkk1 Functions in Forebrain Specification and Axial Mesendoderm Formation. *Dev. Biol.* **217**, 138-152.

Hata, A., Seoane, J., Lagna, G., Montalvo, E., Hemmati-Brivanlou, A. and Massague, J. (2000). OAZ uses distinct DNA- and protein-binding zinc fingers in separate BMP- Smad and Olf signaling pathways. *Cell* **100**, 229-240.

Hemmati-Brivanlou, A., de, l. T. J., Holt, C. and Harland, R. M. (1991). Cephalic expression and molecular characterization of Xenopus En-2. *Development* **111**, 715-724.

Hemmati-Brivanlou, A., Kelly, O. G. and Melton, D. A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell* **77**, 283-295.

Henningfeld, K. A., Rastegar, S., Adler, G. and Knochel, W. (2000). Smad1 and Smad4 are Components of the BMP-4 Induced Transcription Complex of the Xvent-2B Promoter. *J. Biol. Chem.* **275**, 21827-21835.

Hensey, C. and Gautier, J. (1998). Programmed cell death during Xenopus development: a spatio-temporal analysis. *Dev. Biol.* **203**, 36-48.

Hollemann, T., Panitz, F. and Pieler, T. (1998). *In Situ Hybridization Techniques with* Xenopus *Embryos*, pp.279-290. Oxford University Press.

Hoppler, S., Brown, J. D. and Moon, R. T. (1996). Expression of a dominant-negative wnt blocks induction of MyoD in Xenopus embryos. *Genes Dev.* **10**, 2805-2817.

Hsieh, J. C., Kodjabachian, L., Rebbert, M. L., Rattner, A., Smallwood, P. M., Samos, C. H., Nusse, R., Dawid, I. B. and Nathans, J. (1999). A new secreted protein that binds to Wnt proteins and inhibits their activities. *Nature* **398**, 431-436.

Hsu, D. R., Economides, A. N., Wang, X., Eimon, P. M. and Harland, R. M. (1998). The Xenopus dorsalizing factor gremlin identifies a novel family of secreted proteins that antagonize BMP activities. *Mol. Cell* **1**, 673-683.

Iemura, S.-I., Yamamoto, T., Takagi, C., Uchiyama, H., Natsume, T., Shimasaki, S., Sugino, H. and Ueno, N. (1998). Direct binding of follistatin to a complex of bone morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early Xenopus embryos. *Proc. Natl. Acad. Sci. USA* **95**, 9337-9342.

Itoh, K. and Sokol, S. Y. (1999). Axis determination by inhibition of Wnt signaling in Xenopus. *Genes Dev.* **13**, 2328-2336.

Jones, C. M., Lyons, K. M., Lapan, P. M., Wright, C. V. and Hogan, B. L. (1992). DVR-4 (bone morphogenetic protein-4) as a posterior-ventralizing factor in *Xenopus* mesoderm induction. *Development* **115**, 639-647.

Knecht, A. K., Good, P. J., Dawid, I. B. and Harland, R. M. (1995). Dorsal-ventral patterning and differentiation of noggin-induced neural tissue in the absence of mesoderm. *Development* **121**, 1927-1235.

Kroll, K. L. and Amaya, E. (1996). Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development* **122**, 3173-3183.

Krupnik, V. E., Sharp, J. D., Jiang, C., Robison, K., Chickering, T. W., Amaravadi, L., Brown, D. E., Guyot, D., Mays, G., Leiby, K., Chang, B., Duong, T., Goodearl, A. D., Gearing, D. P., Sokol, S. Y. and McCarthy, S. A. (1999). Functional and structural diversity of the human Dickkopf gene family. *Gene* **238**, 301-313.

Kühl, M., Sheldahl, L. C., Malbon, C. C. and Moon, R. T. (2000). Ca^{2+}/calmodulin-dependent protein kinase II is stimulated by Wnt and Frizzled homologs and promotes ventral cell fates in Xenopus. *J. Biol. Chem.* **275**, 12701-12711.

