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Metastasis-associated kinase modulates Wnt signaling to regulate brain patterning and morphogenesis

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Wnt signaling is a major pathway regulating cell fate determination, cell proliferation and cell movements in vertebrate embryos. Distinct branches of this pathway activate β-catenin/TCF target genes and modulate morphogenetic movements in embryonic tissues by reorganizing the cytoskeleton. The selection of different molecular targets in the pathway is driven by multiple phosphorylation events. Here, we report that metastasis-associated kinase (MAK) is a novel regulator of Wnt signaling during morphogenetic movements, and eye and brain development in *Xenopus* embryos. Injected MAK RNA suppressed Wnt transcriptional reporters and activated Jun N-terminal kinase. Furthermore, MAK was recruited to the cell membrane by Frizzled 3, formed a complex with Dishevelled and phosphorylated Dsh in vitro. The regional brain markers *Otx2*, *En2* and *Gbx2* were affected in embryos with modulated MAK activity in a manner consistent with a role for MAK in midbrain-hindbrain boundary formation. Confirming the inhibitory role for this kinase in Wnt/β-catenin signaling, the midbrain patterning defects in embryos depleted of MAK were rescued by the simultaneous depletion of β-catenin. These findings indicate that MAK may function in different developmental processes as a switch between the canonical and non-canonical branches of Wnt signaling.

KEY WORDS: Wnt, *Xenopus*, Dsh, Midbrain, Morphogenesis, Kinase, JNK, SNF-1

INTRODUCTION

Vertebrate embryonic development proceeds through a cascade of inductive interactions, which specify major body axes and generate the three germ layers, ectoderm, mesoderm and endoderm, in the embryo. The Wnt pathway is among the few major signaling pathways that specify cell fates during development and is implicated in cell and tissue polarization (Logan and Nusse, 2004). Before gastrulation, the pathway is used to establish the Spemann organizer, a major inductive center in the embryo, which is responsible for dorsal development and neural induction (Harland and Gerhart, 1997; Moon and Kimelman, 1998; Sokol, 1999; Tao et al., 2005). After the initial induction of the central nervous system, localized sources of Wnt ligands induce and maintain a secondary organizing center at the midbrain-hindbrain boundary, which mediates regional patterning of the brain (Chi et al., 2003; Hidalgo-Sanchez et al., 2005; Liu and Joyner, 2001). Besides organizer specification and brain patterning, Wnt signaling is involved in somitogenesis, eye and neural crest development, cardiogenesis, and endoderm differentiation (Bainter et al., 2001; Cavodeassi et al., 2005; Ciani and Salinas, 2005; Gamse and Sive, 2000; Logan and Nusse, 2004; Wu et al., 2003). Thus, Wnt signaling is re-used at many developmental stages in different embryonic tissues.

The pathway leads to two major outcomes: β-catenin-dependent (canonical) activation of target genes and the regulation of actin cytoskeleton and cell polarity via a poorly understood process (noncanonical signaling). In the canonical pathway, Wnt ligands and their cognate Frizzled receptors signal through Dishevelled to stabilize β-catenin and induce its association with the TCF transcription factors to activate Wnt target genes. By contrast, Wnt signaling to the cytoskeleton occurs through the activation of small GTPases (Habas et al., 2003; Habas et al., 2001), Rho-associated kinases (Marlow et al., 2002; Winter et al., 2001), Jun-N-terminal kinases (Boutros et al., 1998; Lisovsky et al., 2002) and intracellular Ca^{2+} release (Sheldahl et al., 2003; Slusarski et al., 1997). Noncanonical Wnt signaling regulates changes in cell shape and motility during gastrulation, neurulation and organogenesis (Ciani and Salinas, 2005; Saneyoshi et al., 2002; Sokol, 1996; Wallingford et al., 2002; Winklbauer et al., 2001; Zohn et al., 2003). Both canonical and noncanonical branches of pathway involve Dishevelled, a protein phosphorylated in response to Wnt signaling (Yanagawa et al., 1995), although how Dishevelled directs signals to different molecular targets remains unclear (Wallingford and Habas, 2005).

Here, we report that a SNF1 (sucrose non fermenting 1)-related protein kinase, known as metastasis-associated kinase (MAK) (Gardner et al., 2000b; Korobko et al., 1997), phosphorylates Dishevelled, inhibits the canonical Wnt pathway and upregulates non-canonical Wnt signaling during early development. SNF1-related kinases are involved in the metabolic response to nutritional and environmental stress, cell cycle, cell polarity and vertebrate development (Becker and Brendel, 1996; Hardie et al., 1998; Ruiz et al., 1994). MAK is distantly related to the yeast Kin-1 and to the *C. elegans* PAR-1. Kin-1 is crucial for growth polarity and cytoskeletal organization in fission yeast (Drewes and Nurse, 2003; Levin et al., 1987; Tassan and Le Goff, 2004). PAR-1 regulates cell polarity in *C. elegans* and *Drosophila* embryos and mammalian cells (Bohm et al., 1997; Pellettieri and Seydoux, 2002; Shulman et al., 2000; Tomancak et al., 2000) and has been implicated in Wnt signaling (Ossipova et al., 2005; Sun et al., 2001). Our experiments demonstrate that MAK regulates morphogenetic movements, eye development and midbrain patterning in *Xenopus* embryos and suggest that MAK may function as a molecular switch between the canonical and noncanonical Wnt pathways.

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MATERIALS AND METHODS

DNA constructs and RNA synthesis

A plasmid containing a full length *Xenopus* MAK cDNA corresponds to the IMAGE clone 5156600 (Accession Number CA793236), obtained from Open Biosystems. Two different, but highly related *MAK* genes have been reported in *Xenopus* databases (Ruzov et al., 2004). The expression pattern of the second gene (Accession Number AY318878) (Ruzov et al., 2004) differs from what we report here for *xMAK*. To generate pXT7-GFP-xMAK, xMAK cDNA was amplified by PCR with 5′-AAGAATTCGATGC-CGGCTGCCGCTG-3′ and 5′-GCCTCGAGGCAAGGACAAATCTGTT-3′, and subcloned into pXT7-GFP (Itoh et al., 2000) that has been digested with *Eco*RI and *Sal*I. Flag-xMAK constructs were generated by PCR and subcloned into pCS2-Flag. Untagged mMAK cDNA in the pCTX vector (S.Y.S., unpublished) was used as a transcription template. Myc-MAK constructs were generated from the wild-type or kinase-dead (K91>R) mMAK cDNA (Korobko et al., 2004) by PCR with the primers 5′-AACTCGAGATGCCGGCAGCGG-3′ and 5′-AGGTTAACACTGGCC-CTTGACACCGTC-3′ and subcloned into the *Xho*I and *Eco*RV sites of pCTX-Myc. Details of cloning are available upon request. Other expression constructs were pCS2-nβgal (Turner and Weintraub, 1994), Xfz3 (Shi et al., 1998), rFz2, membrane-tethered DsRed (Unterseher et al., 2004), Myc-Xdsh and β-catenin (Sokol, 1996), HA-Xdsh (Itoh et al., 2000), Xwnt8 (Sokol et al., 1991) and ΔN-Fz8 (Lisovsky et al., 2002).

Embryo culture, microinjection, axis induction and extension assays

In vitro fertilization, culture and microinjections of *Xenopus* eggs were essentially as described (Sokol, 1996). Stages were determined according to Nieuwkoop and Faber (Nieuwkoop et al., 1967). Morpholino oligonucleotides were from GeneTools (Oregon) and had the following sequences: MAK MO, 5′-CGGCATCCCCAGTGGTGTAGATCTC-3′; β-Cat MO, 5′-TTTCAACCGTTTCCAAAGAACCAGG-3′; control MO, 5′-ATCGACTTCCTCCGAAACGGACATG-3′. RNAs for microinjection were synthesized using mMessage mMachine kit (Ambion). Axis induction assays were carried out by injecting HA-Xdsh, Xwnt8 or β-catenin RNA into a single vegetal ventral blastomere at the four- to eight-cell stage at indicated doses and assessed when the injected embryos reached stage 36-40. To monitor axis extension defects, MAK RNA, MAK-KD or GFP RNA were injected into two dorsovegetal blastomeres of four-cell embryos (2 ng each injection) and the injected embryos were allowed to develop until sibling embryos reached stage 32. In other cases, sites of injection are specified in corresponding figure legends. For lineage tracing, 20-40 pg of RNA encoding nuclear β-galactosidase (pCS2-nβgal) was injected together with morpholinos or mRNAs. β-Galactosidase activity was visualized with the Red-Gal substrate (Research Organics).

