CORRIGENDUM

Kremen proteins interact with Dickkopf1 to regulate anteroposterior CNS patterning

Davidson, G., Mao, B., del Barco Barrantes, I. and Niehrs, C. *Development* **129**, 5587-5596.

In the reference list and text, one of the references was mis-spelled.

The correct reference is **Lekven**, A. C., Thorpe, C. J., Waxman, J. S. and Moon, R. T. (2001). Zebrafish wnt8 encodes two proteins on a bicistronic transcript and is required for mesoderm and neurectoderm patterning. *Dev. Cell* **1**, 103-114.

The authors apologise to readers for this mistake.

Development 129, 5587-5596

doi:10.1242/dev.00154

Kremen proteins interact with Dickkopf1 to regulate anteroposterior CNS patterning

Gary Davidson, Bingyu Mao, Ivan del Barco Barrantes and Christof Niehrs*

Division of Molecular Embryology, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany
*Author for correspondence (e-mail: niehrs@dkfz-heidelberg.de)

Accepted 9 September 2002

SUMMARY

A gradient of Wnt/β-catenin signalling formed by posteriorising Wnts and anteriorising Wnt antagonists regulates anteroposterior (AP) patterning of the central nervous system (CNS) during *Xenopus* gastrulation. In this process, the secreted Wnt antagonist Dkk1 functions in the Spemann organiser and its anterior derivatives by blocking Wnt receptors of the lipoprotein receptor-related protein (LRP) 5 and 6 class. In addition to LRP6, Dkk1 interacts with another recently identified receptor class, the transmembrane proteins Kremen1 (Krm1) and Kremen2 (Krm2) to synergistically inhibit LRP6. We have investigated the role of Krm1 and Krm2 during early *Xenopus* embryogenesis. Consistent with a role in zygotic Wnt inhibition, overexpressed Krm anteriorises embryos and rescues embryos posteriorised by Wnt8. Antisense morpholino oligonucleotide (Mo) knockdown of Krm1 and Krm2 leads to deficiency of anterior neural development. In this process, Krm proteins functionally interact with Dkk1: (1) in axis duplication assays *krm2* synergises with *dkk1* in inhibiting Wnt/LRP6 signalling; (2) *krm2* rescues microcephalic embryos induced by injection of inhibitory anti-Dkk1 antibodies; and (3) injection of *krm1/2* antisense Mo enhances microcephaly induced by inhibitory anti-Dkk1 antibodies. The results indicate that Krm proteins function in a Wnt inhibition pathway regulating early AP patterning of the CNS.

Key words: Anteroposterior patterning, Head induction, bf1, Wnt, Fz, Wnt/LRP signalling, Wnt/LRP inhibition, Dickkopf, Kremen, *Xenopus*

INTRODUCTION

During early patterning of the vertebrate central nervous system (CNS), neural inducers and modifiers establish a crude anteroposterior (AP) pattern before and during gastrulation that becomes refined at later stages (Shimamura et al., 1995; Lumsden and Krumlauf, 1996; Sasai and de Robertis, 1997; Chang and Hemmati-Brivanlou, 1998; Stern, 2001; Chapman et al., 2002). Instructive signalling through the FGF (Hongo et al., 1999; Shanmugalingam et al., 2000; Streit et al., 2000; Wilson et al., 2000; Shinya et al., 2001) and IGF (Pera et al., 2001; Richard-Parpaillon et al., 2002) pathways are involved in anterior neural specification. Furthermore, inhibition of BMP and nodal signalling is a prerequisite for anterior neural induction, and this antagonism is mediated by secreted anti-BMPs (noggin, chordin and follistatin) (Smith and Harland, 1992; Hemmati-Brivanlou et al., 1994; Sasai et al., 1994; Bachiller et al., 2000) (reviewed by Harland and Gerhart, 1997; Streit and Stern, 1999) and anti-nodals (antivin, Cerberus and lefty) (Fainsod et al., 1997; Meno et al., 1999; Piccolo et al., 1999; Thisse and Thisse, 1999; Thisse et al., 2000) (reviewed by Schier and Shen, 2000).These factors are emitted from the Spemann organiser of amphibia and its equivalents in other vertebrates. As for anterior CNS formation, posterior CNS formation also depends on BMP inhibition, but in addition requires posteriorising signals such as FGF, retinoic acid and Wnt (Gilbert and Saxen, 1993; Doniach and Musci, 1995; McGrew et al., 1995; Nieuwkoop, 1997; Sasai and de Robertis, 1997; Dupe and Lumsden, 2001).

We and others recently showed that a gradient of Wnt/β-catenin signalling regulates anteroposterior (AP) patterning of the entire neural plate during *Xenopus* gastrulation (Kiecker and Niehrs, 2001; Nordstrom et al., 2002). This gradient is high in posterior and low in anterior regions of the embryo, a likely consequence of Wnt and Wnt inhibitor expression domains being predominantly posterior and anterior, respectively. The anterior source of secreted Wnt antagonists is formed in anterior endomesoderm with the expression of *cerberus* (Bouwmeester et al., 1996), *sFRPs* (Leyns et al., 1997; Rattner et al., 1997; Wang et al., 1997) and *dkk1* (Glinka et al., 1998). Indeed, a distinguishing feature of organising centres involved in anterior neural induction in vertebrates is their expression of Wnt antagonists. Three lines of evidence support the theory that Wnt antagonism plays a central role in anterior specification: (1) co-expression of Wnt and BMP antagonists induces ectopic heads including anterior CNS while BMP antagonists alone induce only trunk structures (Glinka et al., 1997; Glinka et al., 1998); (2) overexpression of Wnt inhibitors in *Xenopus* and zebrafish embryos induces enlarged heads and forebrain (Itoh et al., 1995; Hoppler et al., 1996; Pierce and

Kimelman, 1996; Glinka et al., 1997; Leyns et al., 1997; Wang et al., 1997; Deardorff et al., 1998; Glinka et al., 1998; Hsieh et al., 1999; Fekany-Lee et al., 2000; Hashimoto et al., 2000; Heasman et al., 2000; Shinya et al., 2000); (3) in loss-of-function studies Wnt inhibitors were shown to be necessary for formation of anterior neural structures. Inactivation of the secreted Wnt antagonist Dickkopf1 (Dkk1) in *Xenopus* embryos using neutralising antibodies (Glinka et al., 1998; Kazanskaya et al., 2000) or targeted deletion of the *dkk1* gene in mouse (Mukhopadhyay et al., 2001), as well as inactivation of the intracellular Wnt pathway inhibitors *tcf3* and *axin1* in the zebrafish *headless* and *masterblind* mutants, respectively (Kim et al., 2000; Heisenberg et al., 2001; van de Water et al., 2001), all result in microcephalic embryos.

As regulation of Wnt/β-catenin signalling is crucial for AP neural patterning, it is important to understand the regulatory network interacting with Wnt antagonists, because it will have a bearing on the AP patterning process. We focus on the regulation of the Wnt antagonist Dickkopf1 (Dkk1), member of a multigene family of secreted glycoproteins with at least four different members in human (Glinka et al., 1998; Krupnik et al., 1999). *Dkk1* is expressed in the Spemann organiser and the presumptive prechordal plate and acts as a head inducer during vertebrate gastrulation (Glinka et al., 1998; Hashimoto et al., 2000; Kazanskaya et al., 2000; Shinya et al., 2000; Mukhopadhyay et al., 2001). The mechanism of Dkk1 action is unlike that of other extracellular Wnt inhibitors belonging to the sFRP (Leyns et al., 1997; Rattner et al., 1997; Wang et al., 1997), WIF (Hsieh et al., 1999) and Cerberus (Glinka et al., 1997; Piccolo et al., 1999) class, which directly bind and inactivate Wnt proteins. Dkk1 neither interacts with Wnts nor affects Wnt/Fz interactions. Instead, it binds as a high affinity antagonist to Wnt receptors of the lipoprotein receptor-related protein (LRP) 5 and 6 class (Bafico et al., 2001; Mao et al., 2001; Semenov et al., 2001). Owing to its mechanism of action (Wehrli et al., 2000), Dkk1 is a selective inhibitor of the Wnt/β-catenin pathway, and it does not affect the Wnt/planar cell polarity (PCP) pathway that drives convergent extension movements in *Xenopus* (Semenov et al., 2001).

In addition to LRPs, Dkk1 interacts with another recently identified receptor class, the transmembrane proteins Krm1 and Krm2, to synergistically inhibit LRP6 (Mao et al., 2002). Mouse *Krm1* (*Kremen*) had previously been identified as a differentially expressed gene without known function (Nakamura et al., 2001). Murine Krm proteins strongly cooperate with Dkk1 to inhibit Wnt signalling both in the mammalian cell line HEK 293 as well as in *Drosophila* wings, when expressed as heterologous transgenes. Upon binding to Dkk1, Krm proteins are recruited into a complex with LRP6, which leads to rapid endocytosis and removal of this Wnt receptor from the plasma membrane (Mao et al., 2002).