LaBonne, C. and Bronner-Fraser, M. (1998). Neural crest induction in *Xenopus*: evidence for a two-signal model. *Development* **125**, 2403-2414.

Ladher, R., Mohun, T. J., Smith, J. C. and Snape, A. M. (1996). *Xom*: a *Xenopus* homeobox gene that mediates the early effects of BMP-4. *Development* **122**, 2385-2394.

Leyns, L., Bouwmeester, T., Kim, S.-H., Piccolo, S. and De Robertis, E. M. (1997). Frzb-1 is a secreted antagonist of wnt-signals expressed in the Spemann organizer. *Cell* **88**, 747-756.

Li, H., Tierney, C., Wen, L., Wu, J. Y. and Rao, Y. (1997). A single morphogenetic field gives rise to two retina primordia under the influence of the prechordal plate. *Development* **124**, 603-615.

Macdonald, R., Barth, K. A., Xu, Q., Holder, N., Mikkola, I. and Wilson, S. W. (1995). Midline signalling is required for *Pax* gene regulation and patterning of the eyes. *Development* **121**, 3267-3278.

Mangold, O. (1933). Über die Induktionsfähigkeit der verschiedenen Bezirke der Neurula von Urodelen. *Naturwissenschaften* **21**, 761-766.

Marom, K., Fainsod, A. and Steinbeisser, H. (1999). Patterning of the mesoderm involves several threshold responses to BMP- 4 and xwnt-8. *Mech. Dev.* **87**, 33-44.

McGrew, L. L., Hoppler, S. and Moon, R. T. (1997). Wnt and FGF pathways cooperatively pattern anteroposterior neural ectoderm in Xenopus. *Mech. Dev.* **69**, 105-114.

Meersman, G., Verschueren, K., Nelles, L., Blumenstock, C., Kraft, H., Wuytens, G., Remacle, J., Kozak, C. A., Tylzanowsky, P., Niehrs, C. and Huylebroeck, D. (1997). The C-terminal domain of Mad-like signal transducers is sufficient for biological activity in vivo and transcriptional activation. *Mech. Dev.* **61**, 127-140.

Moon, R. T., Brown, J. D., Yang-Snyder, J. A. and Miller, J. R. (1997). Structurally related receptors and antagonists compete for secreted wnt ligands. *Cell* **88**, 725-728.

Newman, C. S., Chia, F. and Krieg, P. A. (1997). The XHex homeobox gene is expressed during development of the vascular endothelium: overexpression leads to an increase in vascular endothelial cell number. *Mech. Dev.* **66**, 83-93.

Niederreither, K., Subbarayan, V., Dolle, P. and Chambon, P. (1999). Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nat. Genet.* **21**, 444-448.

Niehrs, C. (1999). Head in the Wnt - the molecular nature of Spemann's head organizer. *Trends Genet.* **15**, 314-319.

Nieuwkoop, P. D. (1997). Short historical survey of pattern formation in the endo-mesoderm and the neural anlage in the vertebrates: the role of vertical and planar inductive actions. *Cell. Mol. Life Sci.* **53**, 305-318.

Nishita, M., Hashimoto, M. K., Ogata, S., Laurent, M. N., Ueno, N., Shibuya, H. and Cho, K. W. (2000). Interaction between Wnt and TGF-

beta signalling pathways during formation of Spemann's organizer. *Nature* **403**, 781-785.

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

Pannese, M., Polo, C., Andreazzoli, M., Vignali, R., Kablar, B., Barsacchi, G. and Boncinelli, E. (1995). The *Xenopus* homologue of Otx2 is a maternal homeobox gene that demarcates and specifies anterior body regions. *Development* **121**, 707-720.

Pearce, J. J. H., Penny, G. and Rossant, J. (1999). A mouse cerberus/dan-related gene family. *Dev. Biol.* **209**, 98-100.