For animal cap elongation assay, four-cell embryos were injected four times in the animal region of each blastomere with mMAK or mMAK-KD RNA (2 ng per injection), animal caps were dissected at stage 8 to 9, and cultured with or without 50 ng/ml of human recombinant activin A until the sibling embryos reached stage 15.

Transcriptional reporter assays

Embryos were injected into animal pole region of one ventral blastomere at the four-cell stage with 20 pg of the –833pSia-Luc reporter DNA (Fan et al., 1998) per embryo, alone or with the indicated amounts of mRNAs. Lysates were prepared from embryos at stage 10.5 and assayed for luciferase activity as previously described (Fan et al., 1998). For every experimental group, measurements were carried out for triplicate samples, each consisting of five embryos. Values shown are averages±s.d., which are representative of at least three different experiments.

Subcellular localization of MAK-GFP

For subcellular localization of MAK-GFP constructs, RNAs encoding MAK-GFP (500 pg) and mDsRed (2 ng) were co-injected with or without Fz3 or Fz2 RNA (1 ng) into the animal pole region of two- to four-cell embryos. Animal cap explants were dissected at stage 9-9.5, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 35-45 minutes, washed and mounted for observation in the Vectashield mounting medium with DAPI (Vector) as described (Itoh et al., 2005). Fluorescence was visualized on a Zeiss Axiophot microscope with Apotome attachment and images were acquired with Axiocam HR camera.

Whole-mount in situ hybridization

Digoxigenin-labeled antisense RNA probes were synthesized from linearized plasmids, encoding *En2* (Brivanlou and Harland, 1989), *Krox20* (Bradley et al., 1993), *Otx2* (Pannese et al., 1995), *Gbx2* (von Bubnoff et al., 1996) and *xMAK*, using a digoxigenin-labeling mixture (Boehringer Mannheim). Whole-mount in situ hybridization was carried out according to Harlan (Harland, 1991) with modifications as described previously (Hikasa and Sokol, 2004). For in situ hybridization, embryos were rehydrated in 1×PBS, 0.1% Tween 20. The staining reaction was carried out for sense and anti-sense probes for the same duration. 5-Bromo-4-chloro-3-indolyl phosphate (Sigma) and Nitro blue tetrazolium (Sigma) were used for chromogenic reactions. After staining, some embryos were dehydrated, embedded into paraplast and sectioned at 10 μm for image analysis at higher resolution.

RNA isolation and RT-PCR

Total RNA was extracted from embryos or animal caps by proteinase K-phenol extraction as described (Itoh and Sokol, 1997). cDNAs were made from DNase-treated RNA using the Superscript first strand synthesis system (Invitrogen). RT-PCR was performed on total RNA isolated from stage 10.5 embryos as previously described (Itoh and Sokol, 1997). Primers for RT-PCR were: *xMAK*, 5′-ACCAGAAGATGGTCGA-3′, 5′-TTCCAACT-GATGAAACT-3′; *chordin*, 5′-AACTGCCAGGACTGGATGGT-3′, 5′-GGCAGGATTTAGAGTTGCTTC-3′; *Xnr3*, 5′-CGAGTGCAAG-AAGGTGGACA-3′, 5′-ATCTTCATGGGGACACAGGA-3′; *siamois*, 5′-CTCCAGCCACCAGTACCAGATC-3′, 5′-GGGGAGAGTGGAAA-GTGGTTG-3′; *gsc*, 5′-TTCACCGATGAACAACTGGA-3′, 5′-TTCC-ACTTTTGGGCATTTTC-3′; *MyoD*, 5′-AGCTCCAACTGCTCCGAC-GGCATGAA-3′, 5′-AGGAGAGAATCCAGTTGATGGAAACA-3′; *Dkk1*, 5′-CACCAAGCACAGGAGGAA-3′, 5′-TCAGGGAAGACCAGAGCA-3′; *EF-1α*, 5′-CAGATTGGTGCTGGATATGC-3′, 5′-ACTGCCTTG-ATGACTCCTAG-3′; *FGFR*, 5′-TTGAAGTCTGATGCGAGTGA-3′, 5′-GGGTTGTAGCAGTACTCCAT-3′. One quarter of each PCR reaction was electrophoresed in a 5% polyacrylamide gel, stained with ethidium bromide and photographed under ultraviolet light.

Immunoprecipitation and western analysis

Immunoprecipitation and western analyses were carried out with embryo lysates as described (Gloy et al., 2002). To prepare embryo lysates at stage 10+, each blastomere of four-cell embryos was injected with different mRNAs. For immunoprecipitation, 20 μl of 9E10 (anti-Myc) or 12CA5 (anti-HA) hybridoma supernatants (ATCC) were used per sample. Protein amount equivalent to one half embryo was loaded per lane for embryo lysates, and the equivalent of four to nine embryos for immunoprecipitated proteins. Monoclonal M2 antibodies (anti-Flag) and polyclonal anti-β-tubulin antibodies were from Sigma, secondary HRP-conjugated antibodies were from Jackson ImmunoResearch.

In vitro translation, MAK and JNK kinase assays

In vitro translation reactions were performed using rabbit reticulocyte lysates from the Retic Lysate IVT kit (Ambion) or TnT system (Promega) according to manufacturer's instructions. MAK kinase reactions were performed essentially as described (Ossipova et al., 2005). For in vitro blocking studies, 600 pM of MAK MO or COMO were incubated with 0.3 μg of xMAK RNA or mMAK RNA in 10 μl of distilled water for 30 minutes at room temperature, following by in vitro translation reaction for 90 minutes. Half of each reaction was loaded on an SDS-polyacrylamide gel. Radiolabeled lysates were electrophoresed as described (Sambrook et al., 1989), gels were fixed in 50% methanol and 10% acetic acid, dried and exposed to Kodak XAR-5 film for autoradiography.

In vitro JNK kinase assays were performed essentially as described (Lisovsky et al., 2002). Four-cell stage embryos were injected into each cell with GFP, mMAK, or mMAK-KD RNA at 1.5 ng per injection, or ΔN-Fz8 RNA (0.5 ng). Embryos were lysed at stage 14 in 100 μl of buffer containing 40 mM HEPES (pH 7.5), 50 mM KCl, 5 mM EDTA, 5 mM EGTA, 50 mM

β-glycerophosphate, 2 mM DTT, 1 mM sodium vanadate, 50 mM sodium fluoride, 1% Triton X-100, 10 M PMSF, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin and 1 μg/ml antipain. The reaction mixture (25 μl) contained 1 μl of total embryo lysate, 0.5 μg of GST-Jun(1-135) and the kinase buffer (20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM sodium vanadate, 2 mM DTT, 25 mM β-glycerophosphate, 100 M cold ATP). The reactions were allowed to proceed for 30 minutes at 30°C. Proteins were separated by 12% SDS-PAGE, phosphorylated Jun-GST was detected with anti-phospho-Jun-specific antibodies (Cell Signaling Technology).

RESULTS

MAK expression is dynamically regulated during *Xenopus* development

To gain insight into possible developmental functions of MAK in vertebrate embryos, we first analyzed the expression of a *Xenopus* homologue of *MAK (xMAK)* during embryonic development. Reverse transcription-based polymerase chain reaction (RT-PCR) has been carried out using total RNA isolated from different developmental stages (Fig. 1A). No maternal expression of xMAK was detected in eggs and early embryos. Zygotic xMAK transcripts appeared after the onset of zygotic transcription at the midblastula transition, and remained expressed throughout later development, with the highest levels during neurulation (stages 13-19, Fig. 1A).