While this suggests that Krm proteins function in Dkk1-mediated Wnt inhibition, it is unknown what role these transmembrane receptors play physiologically, e.g. during embryogenesis. To investigate the physiological relevance of their interaction with Dkk1 and to study their role during embryogenesis, we have cloned and functionally characterised the *Xenopus* homologues of *krm1* and *krm2*. We show that Krm proteins functionally interact with Dkk1 during Wnt inhibition in *Xenopus* embryos and that they are required for formation of the anterior CNS.

MATERIALS AND METHODS

Embryos, explants, in situ hybridisation

In vitro fertilisation, embryo culture, staging, microinjection, culture of explants and whole-mount in situ hybridisation of *Xenopus* embryos were carried out as described (Gawantka et al., 1995). For vibratome sectioning, embryos were placed in embedding medium (0.4% gelatine, 30% albumin, 20% sucrose in PBS), and, after hardening in presence of 2% gluteraldehyde, sectioned using a VT100E vibratome (Leica). Whole-mount in situ hybridisation of mouse embryos was performed according to previously described procedures (Koop et al., 1996).

Cloning of *Xenopus* Kremens and constructs

Xenopus krm1 and *krm2* cDNA fragments were amplified by RT-PCR using degenerate oligos (forward, AATGGNGCNGAYTAYMGAGG; reverse, CCRCARAARCANGCRTAWCC) and mRNA from stage 18 *Xenopus* embryos. Two 400 bp *krm1* and *krm2* fragments were obtained and used as probes to obtain full-length *krm* cDNAs (Accession Number, AY150813). These were subcloned into pCS2+ to obtain pCS-*Xkrm1* and pCS-*Xkrm2*. C-terminal hemaglutinin-(HA) and V5-tagged pCS-*Xkrm1*HA and pCS-*Xkrm2*V5, as well as N-terminal V5-tagged pCS-V5*Xkrm2* were created by PCR.

Morpholino antisense oligonucleotides

The 5′ nucleotide sequences of additional (pseudo-) alleles for both *Xenopus krm1* and *krm2* genes were obtained using 5′ RACE (GeneRacer kit, Invitrogen). Based on these sequences, antisense oligonucleotides with optimal complementary to both alleles around the ATG start codon were designed: *krm2*, ACCACAGCATCTCCAC-CAACATTGT; *krm1*, TGAAATTGTCCAAATATCCATCACC.

RNA synthesis and western blot analysis

Preparation of mRNA for *Xenopus* injections was carried out using the MegaScript in vitro transcription kit (Ambion), according to manufacturer's instructions. For western blot immunological detection of tagged Krm proteins, either anti-hemagglutinin (HA) (1:10,000, Roche) or anti-V5 (1:10,000, Invitrogen) monoclonal antibodies were used. Chemiluminescence detection (SuperSignal® solution, Pierce) was carried out according to manufacturer's instructions after incubation of blots with anti-mouse IgG-HRP (1:10,000, Pierce).

RT-PCR

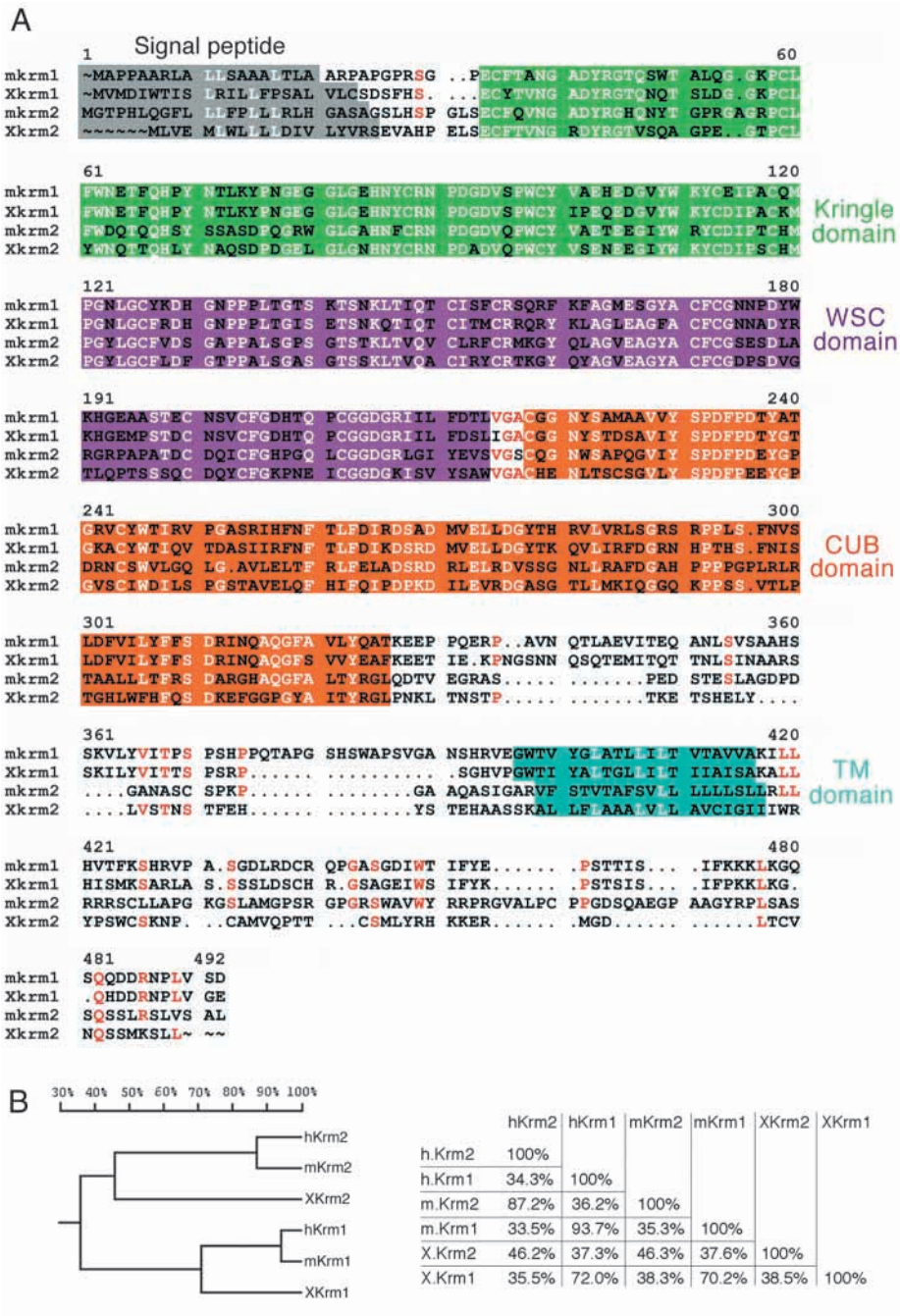
RT-PCR assays were carried out in the linear phase of amplification and with primers as described (Glinka et al., 1997). Other primers were: mouse *Krm1* (forward, GTGCTTCACAGCCAACGGTGCA; reverse, ACGTAGCACCAAGGGCTCACGT); mouse *Krm2* (forwards, AGGGAAACTGGTCGGCTC; reverse, AAGGCACG-GAGTAGGTTGC); *Xenopus krm1* (forward, CACTAGATGGTG-GGAAGCCTTGC; reverse, CCTCCAGCCCAGCTAGCTTGT); and *Xenopus krm2* (forward, CCCGACAATGTTGGTGGAGATGC; reverse, GGTGCCTACGTCTGATGGATCGC).

RESULTS

Isolation of *Xenopus kremen1* and *2*

Full-length cDNA clones of *Xenopus krm1* and *krm2* were isolated from stage 31 head cDNA libraries. The genes are predicted to encode proteins of 452 amino acids/50 kDa (*Xkrm1*) and 421 amino acids/46 kDa (*Xkrm2*). Like their mouse homologues, they contain an extracellular kringle-, WSC- and CUB domain, followed by a transmembrane domain and a cytoplasmic domain (Fig. 1A). Kringles are autonomous

structural domains, found predominantly in blood clotting and fibrinolytic proteins (Patthy et al., 1984; Castellino and McCance, 1997), as well as some other serine proteases (Kurosky et al., 1980; Thery and Stern, 1996; Gschwend et al., 1997). Kringle domains are believed to play a role in binding mediators such as membrane phospholipids (Church et al., 1991) and proteoglycans (Goretzki et al., 2000). WSC domains are present in yeast cell wall integrity and stress response component proteins (Lodder et al., 1999). The CUB domain is involved in protein-protein and glycosaminoglycan-protein interactions, and is found in a number of proteins involved in development and differentiation (Bork and Beckmann, 1993). Although amino acid sequence homologies between vertebrate Krm1 and Krm2 are only between 35-40%, both the occurrence and the order of their domains is conserved in all orthologues. Krm1 homologues are more closely related to each other than are Krm2 homologues (Fig. 1B). The cytoplasmic domains show no homologies to known structural motifs, but there is a high degree of sequence conservation within these domains between Krm1 homologues.



	hKrm2	hKrm1	mKrm2	mKrm1	XKrm2	XKrm1
h.Krm2	100%					
h.Krm1	34.3%	100%				
m.Krm2	87.2%	36.2%	100%			
m.Krm1	33.5%	93.7%	35.3%	100%		
X.Krm2	46.2%	37.3%	46.3%	37.6%	100%	
X.Krm1	35.5%	72.0%	38.3%	70.2%	38.5%	100%

Fig. 1. Sequence comparison of Krm proteins. (A) Alignment of Krm1 and Krm2 protein sequences from *Xenopus* (X) and mouse (m). The Kringle, WSC, CUB and transmembrane (TM) domains are highlighted and conserved amino acids are shown in white (within coloured domains) or red. (B) Krm homology tree and matrix showing overview of homology and amino acid identity, respectively, between the *Xenopus*, mouse and human Krm proteins.