Pera, E. M. and Kessel, M. (1997). Patterning of the chick forebrain anlage by the prechordal plate. *Development* **124**, 41534162.

Peng, H. B. (1991). *Xenopus laevis*: Practical uses in cell and molecular biology. Solutions and protocols. *Methods Cell. Biol.* **36**, 659.

Piccolo, S., Agius, E., Leyns, L., Bhattacharyya, S., Grunz, H., Bouwmeester, T. and De Robertis, E. M. (1999). The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. *Nature* **397**, 707-710.

Piccolo, S., Sasai, Y., Lu, B. and De Robertis, E. M. (1996). Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell* **86**, 589-598.

Poznanski, A. and Keller, R. (1997). The role of planar and early vertical signaling in patterning the expression of Hoxb-1 in Xenopus. *Dev. Biol.* **184**, 351-366.

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

Roelink, H. and Nusse, R. (1991). Expression of two members of the Wnt family during mouse development – restricted temporal and spatial patterns in the developing neural tube. *Genes Dev.* **5**, 381-388.

Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., Ruiz i Altaba, A., Tanabe, Y., Placzek, M., Edlund, T., Jessell, T. M. et al. (1994). Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord. *Cell* **76**, 761-775.

Ruiz i Altaba, A. and Jessell, T. M. (1991a). Retinoic acid modifies mesodermal patterning in early Xenopus embryos. *Genes Dev.* **5**, 175-187.

Ruiz i Altaba, A. and Jessell, T. M. (1991b). Retinoic acid modifies the pattern of cell differentiation in the central nervous system of neurula stage *Xenopus* embryos. *Development* **112**, 945-958.

Ruiz i Altaba, A. (1993). Induction and axial patterning of the neural plate: planar and vertical signals. *J. Neurobiol.* **24**, 1276-1304.

Ruiz i Altaba, A. (1998). Neural patterning. Deconstructing the organizer. *Nature* **391**, 748-749.

Saint-Jeannet, J.-P., He, X., Varmus, H. and Dawid, I. B. (1997). Regulation of dorsal fate in the neuraxis by Wnt-1 and Wnt-3a. *Proc. Natl. Acad. Sci. USA* **94**, 13713-13718.

Salic, A. N., Kroll, K. L., Evans, L. M. and Kirschner, M. W. (1997). Sizzled: a secreted Xwnt8 antagonist expressed in the ventral marginal zone of *Xenopus* embryos. *Development* **124**, 4739-4748.

Sampath, K., Rubinstein, A. L., Cheng, A. M., Liang, J. O., Fekany, K., Solnica, K. L., Korzh, V., Halpern, M. E. and Wright, C. V. (1998). Induction of the zebrafish ventral brain and floorplate requires cyclops/nodal signalling. *Nature* **395**, 185-189.

Sasai, Y. and De Robertis, E. M. (1997). Ectodermal patterning in vertebrate embryos. *Dev. Biol.* **182**, 5-20.

Schier, A. F., Neuhauss, S. C., Harvey, M., Malicki, J., Solnica, K. L., Stainier, D. Y., Zwartkruis, F., Abdelilah, S., Stemple, D. L., Rangini, Z., Yang, H. and Driever, W. (1996). Mutations affecting the development of the embryonic zebrafish brain. *Development* **123**, 165-178.

Schier, A. F., Neuhauss, S. C., Helde, K. A., Talbot, W. S. and Driever, W. (1997). The one-eyed pinhead gene functions in mesoderm and endoderm formation in zebrafish and interacts with no tail. *Development* **124**, 327-342.

Schneider, V. A. and Mercola, M. (1999). Spatially distinct head and heart inducers within the Xenopus organizer region. *Curr. Biol.* **9**, 800-809.

Sharpe, C. and Goldstone, K. (2000). The control of Xenopus embryonic primary neurogenesis is mediated by retinoid signalling in the neurectoderm. *Mech. Dev.* **91**, 69-80.