We next studied the spatial distribution of *xMAK* transcripts by whole-mount in situ hybridization (Fig. 1B-M). Consistent with the RT-PCR data, no signal was detected in stage 7 embryos. At stage 10, *xMAK* expression was predominantly in prospective ectoderm and involuting mesoderm, although the signal may have been weaker in the vegetal region owing to poor probe penetration (Fig. 1C). At neurulation, *xMAK* transcripts were present in the deep (sensorial) layer of epidermal ectoderm and somitogenic mesoderm (Fig. 1E,N,O). x*MAK* transcripts were also detected in the developing eyes and as a well-defined stripe in the brain (Fig. 1D). The comparison with two additional markers, *Engrailed 2* (*En2*), a marker for the midbrain-hindbrain boundary (MHB), and *Krox20*, which is expressed in rhombomeres 3 and 5 (Fig. 1H), revealed that the brain-specific expression of *xMAK* is restricted to the MHB. At later stages, *xMAK* expression was observed in the eyes, branchial arches, the otic vesicle and the tailbud (Fig. 1J,K). No staining was detected with the sense probe used as a negative control (Fig. 1F,G,I,L,M). These results demonstrate that *xMAK* expression is dynamically regulated during development.

Gain-of-function phenotype in embryos overexpressing MAK

To evaluate activity of MAK in a gain-of-function assay, mRNAs encoding tagged MAK or its kinase-dead mutant (MAK-KD) have been injected in the dorsal margin of four-cell embryos (Fig. 2A). We observed that MAK RNA, but not the control GFP RNA, caused strong morphogenetic abnormalities, eye and anterior brain deficiencies in injected embryos. In a typical experiment, MAK RNA produced axis extension defects in 82% of injected embryos (n=27) at stage 38. MAK-KD RNA did not have a significant effect on gastrulation or neurulation movements, indicating that MAK enzymatic activity is necessary for the morphogenetic defects observed. The majority (93%) of MAK RNA-injected embryos had incomplete or absent retinal pigmentation, and some were missing anterior head structures (Fig. 2A). MAK-KD RNA injections also produced mild eye deficiencies at lower frequency (Fig. 2A). These observations suggest that MAK may be involved in the control of morphogenetic movements, head and eye development.

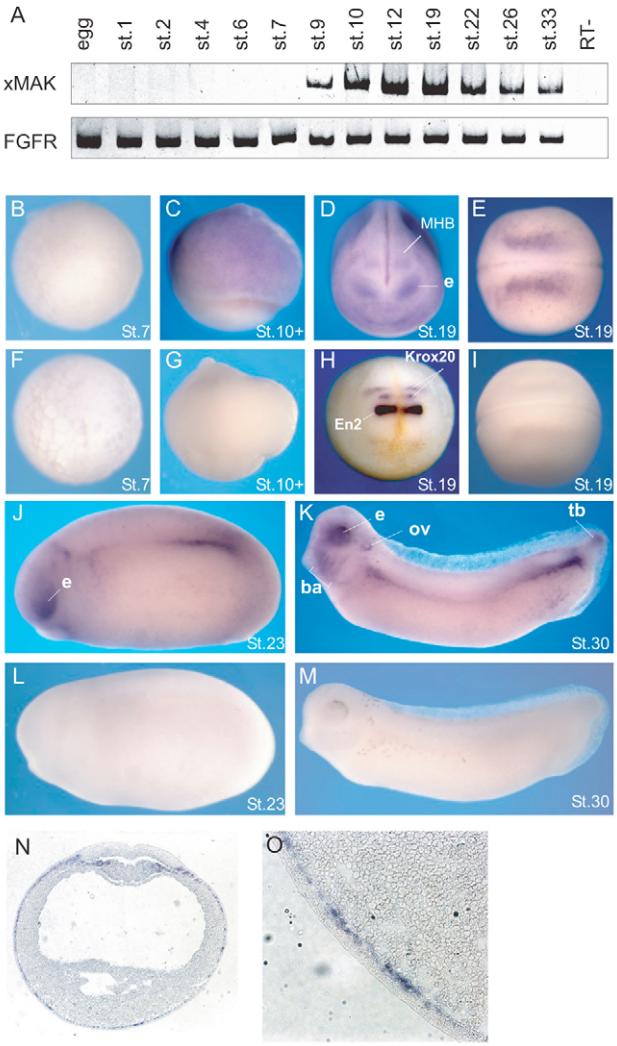


Fig. 1. xMAK expression is dynamically regulated during *Xenopus* development. (**A**) The analysis of xMAK expression at different developmental stages. Total RNA from embryos isolated at different developmental stages was used for RT-PCR. FGFR served as a loading control. RT–, no reverse transcriptase. (**B-M**) Spatial distribution of xMAK RNA revealed by whole-mount in situ hybridization of albino embryos at indicated stages. (B-E,J,K) xMAK antisense probe. (F,G,I,L,M) xMAK sense probe. (D) Anterior view; e, eye; MHB, midbrain-hindbrain boundary. (H) En2 and Krox20 probes, anterior view. En2 is expressed as a bright band at the MHB, located anterior to Krox20, which marks rhombomeres 3 and 5. (B,F) Animal pole view; (C,G) lateral view; (E,I) dorsal view, anterior is towards the left. (J-M) Lateral view, anterior is towards the left. (K) e, eye; ov, otic vesicle; ba, branchial arches; tb, tailbud. (**N**,**O**) A cross-section of a stage 17 neurula embryo after in situ hybridization with xMAK antisense probe. Staining is observed in the deep (sensorial) layer of epidermal ectoderm (magnified view shown in O) and in somitogenic mesoderm.

To evaluate whether MAK selectively interferes with morphogenetic movements or cell fate specification, we analyzed elongation of animal cap explants treated with activin, a common model for convergent extension (Sokol, 1996; Symes and Smith, 1987). Whereas MAK-KD RNA-injected and control uninjected explants elongated in response to activin, explants expressing wild-type MAK failed to elongate under identical culture conditions (Fig.

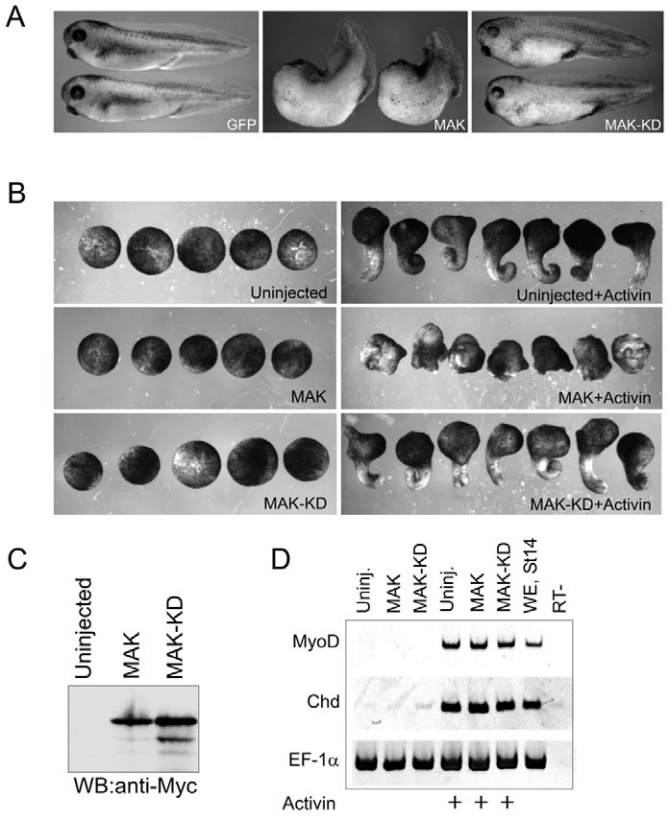


Fig. 2. MAK causes morphogenetic defects and head abnormalities. Four-cell stage embryos were injected into dorsal equatorial region (A, two injections) or animal pole region (B-D, four injections), with RNAs encoding wild-type MAK, MAK KD or GFP, as indicated, at 2 ng per injection. (**A**) Morphological analysis of MAK-expressing embryos. Embryos were fixed at stage 38 and scored for axis elongation and head defects. (**B**) The effect of MAK on morphogenetic movements. Animal caps were isolated from injected midblastula embryos and treated with 50 ng/ml of activin. MAK, but not MAK KD, inhibited animal cap elongation in response to activin. (**C**) Expression levels of Myc-tagged MAK and MAK-KD, revealed by Western blot analysis with anti-Myc antibodies in lysates of injected embryos at stage 10. (**D**) MAK does not inhibit mesoderm markers induced in animal caps by activin. Animal cap induction was as in B. The mesodermal markers *MyoD, Chordin* and *Goosecoid* were assessed in animal caps by RT-PCR, when sibling embryos reached stage 14. Whole embryo (WE) RNA is a positive control. Uninj., animal caps from uninjected embryos. RT–, no reverse transcriptase. EF-1α is a loading control.