Expression of Kremen genes in mouse and *Xenopus* embryos

In the mouse, both *Krm1* and *Krm2* are expressed in a variety of adult tissues, particularly in heart, eye and reproductive organs as seen by RT-PCR (Fig. 2A). *Krm1* and *Krm2* transcripts were first detected at embryonic day (E) 8 by RT-PCR (data not shown). At this time, which corresponds to early headfold stages, *Krm1* (Fig. 2B) and *Krm2* (similar to *Krm1* and therefore not shown) are expressed predominantly in the early anterior neural ridge (arrowheads). *Krm1* and *Krm2* show differential expression in various neural and mesodermal derivatives in midgestation mouse embryos (Fig. 2B) (Nakamura et al., 2001). At E10.5, prominent co-expression of Krm genes is evident in the branchial arches, the apical epidermal ridge (AER) of limb buds and nasal placode, with lower level co-expression seen in somites (Fig. 2C). Additional expression is seen in the forebrain and otic and optic vesicles (*Krm1*) and mesonephros (*Krm2*) (Fig. 2C).

In *Xenopus*, RT-PCR analysis shows that Krm mRNAs are present throughout embryogenesis, because of both maternal contribution (*krm1*) and zygotic expression (Fig. 3A). Zygotic expression starts at early (*krm2*) and late gastrula (*krm1*) and remains relatively constant throughout neurulation and organogenesis (Fig. 3A).

By whole-mount in situ hybridisation, *krm2* expression is observed in the gastrula marginal zone (both deep and superficial, not shown) with exception of the Spemann organiser (Fig. 3G). At early neurula stage, *krm2* expression is seen in two longitudinal stripes along the lateral neural plate (Fig. 3H). Longitudinal (Fig. 3I) and sagittal (Fig. 3J) sections at these stages show staining in anterior mesoderm, but not anterior neuroectoderm. In mid neurulae neural

tubes, expression is seen in the dorsal midline as well as two longitudinal stripes, and in sagittal sections prominent expression is observed in the prechordal plate (Fig. 3K,L). In tailbud embryos expression is seen in hatching gland, branchial arch, dorsal otic vesicle, fin mesenchyme and pronephric duct (Fig. 3M).

krm1 is first detected by in situ hybridisation at neurula stages, when it shows a similar expression pattern to *krm2* (Fig. 3C-E). Staining in sections of early neurulae are similar to those shown for *krm2* (Fig. 3I,J) and therefore not shown. At tailbud stage, as for *krm2*, expression is seen in hatching gland and fin mesenchyme, but *krm1* shows additionally expression in notochord and weakly in somites (Fig. 3F).

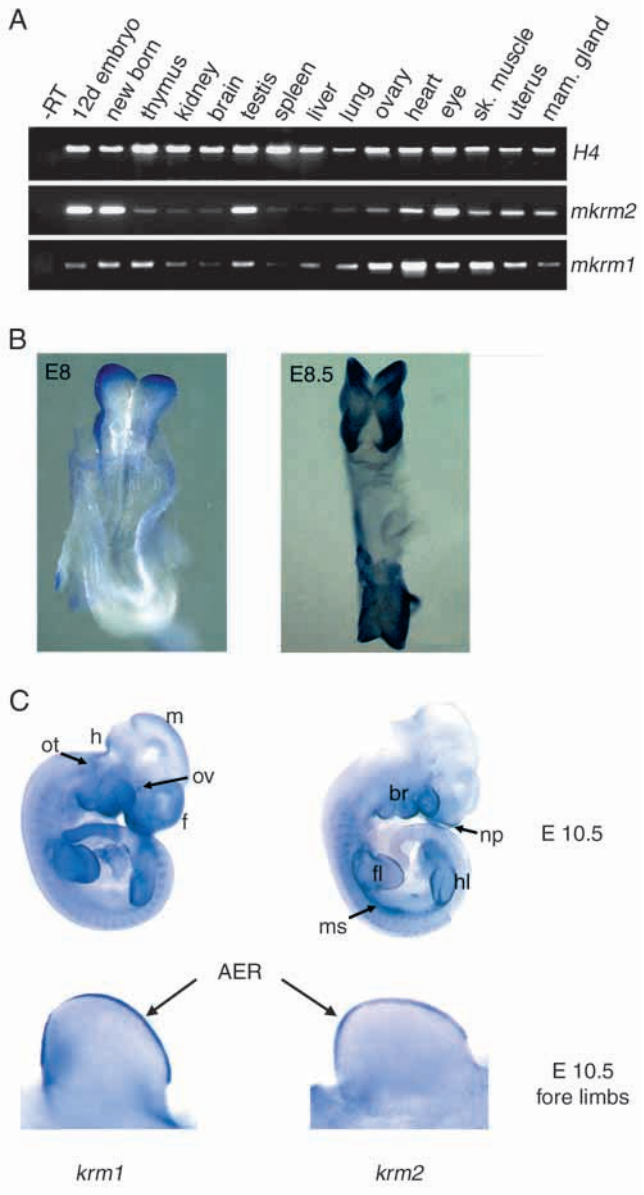


Fig. 2. Expression analysis of *Krm1* and *Krm2* in the mouse. (A) Relative expression levels of mouse *Krm1* and *Krm2* in indicated tissues, as analysed by RT-PCR. Histone H4 was used as reference for sample normalisation. (B) *Krm1* whole-mount in situ hybridisation of early (E8) and late (E8.5) headfold stage mouse embryos. (C) Whole-mount in situ hybridisation of E10.5 mouse embryos for *Krm1* and *Krm2* (top). Dissected anterior limb buds are shown in bottom panels, with arrows indicating staining in the apical ectodermal ridge (AER). br, branchial arches; f, forebrain; fl, forelimb; h, hindbrain; hl, hindlimb; m, midbrain; ms, mesanephros; np, nasal placode; ot, otic vesicle; ov, optic vesicle.

Kremens inhibit Wnt signalling in *Xenopus* embryos

We previously showed that *dkk1* and Krms synergise to inhibit Wnt signalling in HEK 293 cells and in the *Drosophila* wing (Mao et al., 2002). To test if they also functionally interact in *Xenopus* embryos, we carried out axis duplication assays with *dkk1* and *krm*. In these assays, Wnt signalling is read during a period when both endogenous Krm and LRP6 are present (because of maternal contribution), but when Dkk1 is absent. *Xwnt8* mRNA injection induces about 60% secondary embryonic axes and this is effectively inhibited by co-injection of *dkk1* mRNA [Fig. 4A,B,G (columns 1 and 2)], but not *krm1* or *krm2* (data not shown). It is thus unlikely that Krm can function without Dkk1. In contrast to its inhibition of *Wnt8*-induced axis duplication, *dkk1* fails to inhibit *Xwnt8/Lrp6* induced axis duplication [Fig. 4C,D,G (column 3)]. However, co-injection of *krm2* and *dkk1* mRNAs, but not *krm2* alone, leads to complete inhibition of *Wnt8/Lrp6*-induced axis duplication [Fig. 4E,F,G (columns 4 and 5)]. We conclude that Dkk1 and Krm proteins can functionally synergise during inhibition of Wnt signalling in *Xenopus* embryos.