Sharpe, C. R. (1991). Retinoic acid can mimic endogenous signals involved in transformation of the Xenopus nervous system. *Neuron* **7**, 239-247.

Sive, H. L., Draper, B. W., Harland, R. M. and Weintraub, H. (1990). Identification of a retinoic acid-sensitive period during primary axis formation in Xenopus laevis. *Genes Dev.* **4**, 932-942.

Slack, J. (1994). Role of fibroblast growth factors as inducing agents in early embryonic development. *Mol. Reprod. Dev.* **39**, 118-124.

Smith, J. C., Price, B. M., Green, J. B., Weigel, D. and Herrmann, B. G. (1991). Expression of a Xenopus homolog of Brachyury (T) is an immediate-early response to mesoderm induction. *Cell* **67**, 79-87.

Smith, W. C. and Harland, R. M. (1991). Injected Xwnt-8 RNA acts early in Xenopus embryos to promote formation of a vegetal dorsalizing center. *Cell* **67**, 753-765.

Strahle, U., Jesuthasan, S., Blader, P., Garcia, V. P., Hatta, K. and Ingham, P. W. (1997). *one-eyed pinhead* is required for development of the ventral midline of the zebrafish (*Danio rerio*) neural tube. *Genes Funct.* **1**, 131-148.

Suzuki, A., Chang, C., Yingling, J. M., Wang, X. F. and Hemmati-Brivanlou, A. (1997). Smad5 induces ventral fates in Xenopus embryo. *Dev. Biol*. **184**, 402-405.

Suzuki, A., Thies, R. S., Yamaji, N., Song, J. J., Wozney, J. M., Murakami, K. and Ueno, N. (1994). A truncated bone morphogenetic protein receptor affects dorsal-ventral patterning in the early Xenopus embryo. *Proc. Natl. Acad. Sci. USA* **91**, 10255-10259.

Takada, S., Stark, K. L., M.J., S., Vassileva, G. and McMahon, J. A. (1994). Wnt-3a regulates somite and tailbud formation in the mouse embryo. *Genes Dev.* **8**, 174-189.

Thisse, B., Wright, C. V. and Thisse, C. (2000). Activin- and Nodal-related factors control antero-posterior patterning of the zebrafish embryo. *Nature* **403**, 425-428.

Wang, S., Krinks, M., Lin, K., Luyten, F. P. and Moos, M. (1997a). Frzb, a secreted protein expressed in the Spemann organizer, binds and inhibits Wnt-8. *Cell* **88**, 757-766.

Wang, S., Krinks, M. and Moos, M. J. (1997b). Frzb-1, an antagonist of Wnt-1 and Wnt-8, does not block signaling by Wnts −3A, −5A, or −11. *Biochem. Biophys. Res. Commun.* **236**, 502-504.

Wilson, P. A., Lagna, G., Suzuki, A. and Hemmati-Brivanlou, A. (1997). Concentration-dependent patterning of the *Xenopus* ectoderm by BMP4 and its signal transducer Smad1. *Development* **124**, 3177-3184.

Wolda, S. L., Moody, C. J. and Moon, R. T. (1993). Overlapping expression of Xwnt-3A and Xwnt-1 in neural tissue of *Xenopus laevis* embryos. *Dev. Biol.* **155**, 46-57.

Zaraisky, A. G., Lukyanov, S. A., Vasiliev, O. L., Smirnov, Y. V., Belyavsky, A. V. and Kazanskaya, O. V. (1992). A novel homeobox gene expressed in the anterior neural plate of the Xenopus embryo. *Dev. Biol.* **152**, 373-382.

Zimmerman, L. B., De Jesús-Escobar, J.-E. and Harland, R. M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein-4. *Cell* **86**, 599-606.

Zoltewicz, J. S. and Gerhart, J. C. (1997). The Spemann organizer of Xenopus is patterned along its anteroposterior axis at the earliest gastrula stage. *Dev. Biol.* **192**, 482-491.