2B). Both proteins were expressed equally well in injected embryos, demonstrating that the difference in activity is not due to different expression levels (Fig. 2C).

To evaluate whether the effect of MAK on convergent extension movements in animal pole explants may be due to a block in cell responsiveness to activin, we assessed mesodermal markers in animal caps cultured until stage 14 using RT-PCR (Fig. 2D). Neither MAK nor MAK-KD inhibited the induction of *MyoD* and *Chordin* by activin. This observation suggests that MAK has a specific effect on convergent extension, rather than on the ability of animal pole cells to respond to activin. Together, these observations demonstrate that active MAK is a potent regulator of morphogenetic movements and eye development in early embryos.

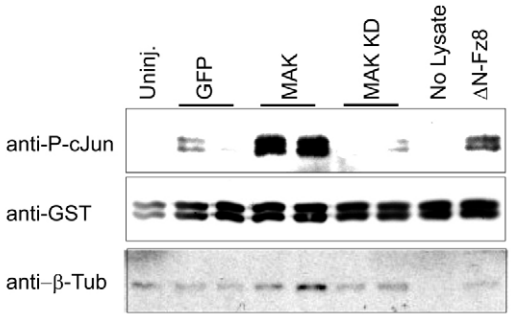


Fig. 3. MAK is an activator of JNK. JNK activity was analyzed in embryonic lysates by in vitro phosphorylation of GST-Jun(1-135). Four-cell embryos were injected marginally four times with GFP, MAK or MAK-KD RNA (1.5 ng per injection), or ΔN-Fz8 RNA (0.5 ng per injection) as a positive control. Lysates were collected at stage 14 for JNK activity determination by western analysis with anti-phospho-Jun antibodies. Assays were carried out in duplicates, with 10 embryos in each experimental group. Anti-GST and anti-β-tubulin antibodies indicate loading.

MAK is an activator of Jun N-terminal kinase

The strong effect of the wild-type MAK on convergent extension movements suggested that MAK may be involved in non-canonical Wnt signaling. Several noncanonical Wnt signaling components, including Dsh, Frizzled, Prickle and Strabismus, have been reported to activate JNK in mammalian cells and *Xenopus* embryonic ectoderm (Boutros et al., 1998; Lisovsky et al., 2002; Park and Moon, 2002; Takeuchi et al., 2003). As JNK activation is often associated with non-canonical Wnt signaling, we measured Jun phosphorylation in lysates of embryos injected with wild type MAK or MAK-KD RNA. We observed that MAK, but not MAK-KD, activated JNK in injected embryos (Fig. 3), suggesting a role for MAK in noncanonical Wnt signaling. Both constructs were expressed at equal levels (data not shown). An activated form of Fz8, previously reported to induce JNK activity (Lisovsky et al., 2002) served as a positive control in this assay. The effects of MAK on morphogenetic movements and the ability to activate JNK are consistent with the postulated function of MAK in noncanonical signaling.

Interactions of MAK with Frizzled and Dsh

To gain further insight into the molecular function of MAK, we studied whether upstream Wnt signaling components influence the distribution of MAK in the cell. For these experiments, we analyzed the subcellular localization of GFP-tagged xMAK in animal cap cells of stage 10 embryos. xMAK-GFP exhibited a punctuated cytoplasmic distribution pattern (Fig. 4A). Co-expression of Frizzled 3 RNA dramatically altered this pattern and recruited xMAK-GFP to the cell membrane. This effect was not observed upon co-expression of Frizzled 2 or 7 RNA (Fig. 4A and data not shown), indicating that the effect of Frizzled 3 is specific. These observations suggest that the subcellular localization of MAK depends on Frizzled signaling.

Dsh is another protein reported to be recruited by Frizzled to the cell membrane, and this recruitment has been considered to be crucial for non-canonical signaling (Axelrod et al., 1998; Boutros et al., 1998; Rothbacher et al., 2000; Yang-Snyder et al., 1996). Therefore, we tested whether MAK can physically associate with Dsh. We found that MAK co-immunoprecipitated with Dsh in lysates of injected embryos (Fig. 4B). To assess whether this association is functional, we performed an immune complex kinase

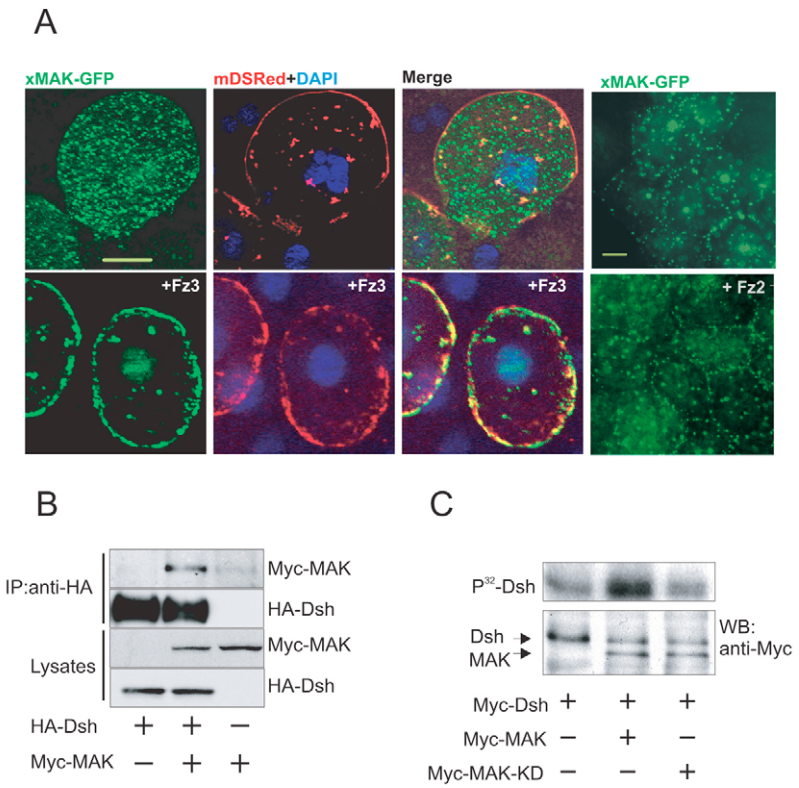


Fig. 4. MAK interacts with Frizzled and Dishevelled. (**A**) Fz3 RNA recruits MAK to the cell membrane in animal pole cells. xMAK-GFP RNA was injected either with membrane-targeted dsRed RNA or with dsRed and Fz3 or Fz2 RNAs and GFP fluorescence was visualized in animal pole cells isolated from injected embryos at stage 10. xMAK-GFP is distributed in a punctate cytoplasmic pattern. Membrane-targeted dsRed RNA allows visualization of the cell membrane. Upon Fz3 RNA co-expression, xMAK-GFP is recruited to the cell membrane. The bottom right panel shows lack of effect of Fz2 RNA on xMAK-GFP localization (compare with top right panel). Bars on top left and top right panels are 5 μm. (**B**) MAK co-immunoprecipitates with Dsh. Lysates of embryos injected with Myc-MAK RNA (2 ng) and/or with HA-Dsh RNA (4 ng) into each cell of four-cell embryos were collected at stage 10 and immunoprecipitated with anti-HA antibodies. Western analysis with anti-Myc antibodies reveals a complex of Dsh and MAK. (**C**) In vitro phosphorylation of Dsh by MAK. Autoradiography (upper panel) reveals phosphorylation of in vitro translated Myc-Dsh that was co-immunoprecipitated with Myc-MAK or Myc-MAK-KD from in vitro translated lysates. Lower panel shows protein levels.

assay in vitro and demonstrated that MAK can phosphorylate Dsh (Fig. 4C). To exclude a possibility that another kinase is responsible for Dsh phosphorylation, we used MAK-KD as a control. As expected, the MAK-KD mutant did not have any kinase activity towards Dsh. This finding shows that Dsh may represent a molecular substrate for MAK in vivo.