A hallmark of Dkk1 is its ability to induce enlarged head structures in *Xenopus* embryos when overexpressed. This is because Dkk1 functions to induce head formation by interfering with posteriorising Wnt signals during gastrulation (Niehrs, 1999). Thus, if Dkk1 acts via Krm to affect Wnt signalling, then Krm overexpression itself may mimic the effects of Dkk1. To test this, *krm2* mRNA was microinjected into four to eight-cell stage embryos. This resulted in anteriorised embryos, with large heads and cement glands, similar to what is observed after *dkk1* overexpression (Fig. 4H-J). Consistent with the anteriorised phenotype, animal caps from *krm2* mRNA injected embryos, like those injected with *dkk1* mRNA, show upregulation of the anterior neural markers *otx2* (Blitz and Cho, 1995) and *XAG1* (Sive et al., 1989), and the pan neural marker *NCAM* (Tonissen and Krieg, 1993) (Fig. 4K). To test if this anteriorisation is due to inhibition of posteriorising Wnt signalling, embryos were microinjected with pCSKA-*Xwnt8* DNA, which induces microcephalic embryos, lacking eyes and cement gland (Christian and Moon, 1993) (Fig. 4L,M). When pCSKA-*Xwnt8* is co-injected with *krm2*, normal head formation is restored (Fig. 4N). Thus, similar to *dkk1*, *krm2* overexpression dorsoanteriorises early *Xenopus* embryos and it does so by inhibiting posteriorising Wnt signals.

If Krm proteins act as receptors for Dkk1 to inhibit Wnt/LRP signalling in *Xenopus*, one would expect excess Krm to compensate for a reduction in Dkk1 activity. This is indeed the case, as shown by the ability of injected *krm2* mRNA to rescue embryos posteriorised by inhibitory anti-Dkk1 antibody (Fig. 4O-Q). *krm1* mRNA also rescues this Dkk1 loss-of-function phenotype (data not shown). In the reverse situation, *dkk1* overexpression shows partial rescue of the phenotype elicited by *krm1/2* antisense morpholino (Mo) (see below) injected embryos (data not shown).

Kremens are required for anterior neural development

Dkk1 is essential for formation of the anterior CNS, both in *Xenopus* and mouse (Glinka et al., 1998; Mukhopadhyay et al., 2001). To test if Krms are likewise required for *Xenopus* anterior CNS development we first injected mRNA encoding a soluble form of Krm2, containing all extracellular domains but lacking transmembrane and intracellular regions, as we reasoned it might function as a dominant negative. However, this was not the case (data not shown), suggesting that membrane attachment of Krm proteins is important for mediating Wnt/LRP inhibition by Dkk1.

We therefore injected morpholino-antisense oligonucleotides (Mo), which function as specific translational inhibitors in both *Xenopus* and zebrafish embryos (Heasman et al., 2000; Nasevicius and Ekker, 2000; Heasman, 2002). When co-injected into *Xenopus* embryos, *krm1* and *krm2* Mo specifically inhibited translation of their cognate mRNA target without affecting translation of the respective orthologue (Fig. 5A). Phenotypically, *krm1*-Mo injection has no effect, and *krm2*-Mo injection yields mild anterior defects (not shown). However, as *krm1* and *krm2* are co-expressed during early *Xenopus* embryogenesis, they may function redundantly. Indeed, co-injection of *krm1* and *krm2*-Mo (*krm1/2*-Mo) results in microcephaly (Fig. 5B,C). In addition, axial malformations such as bent and shortened trunks were observed. These defects could be partially rescued by co-injection of plasmid DNA encoding N-terminally modified *krm2* DNA (lacking the antisense-Mo target sequence; Fig. 5D), indicating that the phenotype was specific. *Krm1/2*-Mo injected embryos showed reduced expression of the forebrain marker *bf1* (Bourguignon et al., 1998) (Fig. 5E-H), but the midbrain marker *en2* appeared normal (Fig. 5G,H).

If Krms function downstream of Dkk1 to inhibit Wnt signalling during head induction, *krm1/2*-Mo should enhance the phenotype produced by a reduction of Dkk1 activity. This is indeed the case (Fig. 5I-P). Co-injection of limiting amounts of an inhibitory anti-Dkk1 antibody (Glinka et al., 1998) with *krm1/2*-Mo results in embryos with head defects far more severe than seen in either *krm1/2*-Mo or anti-Dkk1 antibody-injected embryos. In situ hybridisation for *bf1* shows that reduction of prospective forebrain territory in these embryos at neurula stage parallels the phenotypic deficiencies (Fig. 5Q-T). Together, these data indicate that Krm proteins functionally interact with Dkk1 to inhibit posteriorising Wnts during embryonic head development.

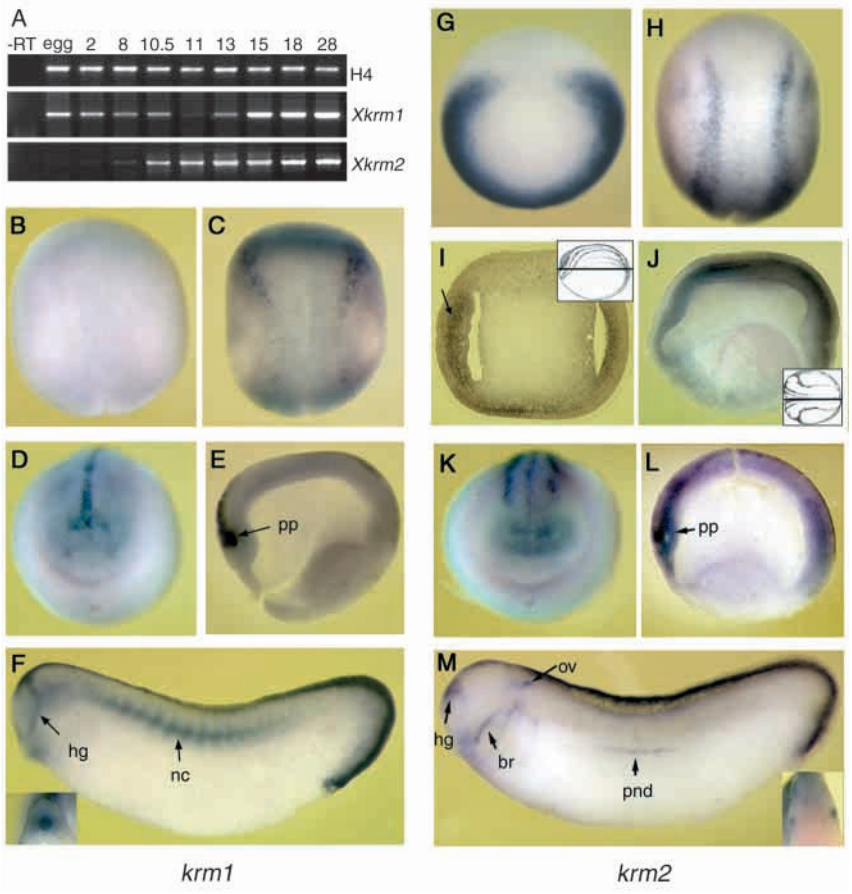


Fig. 3. Expression of Krm genes during *Xenopus* embryogenesis. (A) Developmental timecourse expression, as analysed by RT-PCR, at the indicated stages. Histone H4 was used for cDNA sample normalisation. (B-F) Spatial expression pattern of *krm1* in *Xenopus* embryos, as analysed by whole-mount in situ hybridisation. (B) Control hybridisation of a stage 14 embryo using *krm1* sense riboprobe. (C) Stage 14 embryo showing lateral neural plate expression, strongest in the anterior region. (D) Frontal view of late neurula, dorsal towards the top. (E) Sagittal midline cut of embryo shown in I, revealing expression in prechordal plate (pp). (F) Tailbud embryo showing *krm1* expression in fin mesenchyme, hatching gland (hg) and notochord (nc, see also inset of cross-section). (G-M) Spatial expression pattern of *krm2*. (G) Mid gastrula (stage 11) embryo showing expression in marginal zone but absence from dorsal region. Vegetal view, dorsal towards the top. (H) Early-mid neurula (stage 14) embryo showing lateral neural plate expression. Dorsal view, anterior towards the top. (I,J) *krm2* expression in anterior mesoderm. Vibrotome section (50 μm) of horizontally cut stage15 embryos (I) and sagittally cut stage14 embryos (J). The inserts show the plane of the section, indicated by a horizontal line. Arrow in I indicates expression in anterior mesoderm. (K) Frontal view of late neurula embryo (stage 18) showing anterior expression pattern. Dorsal towards the top. (L) Sagittal midline cut of embryo shown in K, revealing expression in prechordal plate (pp) tissue. Anterior is towards the left, dorsal is towards the top. (M) Lateral view of tailbud (stage 28) embryo showing expression in fin mesenchyme, dorsal part of otic vesicle (ov), hatching gland (hg), branchial arches (br) and pronephric duct (pnd, see also inset in cross-section).