Together, these experiments show that MAK is recruited to the cell membrane by Frizzled 3 and can associate with and phosphorylate Dsh. These observations provide a possible mechanism for MAK action in the Wnt pathway and during early development.

MAK plays essential roles in axis elongation and eye development

To further investigate a role for MAK in development in loss-of-function studies, an antisense morpholino oligonucleotide (MAK MO) has been designed to suppress MAK RNA translation in a sequence-specific manner. Indeed, MAK MO, but not a control MO (COMO), inhibited in vitro translation of *xMAK* RNA in rabbit reticulocyte lysates (Fig. 5A). MAK MO did not suppress the translation of *mMAK* RNA that lacks MO target sequence. More importantly, MAK MO caused a reduction of xMAK protein levels in vivo in injected embryos (Fig. 5B).

To assess a developmental role for xMAK, we examined the phenotype of embryos injected with MAK MO into both dorsal blastomeres at the four-cell stage. At stage 38, the majority of MAK-depleted embryos, but not those injected with the same dose of COMO, exhibited shortened trunks and tails and severe retinal defects (Fig. 5C, Table 1). The requirement of MAK for eye development correlates with the observed expression of MAK in the eye field (Fig. 1D). The effect of MAK MO on retinal development was cell-autonomous (Fig. 5D,E). The eye defect was dose dependent, as lower doses of MAK MO had a less pronounced effect on eye pigmentation (data not shown), and it was partially rescued by co-injection of *mMAK* mRNA, lacking MO target sequence (Fig. 5G,H). These observations indicate that the effect of MAK MO is specific and demonstrate a role for MAK in axis elongation and eye development. Thus, the same processes are affected in both gain-of-function and loss-of-function experiments, indicating that MAK levels are under a tight control during normal development.

MAK inhibits Wnt signaling

Our data indicate that MAK is an activator of JNK and may be involved in noncanonical Wnt signaling, which is essential for the control of morphogenetic movements and eye development. We next wanted to assess whether MAK may regulate canonical Wnt signaling, which is crucial for early dorsal development in vertebrate embryos (Harland and Gerhart, 1997). To test this possibility, we assessed the effect of MAK on secondary axis induction by Dsh (Sokol et al., 1995). In a representative experiment, Dsh RNA induced complete body axes with eyes and head structures in 81% of injected embryos (n=27, Fig. 6A,B). By contrast, when *MAK* RNA was co-injected, only 16% embryos

Table 1. Effects of MAK MO on axis elongation and eye development

Group	*n*	Number of embryos with short axes (% of total)	Average eye index
MAK MO	43	43 (100)	0.63
CO MO	35	6 (17)	2.7
Uninjected	44	0	2.92

Four-cell embryos were injected with MOs into the equatorial region of two dorsal blastomeres (80 ng each blastomere). Phenotypes were scored at stage 38. *n*, number of scored embryos. Embryos were scored with short axes if their length was less that 80% of that of control uninjected embryos. The eye index was measured as described in Fig. 5 legend. The data were combined from two separate experiments.

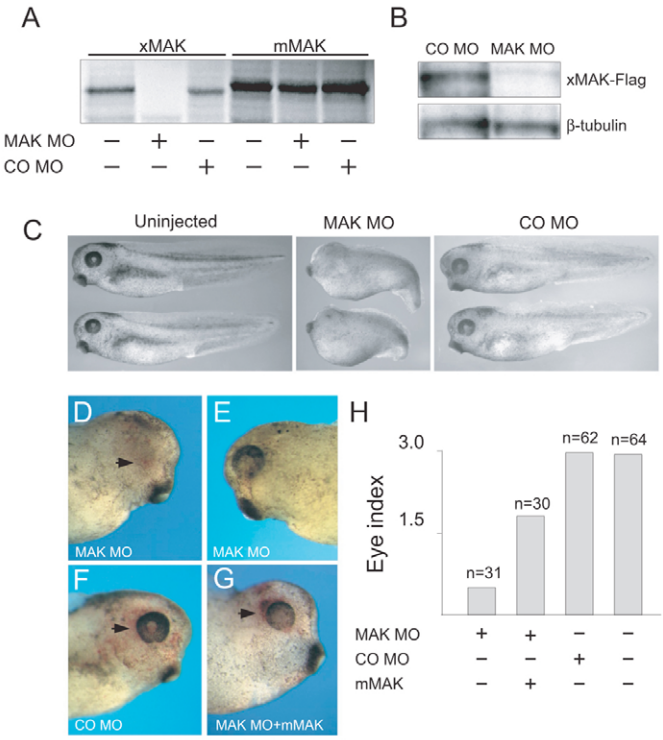


Fig. 5. Axis elongation and eye defects in MAK-depleted embryos. (**A**) xMAK MO specifically inhibits in vitro translation of xMAK RNA. Autoradiography of in vitro translated ^{35}S-methionine-labeled proteins is shown. No suppression is observed for mMAK RNA, which lacks morpholino target sequence. (**B**) MAK MO inhibits xMAK translation in vivo. Western analysis with anti-Flag antibodies shows that MAK MO, but not COMO, effectively downregulated levels of C-terminally tagged xMAK in injected embryos. Loading is controlled with anti-β-tubulin. (**C**) MAK MO or COMO was injected into two dorsal blastomeres of four-cell embryos. At stage 38, MAK-depleted embryos had shortened axes and eye deficiencies (see also Table 2). (**D-H**) Eye defects caused by MAK MO injection can be partially rescued by mouse MAK RNA in stage 38 embryos. Eight-cell embryos were injected into one animal dorsal blastomere with nβgal RNA as a lineage tracer, together with MAK MO (D,E), COMO (F) or MAK MO and mouse MAK RNA (G). (D,E) Both sides of the same injected embryo. Red staining (arrowheads) reflects lineage tracing. (H) The average eye index was calculated for each group of embryos at stage 38 as follows. 0, no visible eye; 1, severely disrupted retina with little pigmentation; 2, small or partially pigmented eye; 3, wild-type eye.

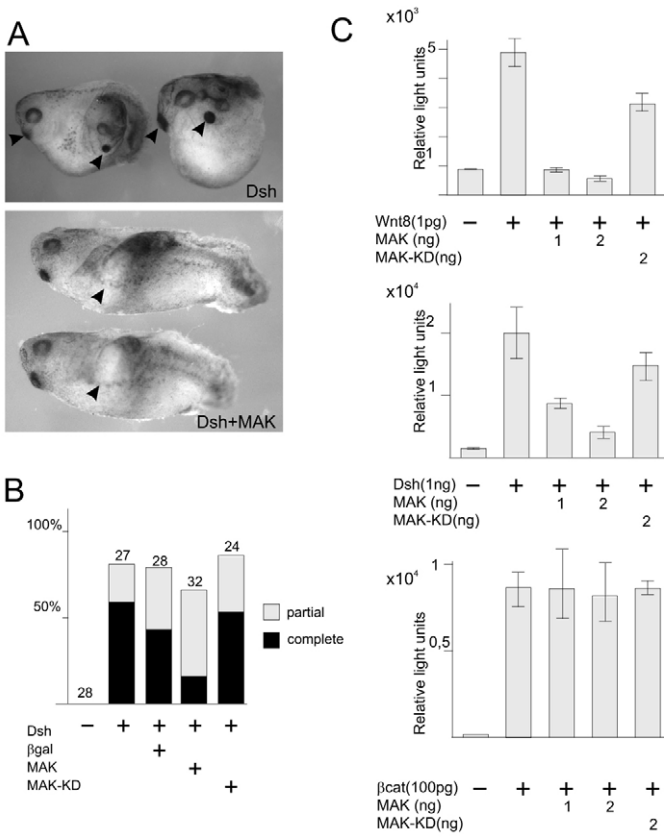


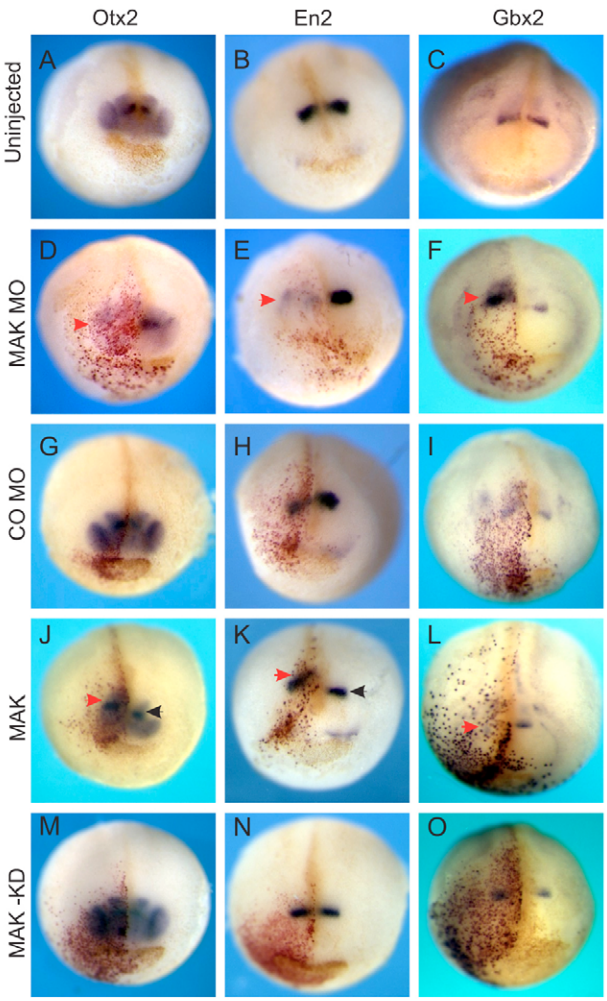
Fig. 6. MAK inhibits Wnt signaling. (**A**,**B**) Axis-inducing activity of Dsh is suppressed by MAK, but not MAK-KD RNA. (A) Indicated RNAs (2 ng) were injected into a single ventral cell of four-cell embryos and secondary axes were assessed at stages 35-38. (B) Summary of axis induction assays, demonstrating that MAK, but not MAK-KD, inhibits Dsh signaling. Partial axes contained secondary neural tubes, usually up to the hindbrain level, without eyes, complete secondary axes contained eyes and/or cement glands. (**C**) MAK inhibits *pSiaLuc* reporter activation induced by Xwnt8 and Dsh, but not by β-catenin. Luciferase activity was determined in lysates of embryos injected with 20 pg of *pSiaLuc* and indicated mRNAs. Results shown in relative light units represent means of three samples±s.d., with five embryos in each sample.

(*n*=32) had complete secondary axes with head structures. Co-injection of the control *nβgal* RNA or *MAK-KD* RNA did not significantly alter the axis-inducing activity of Dsh (Fig. 6B). Consistent with these results, *MAK*, but not *MAK-KD*, RNA dose-dependently suppressed Dsh-mediated induction of the *pSiaLuc* reporter, a direct readout of the canonical pathway (Fig. 6C) (Fan et al., 1998).

To position MAK in the Wnt/β-catenin pathway, *MAK* RNA was co-injected with Xwnt8 RNA and with β-catenin RNA. Whereas MAK efficiently blocked Xwnt8 signaling activity, no effect on β-catenin-dependent reporter stimulation has been observed (Fig. 6C). These findings indicate that MAK is an efficient inhibitor of Wnt signaling, and this inhibition requires MAK kinase activity. Moreover, MAK appears to inhibit Wnt signaling upstream or parallel to β-catenin.

MAK is involved in regional brain patterning

At neurula stages, *xMAK* transcripts are present in the isthmus, suggesting a role for MAK in regional brain patterning. To test this possibility, we analyzed embryos with altered levels of MAK by in situ hybridization with region-specific brain markers. MAK MO or MAK RNA was injected into a single dorsal-animal blastomere of the four to eight-cell embryo. At stage 20, the injected embryos were fixed and hybridized with probes for the anterior brain marker *Otx2* (Blitz and Cho, 1995; Pannese et al., 1995), the midbrain-hindbrain boundary marker *En2* (Brivanlou and Harland, 1989; Joyner and Martin, 1987) and the hindbrain marker *Gbx2* (Millet et al., 1999; von Bubnoff et al., 1996). Embryos were scored based on the difference in marker intensity between injected and uninjected sides (Fig. 7A-O). We have found that MAK MO, but not the control MO, caused unilateral suppression or loss of *Otx2* and *En2* in 71% (*n*=58) and 28% (*n*=50) of injected embryos, respectively (Table 2). This effect was accompanied by the upregulation of the posterior neural marker *Gbx2* in 71% (*n*=62) of injected embryos. These results demonstrate a posteriorizing effect of MAK MO on the developing midbrain-hindbrain boundary.



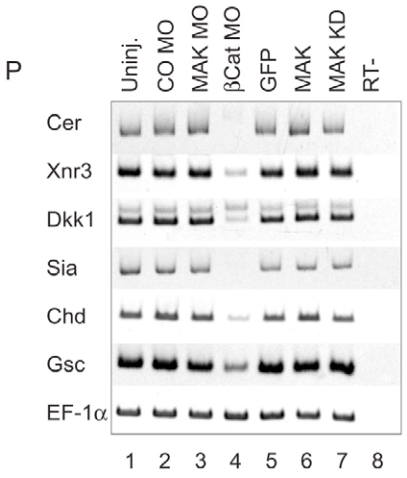


Fig. 7. MAK functions in regional brain patterning, but does not affect organizer markers. (**A-O**) MAK regulates midbrain-hindbrain boundary. Dorsal-animal region of four-cell embryos was injected with 50 ng of MOs or 2 ng of MAK RNAs, as indicated, together with nβgal RNA as a lineage tracer. At stage 20, *Otx2*, *En2* and *Gbx2* were assessed by whole-mount in situ hybridization. MAK MO inhibited *Otx2* and *En2* (D,E), but upregulated *Gbx2* (F). MAK RNA expanded and posteriorly shifted *Otx2* and *En2* on the injected side (J,K), and inhibited *Gbx2* (L). Red arrows indicate altered expression; black arrows indicate marker expression on the uninjected side. (**P**) MAK depletion or overexpression does not affect organizer marker expression. Two to four-cell embryos were injected twice in the dorsal margin with MAK MO or COMO (100 ng), β-catenin MO (30 ng) or indicated RNA (4 ng). RT-PCR was carried out with total RNA from stage 10 embryos. DNA fragments were separated in 6% SDS-polyacrylamide gel. EF-1α is a loading control. Uninj, uninjected embryos. RT–, no reverse transcriptase.

In a complementary gain-of-function experiment, embryos have been injected with wild-type MAK RNA and MAK-KD RNA. MAK RNA caused an effect opposite to that observed for MAK MO. MAK RNA inhibited *Gbx2* in 53% (*n*=32) of injected embryos, whereas *Otx2* and *En2* expression bands shifted to a more posterior position in 38% (*n*=42) and 37% (*n*=41) of injected embryos, respectively (Table 2), suggesting that MAK anteriorizes neural tissue. Together with the observed expression of MAK at the midbrain-hindbrain boundary (Fig. 1D), these findings are consistent with the hypothesis that MAK is involved in the establishment of the isthmic organizer (Millet et al., 1999; Wurst and Bally-Cuif, 2001).

As MAK regulates the Wnt pathway, and this pathway is essential for Spemann organizer function, the observed effects of MAK on brain development may be a secondary consequence of the impaired organizer function. We evaluated this possibility by comparing expression levels of several organizer markers (Stennard et al., 1997) in injected embryos at the beginning of gastrulation. RT-PCR analysis did not reveal any significant changes for *Chordin* (*Chd*), *Goosecoid* (*Gsc*), *Xenopus nodal-related 3* (*Xnr3*), *Siamois* (*Sia*), *Dickkopf1* (*Dkk1*) *and Cerberus* (*Cer*) in embryos depleted of MAK or overexpressing MAK (Fig. 7P). By contrast, β-catenin MO significantly downregulated organizer markers, demonstrating high sensitivity of this assay. These observations suggest that MAK functions to establish the midbrain-hindbrain boundary (MHB) by influencing neural tissue directly, rather than by affecting the organizer.