DISCUSSION

Krm and Dkk1 interact during *Xenopus* development

The head inducer Dkk1 functions as a secreted Wnt inhibitor that binds to and blocks signalling by the Wnt receptor LRP5/6 (Bafico et al., 2001; Mao et al., 2001; Semenov et al., 2001), thereby antagonising posteriorising Wnts during patterning of the vertebrate AP axis. However, the recent finding that the

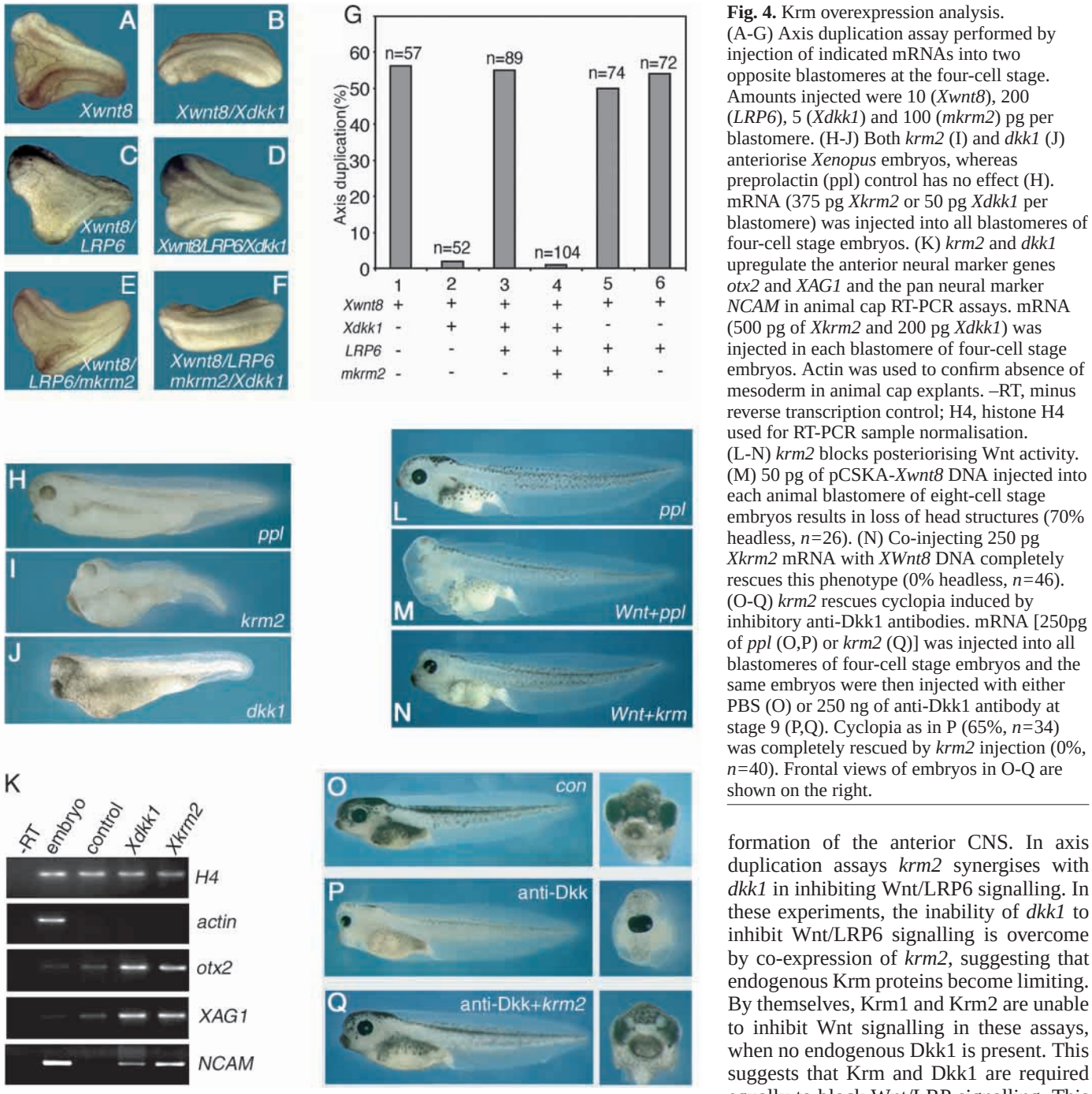


Fig. 4. Krm overexpression analysis. (A-G) Axis duplication assay performed by injection of indicated mRNAs into two opposite blastomeres at the four-cell stage. Amounts injected were 10 (*Xwnt8*), 200 (*LRP6*), 5 (*Xdkk1*) and 100 (*mkrm2*) pg per blastomere. (H-J) Both *krm2* (I) and *dkk1* (J) anteriorise *Xenopus* embryos, whereas preprolactin (ppl) control has no effect (H). mRNA (375 pg *Xkrm2* or 50 pg *Xdkk1* per blastomere) was injected into all blastomeres of four-cell stage embryos. (K) *krm2* and *dkk1* upregulate the anterior neural marker genes *otx2* and *XAG1* and the pan neural marker *NCAM* in animal cap RT-PCR assays. mRNA (500 pg of *Xkrm2* and 200 pg *Xdkk1*) was injected in each blastomere of four-cell stage embryos. Actin was used to confirm absence of mesoderm in animal cap explants. –RT, minus reverse transcription control; H4, histone H4 used for RT-PCR sample normalisation. (L-N) *krm2* blocks posteriorising Wnt activity. (M) 50 pg of pCSKA-*Xwnt8* DNA injected into each animal blastomere of eight-cell stage embryos results in loss of head structures (70% headless, *n*=26). (N) Co-injecting 250 pg *Xkrm2* mRNA with *XWnt8* DNA completely rescues this phenotype (0% headless, *n*=46). (O-Q) *krm2* rescues cyclopia induced by inhibitory anti-Dkk1 antibodies. mRNA [250pg of *ppl* (O,P) or *krm2* (Q)] was injected into all blastomeres of four-cell stage embryos and the same embryos were then injected with either PBS (O) or 250 ng of anti-Dkk1 antibody at stage 9 (P,Q). Cyclopia as in P (65%, *n*=34) was completely rescued by *krm2* injection (0%, *n*=40). Frontal views of embryos in O-Q are shown on the right.

transmembrane proteins Krm1 and 2 can bind to Dkk1 (Mao et al., 2002) raised the possibility that Dkk1 alone is not sufficient to inhibit Wnt/LRP signalling. Although their functional interaction in cell culture, as well as in *Drosophila*, is consistent with Krm proteins enhancing the Wnt inhibitory effect of Dkk1, it is not known if they are physiologically required for the action of Dkk1 and, if so, how they contribute to AP patterning.

To address these questions, we have isolated and characterised *Xenopus* Krm genes, and have shown that they functionally interact with *dkk1* in vivo. Furthermore we provide evidence that this interaction is required for the formation of the anterior CNS. In axis duplication assays *krm2* synergises with *dkk1* in inhibiting Wnt/LRP6 signalling. In these experiments, the inability of *dkk1* to inhibit Wnt/LRP6 signalling is overcome by co-expression of *krm2*, suggesting that endogenous Krm proteins become limiting. By themselves, Krm1 and Krm2 are unable to inhibit Wnt signalling in these assays, when no endogenous Dkk1 is present. This suggests that Krm and Dkk1 are required equally to block Wnt/LRP signalling. This conclusion is also supported by the findings that both the Dkk1 antibody and *krm1/2* Mo phenotypes can be rescued by overexpressed *krm* and *dkk1*, respectively. Furthermore, the synergistic effect of combined Krm and Dkk1 loss-of-function indicate that Krm proteins are physiologically relevant receptors that mediate Dkk1 inhibition of Wnt/β-catenin signalling.

Krm1 and Krm2 are thus transmembrane inhibitors of a Wnt/β-catenin signalling pathway, the significance of which in vertebrate AP patterning is emerging. Highlighting the role of Wnt/β-catenin signalling in posterior specification, inactivation of members of the Wnt1 class of ligands, both in the mouse (Takada et al., 1994) and zebrafish (Erter et al., 2001; Levken

et al., 2001), as well as of the murine Wnt/β-catenin specific transducer *Lrp6* (Pinson et al., 2000), leads to posterior defects. In addition, combined loss of the murine *Tcf1* and *Lef1* transcription factor genes, which mediate downstream Wnt/β-catenin target gene activation, results in similar posterior deficiencies (Galceran et al., 1999). In contrast to mutations in stimulatory components of the Wnt/β-catenin pathway, mutations in both zebrafish *axin* (*masterblind*) and *tcf3* (*headless*) genes, which encode intracellular inhibitory components of the Wnt/β-catenin pathway, result in anterior neural deficiencies limited to the forebrain and its derivatives (Kim et al., 2000; Heisenberg et al., 2001; van de Water et al., 2001). The latter mutant phenotypes are similar to the loss-of-function of Dkk1 in frog and mouse (Glinka et al., 1998; Mukhopadhyay et al., 2001). Thus, studies from loss-of-function mutations provides substantial genetic evidence to

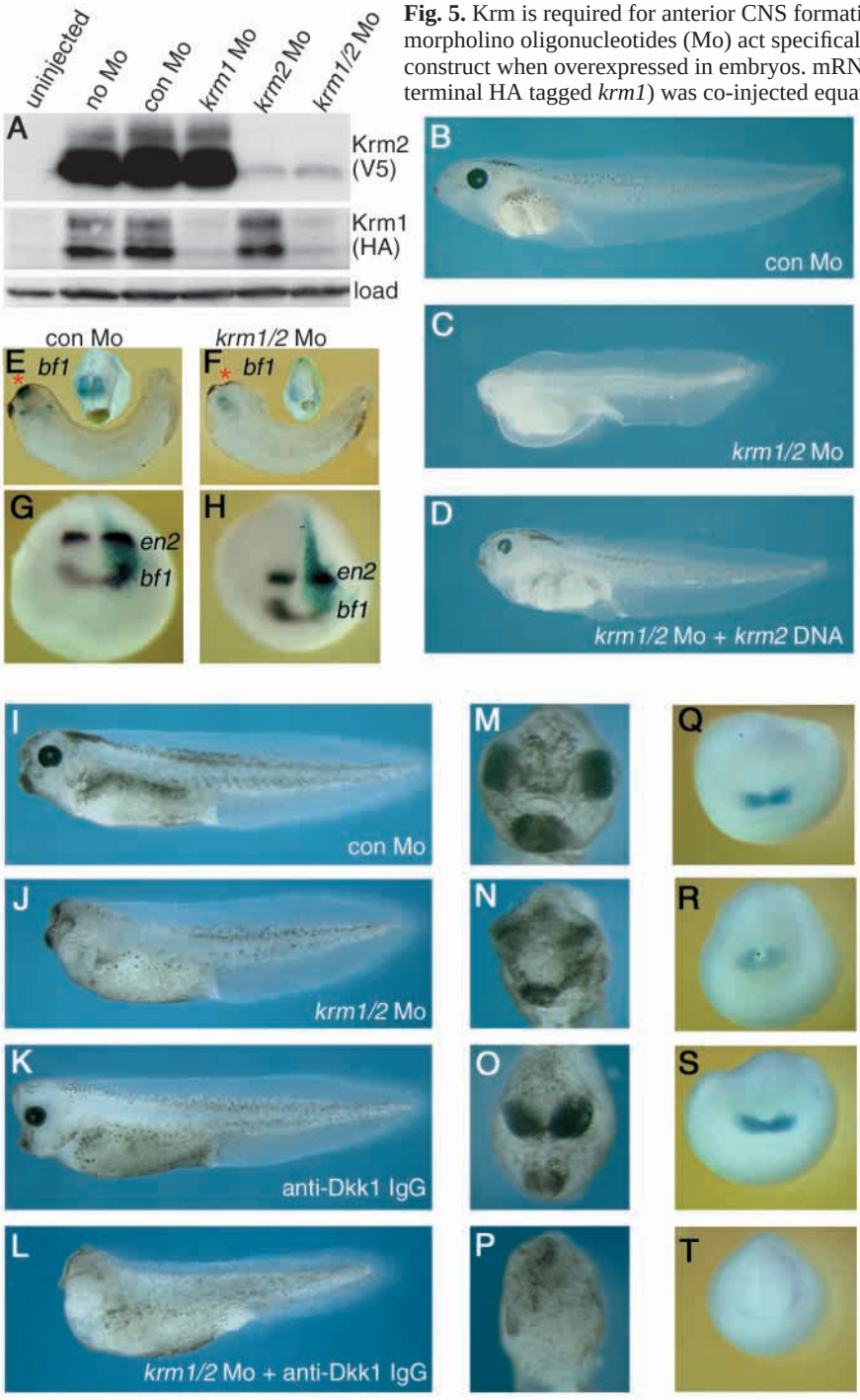


Fig. 5. Krm is required for anterior CNS formation during *Xenopus* embryogenesis. (A) Krm morpholino oligonucleotides (Mo) act specifically to inhibit translation of their cognate cDNA construct when overexpressed in embryos. mRNA (750pg of both C-terminal V5 tagged *krm2* and C-terminal HA tagged *krm1*) was co-injected equatorially into both blastomeres of two-cell stage embryos. The same embryos were then injected with 2.5 ng of the indicated morpholinos in all four vegetal blastomeres at the eight-cell stage and harvested at stage 11. Tagged Krm proteins were visualised by western blot analysis using either anti-V5 IgG (Krm2, top panel) or anti-HA (Krm1, middle panel). A crossreacting protein band from the anti-HA western is shown as a loading control (bottom panel). (B-D) Krm proteins are required for normal head formation. All four animal blastomeres of eight-cell stage embryos were injected with either 5 ng of control Mo (B), 2.5 ng each of *krm1* + *krm2* (*krm1/2*) Mo (C), or co-injected with *krm1/2* Mo and 100pg *krm2* DNA (D) and allowed to develop for 4 days. (B) Embryos injected with control Mo show no abnormalities. (C) Embryos injected with *krm1/2* Mo show microcephaly and slight shortening of the trunk/tail region (85%, *n*=400). (D) Rescue of *krm1/2* Mo phenotype by co-injection of pCS-*Xkrm2* DNA. Rescue, similar to that shown in D, was seen in 25% (*n*=300) of co-injected embryos. (E-H) Krm is required for formation of anterior neural tissue. (E,F) *bf1* in situ hybridisation of stage 25 embryos marks clear reduction of forebrain tissue (red asterisk) in *krm1/2* Mo injected embryo. Frontal views of head region are included at centre-top of panels. (G,H) Double *bf1*/*en2* in situ hybridisation of stage 16 embryos injected in one dorsal blastomere at the four-cell stage with *lacZ* mRNA (used as tracer) and either 5 ng control Mo (G) or *krm1/2* Mo (H). A reduction of the *bf1* expression, but normal *en2* expression was seen in 40% (*n*=60) of *krm1/2* Mo-injected embryos. (I-T) Krm and Dkk1 cooperate in head formation. Embryos were injected in all four animal blastomeres at the eight-cell stage with 5 ng control Mo (I,M) or 2.5 ng each of *krm1/2* Mo (J,N and L,P). At stage 9, the same embryos were injected with either PBS (I,M and J,N) or 100 ng anti-Dkk1 antibody (K,O and L,P) into the blastocoel and allowed to develop for 3 days. Note the similarity in phenotypes for *krm1/2* Mo and anti-Dkk antibody injections (compare especially N and O with M) and their synergy when combined (L,P). (M-P) The corresponding frontal views of embryos shown laterally in I-L. No headless embryos (*n*=50-110) were observed in I-K, but 40% (*n*=70) were headless in L. (Q-S) *bf1* in situ hybridisation of late neurula embryos injected as described above with control Mo (Q), *krm1/2* Mo (R), anti-Dkk1 (S) and *krm1/2* Mo + anti-Dkk1 (T). Compared with the controls (Q), reduction/loss of *bf1* expression domain was seen in 40/0% (R, *n*=200), 15/0% (S, *n*=35) and 40/60% (T, *n*=25) of embryos.

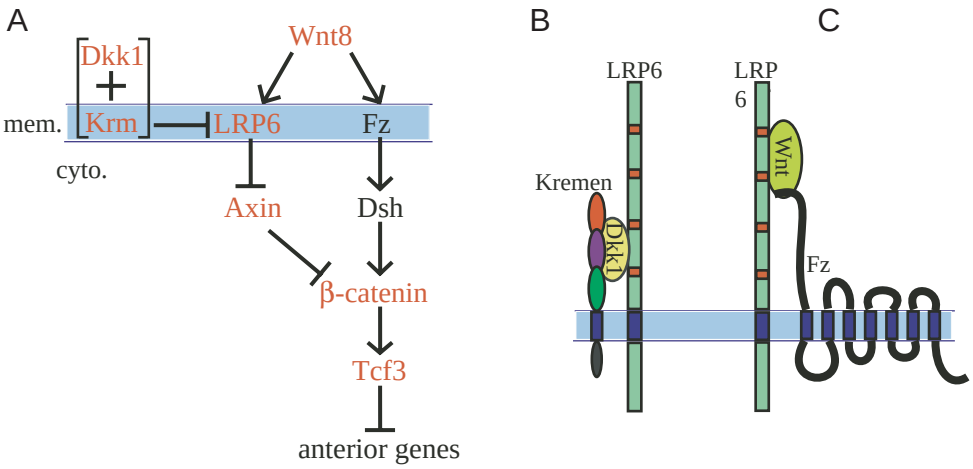


Fig. 6. Canonical Wnt pathway inhibition in AP patterning. (A) Epistatic hierarchy of Wnt/β-catenin signalling pathway components involved in vertebrate AP patterning. Components in red represent factors for which loss-of-function studies have provided direct evidence for a role in AP neural patterning: Dkk1 (Glinka et al., 1998; Mukhopadhyay et al., 2001), Krm (present study), Wnt8 (Erter et al., 2001; Levken et al., 2001), LRP6 (Pinson et al., 2000), Axin (Heisenberg et al., 2001; van de Water et al., 2001), β-catenin (Heasman et al., 2000) and Tcf3 (Kim et al., 2000). For clarity, some components of the pathway have been omitted. (B,C) Proposed molecular interactions for membrane linked Wnt pathway components. Krm, Dkk1 and LRP6 form a ternary complex (B), which disrupts Wnt/LRP6 signalling (C). Proteoglycans have been omitted for simplicity.

support a conserved and essential role for Wnt/β-catenin signalling in vertebrate AP patterning and inhibition of this pathway during anterior CNS formation. The data presented here are consistent with the proposal that Krm proteins are required co-receptors for Dkk1 to inhibit Wnt/LRP signalling and apparently independent of Dishevelled (Dsh) (Li et al., 2002), thereby promoting anterior CNS formation (Fig. 6).