MAK regulates the midbrain-hindbrain boundary by inhibiting β-catenin signaling

The formation of the isthmic organizer at the midbrain-hindbrain boundary is known to require canonical Wnt signaling (Hidalgo-Sanchez et al., 2005; Kunz et al., 2004). Interestingly, the observed effect of MAK on the midbrain-hindbrain boundary closely resembles the effect of β-catenin MO (Wu et al., 2005). This similarity and the potential inhibitory role for MAK in Wnt signaling (Fig. 6) indicate that MAK may regulate midbrain-hindbrain boundary by inhibiting the canonical pathway.

To test this hypothesis, we attempted to rescue the effect of MAK MO by depleting β-catenin. We studied the expression of *Otx2* and *Gbx2* by in situ hybridization in embryos co-injected into a single animal dorsal blastomere with MAK MO and β-catenin MO (Fig. 8). Marker expression was scored by comparing injected and uninjected sides of stage 20 embryos, which have been identified by lineage tracing with β-galactosidase. MAK MO induced Gbx2 in 86% (*n*=14) of injected embryos and downregulated *Otx2* in 70% (*n*=23) of injected embryos. Upon β-catenin MO co-injection, the induction of *Gbx2* was suppressed, whereas *Otx2* levels did not change in the majority (65%, *n*=40) of injected embryos. We conclude that β-catenin MO rescued normal brain patterning in MAK-depleted embryos. Thus, the effects of MAK depletion on

Table 2. A role for MAK in midbrain hindbrain boundary formation

Group	Gene	*n*	Same level, *n* (%)	Reduced, *n* (%)	Induced, *n* (%)	Posterior shift, *n* (%)
MAK MO	*Otx2*	58	16 (27)	41 (71)	1 (2)	
	En2	50	36 (72)	14 (28)	0	
	Gbx2	62	11 (18)	7 (11)	44 (71)	
CO MO	*Otx2*	44	44 (96)	0	0	
	En2	49	48 (98)	1(2)	0	
	Gbx2	44	43 (98)	0	1 (2)	
Uninjected	*Otx2*	30	30 (100)			
	En2	23	23 (100)			
	Gbx2	34	34 (100)			
MAK	*Otx2*	47	15 (36)	6 (19)	10 (24)	16 (38)
	En2	42	16 (39)	9 (22)	2 (5)	15 (37)
	Gbx2	32	13 (40)	17(53)	2 (6)	0
MAK KD	*Otx2*	30	30 (100)			
	En2	21	21 (100)			
	Gbx2	30	28 (94)	1 (3)	1 (3)	

Embryos were injected with 50 ng of MOs or 2 ng of MAK RNAs into one dorsal animal blastomere at the four/eight-cell stage. At stage 19, embryos were fixed and expression of *Otx2, En2* and *Gbx2* were analyzed by in situ hybridization. Marker expression was assessed by comparing the injected and uninjected sides. *n* is number of scored embryos; percentage of affected embryos is shown in parentheses.

Otx2 and *Gbx2* expression are probably due to canonical pathway upregulation at the midbrain-hindbrain boundary, which is consistent with an inhibitory role for MAK in Wnt/β-catenin signaling during midbrain patterning.

DISCUSSION

This study identifies MAK, a member of the SNF1 kinase family, as a novel modulator of Wnt signaling. By the onset of gastrulation, MAK is present in mesodermal and ectodermal tissues in a broad pattern. At neurula stages, the major regions of MAK expression are in the developing eyes, the isthmus and the somites. Consistent with this pattern, our loss- and gain-of-function experiments suggest that during early development MAK is required for convergent extension movements, eye development and the midbrain-hindbrain boundary specification. We note that all these developmental processes are known to be controlled by Wnt signaling (Cavodeassi et al., 2005; Liu and Joyner, 2001; Sokol, 2000). In mouse embryos, MAK has been implicated in mammary gland development, another process regulated by Wnt signaling (Gardner et al., 2000a). In our experiments, Frizzled 3 dramatically altered MAK intracellular localization by recruiting it to the cell membrane (Fig. 4A), demonstrating that MAK can respond to Wnt signaling. The effects of MAK on convergent extension and on canonical Wnt signaling require its kinase activity, suggesting that phosphorylation of a putative MAK substrate plays a major role in signaling outcome. In a search for possible MAK substrates, we found that MAK associates with Dsh and is able to phosphorylate Dsh in vitro. As Dsh has been implicated in gastrulation movements (Sokol, 1996) and brain patterning (Hamblet et al., 2002; Itoh and Sokol, 1997), it may be a relevant molecular target of MAK during these developmental processes.

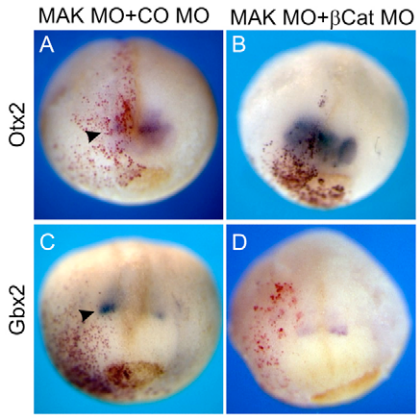


Fig. 8. Depletion of β-catenin restores brain defects caused by MAK MO. Embryos were injected with MAK MO (50 ng) and COMO (8 ng, **A**,**C**) or β-catenin MO (8 ng, **B**,**D**) into one dorsal animal blastomere at the four- to eight-cell stage. Whole-mount in situ hybridization of stage 19 embryos demonstrates that the effects of MAK MO on *Otx2* and *Gbx2* expression were partially reversed by β-catenin MO (see also Table 2). Arrowheads in A and C indicate altered marker expression in MAK-depleted embryos. Nuclear βgal RNA served as a lineage tracer.

The involvement of MAK in midbrain-hindbrain boundary regulation is most probably due to the inhibition of the canonical β-catenin signaling in the isthmus, as β-catenin MO suppresses the effect of MAK MO (Fig. 8). By contrast, inhibition of convergent extension movements in activin-treated explants overexpressing MAK and in embryos injected with MAK RNA or MAK MO (Figs 2, 5) may relate to a noncanonical branch of Wnt signaling. In agreement with this hypothesis, MAK stimulates JNK activity, a common downstream target of noncanonical Wnt signaling (Veeman et al., 2003). Together, these experiments indicate that MAK functions to stimulate non-canonical Wnt signaling, but negatively regulates the canonical pathway. Thus, MAK acts in a manner opposite to that of PAR-1, a related SNF1-like kinase, which activates the canonical pathway but suppresses the noncanonical pathway by downregulating JNK (Ossipova et al., 2005; Sun et al., 2001).

In the isthmic organizer, canonical Wnt signaling may be responsible for the transcriptional activation of several target genes, including those encoding Wnt pathway components, e.g. Wnt1 (McMahon and Bradley, 1990), Axin (Hedgepeth et al., 1999), LEF1/TCF (Kunz et al., 2004; Molenaar et al., 1998) and Frodo (Gillhouse et al., 2004). Consistent with the hypothesis that MAK modulates Wnt signaling in the brain, the loss-of-function

experiments with embryos injected with MAK MO resulted in inhibition of the anterior brain marker *Otx2* and the isthmus marker *En2*, but caused upregulation of *Gbx2*, the posterior brain marker (Fig. 7). MAK injections did not substantially upregulate *Otx2* and *En2*, but shifted these markers more posteriorly (Fig. 7J,K). Together with the lack of effect of MAK on organizer markers (Fig. 7P), this indicates that MAK regulates midbrain-hindbrain boundary specification, rather than causes a more general effect on anteroposterior patterning or Spemann organizer formation. In these experiments, MAK phenocopies the effect of β-catenin MO injection (Wu et al., 2005), confirming our hypothesis that it is due to MAK-mediated inhibition of canonical Wnt signaling. The restricted effect of MAK on the midbrain-hindbrain boundary suggests that the isthmic organizer differs from other brain regions in the responsiveness to Wnt/β-catenin signaling, perhaps by expressing region-specific competence factors. This hypothesis is supported by our observation that brain-specific expression of xMAK is restricted to the midbrain-hindbrain boundary.