Role of Kremen in embryonic development

krm1 is expressed maternally, and in both mouse and frog it is expressed in early anterior neural folds. Furthermore, both *Xenopus krm1* and *krm2* are co-expressed with *dkk1* in the prechordal plate underlying the anterior neurectoderm. These expression domains are consistent with a role of Krm proteins during early anterior development. Maternal *krm1* mRNA suggests that there is also maternal protein which would not be affected by morpholino knockdown. Hence, the observed phenotype may be hypomorphic. Similar to the loss-of-function of Dkk1 in frog and mouse (Glinka et al., 1998; Mukhopadhyay et al., 2001), the observed neural deficiencies are limited to the forebrain and its derivatives, as in cases of mutations of intracellular Wnt inhibitors (Kim et al., 2000; Heisenberg et al., 2001; van de Water et al., 2001). Conversely, zebrafish *wnt8* mutants show expanded forebrain (Levken et al., 2001), indicating that this region of the CNS is most sensitive to Wnt/β-catenin signalling, while more posterior CNS structures may become affected only in compound mutants of Wnt inhibitors.

At tadpole stages, *dkk1*, *krm1* and *krm2* show complex expression patterns, with co-expression observed in the otic vesicle, fin mesenchyme and proctodeum, where the genes may interact. During mouse organogenesis, *dkk1*, *krm1* and *krm2* are co-expressed in the apical ectodermal ridge of mouse limb buds (Monaghan et al., 1999; Nakamura et al., 2001) (and results presented here). As Dkk1 is required for limb formation (Mukhopadhyay et al., 2001), it may interact with Krm proteins in this context to inhibit the Wnt receptor LRP6, which is ubiquitously expressed during embryogenesis (Pinson et al., 2000). However, although there are several sites of *dkk1/krm* co-expression, it cannot be excluded that Dkk1 can also function independently of Krm1 and Krm2, e.g. by recruiting yet unknown co-receptors. Likewise, their multidomain ECDs and the intracellular domain raise the likely possibility that Krm proteins have functions in addition to mediating Dkk1 action. The prominent expression, e.g. in trunk mesoderm, where Dkk genes are not expressed and mild trunk defects observed following morpholino knockdown would be consistent with such additional functions. One other potentially relevant, high-affinity ligand for Krm1 and Krm2 is Dkk2, which is co-expressed with *krm1* in branchial arch, otic and optic vesicles and limb bud (Monaghan et al., 1999; Wu et al., 2000; Nakamura et al., 2001).

We thank Dana Hoppe for technical assistance, Marlene Rau for help with in situ hybridisation and Ralf Klaeren for help with sequencing.

REFERENCES

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. et al. (2000). The organizer factors Chordin and Noggin are required for mouse forebrain development. *Nature* **403**, 658-661.

Bafico, A., Liu, G., Yaniv, A., Gazit, A. and Aaronson, S. A. (2001). Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. *Nat. Cell Biol.* **3**, 683-686.

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.

Bork, P. and Beckmann, G. (1993). The CUB domain. A widespread module in developmentally regulated proteins. *J. Mol. Biol.* **231**, 539-545.

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.

Castellino, F. J. and McCance, S. G. (1997). The kringle domains of human plasminogen. *Ciba Found. Symp.* **212**, 46-60.

Chang, C. and Hemmati-Brivanlou, A. (1998). Cell fate determination in embryonic ectoderm. *J. Neurobiol.* **36**, 128-151.

Chapman, S. C., Schubert, F. R., Schoenwolf, G. C. and Lumsden, A. (2002). Analysis of spatial and temporal gene expression patterns in blastula and gastrula stage chick embryos. *Dev. Biol.* **245**, 187-199.

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.

Church, W. R., Ouellette, L. A. and Messier, T. L. (1991). Modulation of human prothrombin activation on phospholipid vesicles and platelets using monoclonal antibodies to prothrombin fragment 2. *J. Biol. Chem.* **266**, 8384-8391.

Deardorff, M. A., Tan, C., Conrad, L. J. and Klein, P. S. (1998). Frizzled-8 is expressed in the Spemann organizer and plays a role in early morphogenesis. *Development* **125**, 2687-2700.

Doniach, T. and Musci, T. J. (1995). Induction of anteroposterior neural pattern in Xenopus: evidence for a quantitative mechanism. *Mech. Dev.* **53**, 403-413.

Dupe, V. and Lumsden, A. (2001). Hindbrain patterning involves graded responses to retinoic acid signalling. *Development* **128**, 2199-2208.

Erter, C. E., Wilm, T. P., Basler, N., Wright, C. V. and Solnica-Krezel, L. (2001). Wnt8 is required in lateral mesendodermal precursors for neural posteriorization in vivo. *Development* **128**, 3571-3583.

Fainsod, A., Deißler, K., Yelin, R., Marom, M., Epstein, M., Pillemer, G., Steinbeisser, H. and Blum, M. (1997). The dorsalizing and neural inducing gene follistatin is an antagonist of Bmp-4. *Mech. Dev.* **63**, 39-50.

Fekany-Lee, K., Gonzalez, E., Miller-Bertoglio, V. and Solnica-Krezel, L. (2000). The homeobox gene bozozok promotes anterior neuroectoderm formation in zebrafish through negative regulation of BMP2/4 and Wnt pathways. *Development* **127**, 2333-2345.

Galceran, J., Farinas, I., Depew, M. J., Clevers, H. and Grosschedl, R. (1999). Wnt3a$^{-/-}$ like phenotype and limb deficiency in Lef1$^{-/-}$Tcf1$^{-/-}$ mice. *Genes Dev.* **13**, 709-717.

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.

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

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.

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.

Goretzki, L., Lombardo, C. R. and Stallcup, W. B. (2000). Binding of the NG2 proteoglycan to kringle domains modulates the functional properties of angiostatin and plasmin(ogen). *J. Biol. Chem.* **275**, 28625-28633.

Gschwend, T. P., Krueger, S. R., Kozlov, S. V., Wolfer, D. P. and Sonderegger, P. (1997). Neurotrypsin, a novel multidomain serine protease expressed in the nervous system. *Mol. Cell. Neurosci.* **9**, 207-219.

Harland, R. and Gerhart, J. (1997). Formation and function of Spemann's organizer. *Annu. Rev. Cell 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.

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

Heasman, J., Kofron, M. and Wylie, C. (2000). Beta-catenin signaling activity dissected in the early Xenopus embryo: a novel antisense approach. *Dev. Biol.* **222**, 124-134.

Heisenberg, C. P., Houart, C., Take-Uchi, M., Rauch, G. J., Young, N., Coutinho, P., Masai, I., Caneparo, L., Concha, M. L., Geisler, R. et al. (2001). A mutation in the Gsk3-binding domain of zebrafish Masterblind/Axin1 leads to a fate transformation of telencephalon and eyes to diencephalon. *Genes Dev.* **15**, 1427-1434.

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.

Hongo, I., Kengaku, M. and Okamoto, H. (1999). FGF signaling and the anterior neural induction in Xenopus. *Dev Biol.* **216**, 561-581.

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.

Itoh, K., Tang, T. L., Neel, B. G. and Sokol, S. Y. (1995). Specific modulation of ectodermal cell fates in Xenopus embryos by glycogen synthase kinase. *Development* **121**, 3979-3988.

Kazanskaya, O., Glinka, A. and Niehrs, C. (2000). The role of Xenopus dickkopf1 in prechordal plate specification and neural patterning. *Development* **127**, 4981-4992.

Kiecker, C. and Niehrs, C. (2001). A morphogen gradient of Wnt/beta-catenin signaling regulates anteroposterior neural patterning in Xenopus. *Development* (in press).

Kim, C.-H., Oda, T., Itoh, M., Jiang, D. I., Artinger, K., Chandrasekharappa, S. C., Driever, W. and Chitnis, A. B. (2000). Repressor activity of Headless/Tcf3 is essential for vertebrate head formation. *Nature* **407**, 916-920.

Koop, K. E., MacDonald, L. M. and Lobe, C. G. (1996). Transcripts of Grg4, a murine groucho-related gene, are detected in adjacent tissues to other murine neurogenic gene homologues during embryonic development. *Mech. Dev.* **59**, 73-87.

Krupnik, V. E., Sharp, J. D., Jiang, C., Robison, K., Chickering, T. W., Amaravadi, L., Brown, D. E., Guyot, D., Mays, G., Leiby, K. et al. (1999). Functional and structural diversity of the human Dickkopf gene family. *Gene* **238**, 301-313.

Kurosky, A., Barnett, D. R., Lee, T. H., Touchstone, B., Hay, R. E., Arnott, M. S., Bowman, B. H. and Fitch, W. M. (1980). Covalent structure of human haptoglobin: a serine protease homolog. *Proc. Natl. Acad. Sci. USA* **77**, 3388-3392.

Levken, A. C., Thorpe, C. J., Waxman, J. S. and Moon, R. T. (2001). Zebrafish wnt8 encodes two proteins on a bicistronic transcript and is required for mesoderm and neurectoderm patterning. *Dev. Cell* **1**, 103-114.

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, L., Mao, J., Sun, L., Liu, W. and Wu, D. (2002). Second cysteine-rich domain of Dickkopf-2 activates canonical Wnt signaling pathway via LRP-6 independently of dishevelled. *J. Biol. Chem.* **277**, 5977-5981.

Lodder, A. L., Lee, T. K. and Ballester, R. (1999). Characterization of the Wsc1 protein, a putative receptor in the stress response of Saccharomyces cerevisiae. *Genetics* **152**, 1487-1499.

Lumsden, A. and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. *Science* **274**, 1109-1115.

Mao, B., Wu, W., Davidson, G., Marhold, J., Li, M., Mechler, B. M., Delius, H., Hoppe, D., Stannek, P., Walter, C. et al. (2002). Kremen proteins are Dickkopf receptors that regulate Wnt/B-catenin signalling. *Nature* **417**, 664-667.

Mao, B., Wu, W., Li, Y., Hoppe, D., Stannek, P., Glinka, A. and Niehrs, C. (2001). LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. *Nature* **411**, 321-325.

McGrew, L. L., Lai, C. J. and Moon, R. T. (1995). Specification of the anteroposterior neural axis through synergistic interaction of the Wnt signaling cascade with noggin and follistatin. *Dev. Biol.* **172**, 337-342.

Meno, C., Gritsman, K., Ohishi, S., Ohfuji, Y., Heckscher, E., Mochida, K., Shimono, A., Kondoh, H., Talbot, W. S., Robertson, E. J. et al. (1999). Mouse Lefty2 and zebrafish antivin are feedback inhibitors of nodal signaling during vertebrate gastrulation. *Mol. Cell* **4**, 287-298.

Monaghan, A. P., Kioschis, P., Wu, W., Zuniga, A., Bock, D., Poustka, A., Delius, H. and Niehrs, C. (1999). Dickkopf genes are co-ordinately expressed in mesodermal lineages. *Mech. Dev.* **87**, 45-56.

Mukhopadhyay, M., Shtrom, S., Rodriguez-Esteban, C., Chen, L., Tsuku, T., Gomer, L., Dorward, D. W., Glinka, A., Grinberg, A., Huang, S.-P. et al. (2001). Dickkopf1 is required for embryonic head induction and limb morphogenesis in the mouse. *Dev. Cell* **1**, 423-434.

Nakamura, T., Aoki, S., Kitajima, K., Takahashi, T. and Matsumoto, K. (2001). Molecular cloning and characterization of Kremen, a novel kringle-containing transmembrane protein. *Biochim. Biophys. Acta* **1518**, 63-72.

Nasevicius, A. and Ekker, S. C. (2000). Effective targeted gene 'knockdown' in zebrafish. *Nat. Genet.* **26**, 216-220.

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.

Nordstrom, U., Jessell, T. M. and Edlund, T. (2002). Progressive induction of caudal neural character by graded Wnt signaling. *Nat. Neurosci.* **13**, 13.

Patthy, L., Trexler, M., Vali, Z., Banyai, L. and Varadi, A. (1984). Kringles: modules specialized for protein binding. Homology of the gelatin-binding

region of fibronectin with the kringle structures of proteases. *FEBS Lett.* **171**, 131-136.

Pera, E. M., Wessely, O., Li, S. Y. and de Robertis, E. M. (2001). Neural and head induction by insulin-like growth factor signals. *Dev. Cell* **1**, 655-665.

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.

Pierce, S. B. and Kimelman, D. (1996). Overexpression of Xgsk-3 disrupts anterior ectodermal patterning in Xenopus. *Dev. Biol.* **175**, 256-264.

Pinson, K. I., Brennan, J., Monkley, S., Avery, B. J. and Skarnes, W. C. (2000). An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* **407**, 535-538.

Rattner, A., Hsieh, J.-C., Smallwood, P. M., Gilbert, D., Copeland, N. G., Jenkins, N. A. and Nathans, J. (1997). A family of secreted proteins contains homology to the cysteine-rich ligand binding domain of frizzled receptors. *Proc. Natl. Acad. Sci. USA* **94**, 2859-2863.

Richard-Parpaillon, L., Heligon, C., Chesnel, F., Boujard, D. and Philpott, A. (2002). The IGF pathway regulates head formation by inhibiting Wnt signaling in Xenopus. *Dev. Biol.* **244**, 407-417.

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

Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L. K. and de Robertis, E. M. (1994). Xenopus chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* **79**, 779-790.

Schier, A. F. and Shen, M. M. (2000). Nodal signalling in vertebrate development. *Nature* **403**, 385-389.

Semenov, M. V., Tamai, K., Brott, B. K., Kuhl, M., Sokol, S. and He, X. (2001). Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. *Curr. Biol.* **11**, 951-961.

Shanmugalingam, S., Houart, C., Picker, A., Reifers, F., Macdonald, R., Barth, A., Griffin, K., Brand, M. and Wilson, S. W. (2000). Ace/Fgf8 is required for forebrain commissure formation and patterning of the telencephalon. *Development* **127**, 2549-2561.

Shimamura, K., Hartigan, D. J., Martinez, S., Puelles, L. and Rubenstein, J. L. (1995). Longitudinal organization of the anterior neural plate and neural tube. *Development* **121**, 3923-3933.

Shinya, M., Eschbach, C., Clark, M., Lehrach, H. and Furutani-Seiki, M. (2000). Zebrafish Dkk1, induced by the pre-MBT Wnt signaling, is secreted from the prechordal plate and patterns the anterior neural plate. *Mech. Dev.* **98**, 3-17.

Shinya, M., Koshida, S., Sawada, A., Kuroiwa, A. and Takeda, H. (2001). Fgf signalling through MAPK cascade is required for development of the subpallial telencephalon in zebrafish embryos. *Development* **128**, 4153-4164.

Sive, H., Hattori, K. and Weintraub, H. (1989). Progressive determination during formation of the anteroposterior axis in Xenopus laevis. *Cell* **58**, 171-180.

Smith, W. C. and Harland, R. M. (1992). Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. *Cell* **70**, 829-840.

Stern, C. D. (2001). Initial patterning of the central nervous system: How many organizers? *Nat. Rev. Neurosci.* **2**, 92-98.

Streit, A., Berliner, A. J., Papanayotou, C., Sirulnik, A. and Stern, C. D. (2000). Initiation of neural induction by FGF signalling before gastrulation. *Nature* **406**, 74-78.

Streit, A. and Stern, C. D. (1999). Neural induction. A bird's eye view. *Trends Genet* **15**, 20-24.

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

Thery, C. and Stern, C. D. (1996). Roles of kringle domain-containing serine proteases in epithelial-mesenchymal transitions during embryonic development. *Acta Anat.* **156**, 162-172.

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.

Thisse, C. and Thisse, B. (1999). Antivin, a novel and divergent member of the TGFbeta superfamily, negatively regulates mesoderm induction. *Development* **126**, 229-240.

Tonissen, K. F. and Krieg, P. A. (1993). Two neural-cell adhesion molecule (NCAM)-encoding genes in Xenopus laevis are expressed during development and in adult tissues. *Gene* **127**, 243-247.

van de Water, S., van de Wetering, M., Joore, J., Esseling, J., Bink, R., Clevers, H. and Zivkovic, D. (2001). Ectopic Wnt signal determines the eyeless phenotype of zebrafish masterblind mutant. *Development* **128**, 3877-3888.

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

Wehrli, M., Dougan, S. T., Caldwell, K., O'Keefe, L., Schwartz, S., Vaizel-Ohayon, D., Schejter, E., Tomlinson, A. and DiNardo, S. (2000). arrow encodes an LDL-receptor-related protein essential for Wingless signalling. *Nature* **407**, 527-530.

Wilson, S. I., Graziano, E., Harland, R., Jessell, T. M. and Edlund, T. (2000). An early requirement for FGF signalling in the acquisition of neural cell fate in the chick embryo. *Curr. Biol.* **10**, 421-429.

Wu, W., Glinka, A., Delius, H. and Niehrs, C. (2000). Mutual antagonism between dickkopf1 and -2 regulates Wnt/beta-catenin signalling. *Curr. Biol.* **10**, 1611-1614.