MAK does not seem to be able to affect dorsal mesoderm markers when overexpressed in the organizer (Fig. 7P). This finding suggests that MAK is a selective inhibitor of Wnt signaling in MHB, but not at the earlier developmental stage. One reason is that MAK is not present in fertilized eggs and does not function in early axial patterning. It is also possible that the upstream components of Wnt signaling including MAK are not involved in organizer formation in early embryogenesis (Harland and Gerhart, 1997; Sokol, 1996), whereas they may be crucial at an earlier stage, e.g. during oocyte development (Tao et al., 2005). As secreted Wnt antagonists and Dsh interfering mutants do not influence organizer development (Glinka et al., 1998), it has been hypothesized that the pathway is activated by a ligand-independent mechanism (Sokol, 1996).

At present, how MAK redirects Wnt signaling from the canonical to the noncanonical mode is unclear. A simple hypothesis is that it does so by phosphorylation of Dsh. Thus, the identification of MAK-specific phosphorylation sites in Dsh will be important in future studies of MAK. Alternatively, MAK may have other molecular targets that are crucial for signaling, as other protein kinases, including casein kinase 1 and GSK3, function in Wnt signaling by phosphorylating multiple substrates (Lee et al., 2001; Zeng et al., 2005).

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References

Axelrod, J. D., Miller, J. R., Shulman, J. M., Moon, R. T. and Perrimon, N. (1998). Differential recruitment of Dishevelled provides signaling specificity in the planar cell polarity and Wingless signaling pathways. *Genes Dev.* **12**, 2610-2622.

Bainter, J. J., Boos, A. and Kroll, K. L. (2001). Neural induction takes a transcriptional twist. *Dev. Dyn.* **222**, 315-327.

Becker, J. and Brendel, M. (1996). Molecular characterization of the xerC gene of Lactobacillus leichmannii encoding a site-specific recombinase and two adjacent heat shock genes. *Curr. Microbiol.* **32**, 232-236.

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

Bohm, H., Brinkmann, V., Drab, M., Henske, A. and Kurzchalia, T. V. (1997). Mammalian homologues of C. elegans PAR-1 are asymmetrically localized in epithelial cells and may influence their polarity. *Curr. Biol.* **7**, 603-606.

Boutros, M., Paricio, N., Strutt, D. I. and Mlodzik, M. (1998). Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. *Cell* **94**, 109-118.

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.

Brivanlou, A. H. and Harland, R. M. (1989). Expression of an engrailed-related protein is induced in the anterior neural ectoderm of early *Xenopus* embryos. *Development* **106**, 611-617.

Cavodeassi, F., Carreira-Barbosa, F., Young, R. M., Concha, M. L., Allende, M. L., Houart, C., Tada, M. and Wilson, S. W. (2005). Early stages of zebrafish eye formation require the coordinated activity of Wnt11, Fz5, and the Wnt/beta-catenin pathway. *Neuron* **47**, 43-56.

Chi, C. L., Martinez, S., Wurst, W. and Martin, G. R. (2003). The isthmic organizer signal FGF8 is required for cell survival in the prospective midbrain and cerebellum. *Development* **130**, 2633-2644.

Ciani, L. and Salinas, P. C. (2005). WNTs in the vertebrate nervous system: from patterning to neuronal connectivity. *Nat. Rev. Neurosci.* **6**, 351-362.

Drewes, G. and Nurse, P. (2003). The protein kinase kin1, the fission yeast orthologue of mammalian MARK/PAR-1, localises to new cell ends after mitosis and is important for bipolar growth. *FEBS Lett.* **554**, 45-49.

Fan, M. J., Gruning, W., Walz, G. and Sokol, S. Y. (1998). Wnt signaling and transcriptional control of Siamois in *Xenopus* embryos. *Proc. Natl. Acad. Sci. USA* **95**, 5626-5631.

Gamse, J. and Sive, H. (2000). Vertebrate anteroposterior patterning: the *Xenopus* neurectoderm as a paradigm. *BioEssays* **22**, 976-986.

Gardner, H. P., Belka, G. K., Wertheim, G. B., Hartman, J. L., Ha, S. I., Gimotty, P. A., Marquis, S. T. and Chodosh, L. A. (2000a). Developmental role of the SNF1-related kinase Hunk in pregnancy-induced changes in the mammary gland. *Development* **127**, 4493-4509.

Gardner, H. P., Wertheim, G. B., Ha, S. I., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Marquis, S. T. and Chodosh, L. A. (2000b). Cloning and characterization of Hunk, a novel mammalian SNF1-related protein kinase. *Genomics* **63**, 46-59.

Gillhouse, M., Wagner Nyholm, M., Hikasa, H., Sokol, S. Y. and Grinblat, Y. (2004). Two Frodo/Dapper homologs are expressed in the developing brain and mesoderm of zebrafish. *Dev. Dyn.* **230**, 403-409.

Glinka, A., Wu, W., Delius, H., Monaghan, A. P., 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.

Gloy, J., Hikasa, H. and Sokol, S. Y. (2002). Frodo interacts with Dishevelled to transduce Wnt signals. *Nat. Cell Biol.* **4**, 351-357.

Habas, R., Kato, Y. and He, X. (2001). Wnt/Frizzled activation of Rho regulates vertebrate gastrulation and requires a novel Formin homology protein Daam1. *Cell* **107**, 843-854.

Habas, R., Dawid, I. B. and He, X. (2003). Coactivation of Rac and Rho by Wnt/Frizzled signaling is required for vertebrate gastrulation. *Genes Dev.* **17**, 295-309.

Hamblet, N. S., Lijam, N., Ruiz-Lozano, P., Wang, J., Yang, Y., Luo, Z., Mei, L., Chien, K. R., Sussman, D. J. and Wynshaw-Boris, A. (2002). Dishevelled 2 is essential for cardiac outflow tract development, somite segmentation and neural tube closure. *Development* **129**, 5827-5838.

Hardie, D. G., Carling, D. and Carlson, M. (1998). The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? *Annu. Rev. Biochem.* **67**, 821-855.

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

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

Hedgepeth, C. M., Deardorff, M. A. and Klein, P. S. (1999). *Xenopus* axin interacts with glycogen synthase kinase-3 beta and is expressed in the anterior midbrain. *Mech. Dev.* **80**, 147-151.

Hidalgo-Sanchez, M., Millet, S., Bloch-Gallego, E. and Alvarado-Mallart, R. M. (2005). Specification of the meso-isthmo-cerebellar region: the Otx2/Gbx2 boundary. *Brain Res. Brain Res. Rev.* **49**, 134-149.

Hikasa, H. and Sokol, S. Y. (2004). The involvement of Frodo in TCF-dependent signaling and neural tissue development. *Development* **131**, 4725-4734.

Itoh, K. and Sokol, S. Y. (1997). Graded amounts of *Xenopus* dishevelled specify discrete anteroposterior cell fates in prospective ectoderm. *Mech. Dev.* **61**, 113-125.

Itoh, K., Antipova, A., Ratcliffe, M. J. and Sokol, S. (2000). Interaction of dishevelled and *Xenopus* axin-related protein is required for wnt signal transduction. *Mol. Cell. Biol.* **20**, 2228-2238.

Itoh, K., Brott, B. K., Bae, G. U., Ratcliffe, M. J. and Sokol, S. Y. (2005). Nuclear localization is required for Dishevelled function in Wnt/beta-catenin signaling. *J Biol.* **4**, 3.

Joyner, A. L. and Martin, G. R. (1987). En-1 and En-2, two mouse genes with sequence homology to the Drosophila engrailed gene: expression during embryogenesis. *Genes Dev.* **1**, 29-38.

Korobko, E. V., Kiselev, S. L. and Korobko, I. V. (2004). Subcellular localization of MAK-V/Hunk protein kinase expressed in COS-1 cells. *Cell Biol. Int.* **28**, 49-56.

Korobko, I. V., Kabishev, A. A. and Kiselev, S. L. (1997). [Identification of the new protein kinase specifically transcribed in mouse tumors with high metastatic potential]. *Dokl. Akad. Nauk.* **354**, 554-556.

Kunz, M., Herrmann, M., Wedlich, D. and Gradl, D. (2004). Autoregulation of canonical Wnt signaling controls midbrain development. *Dev. Biol.* **273**, 390-401.
Lee, E., Salic, A. and Kirschner, M. W. (2001). Physiological regulation of [beta]-catenin stability by Tcf3 and CK1epsilon. *J. Cell Biol.* **154**, 983-993.
Levin, D. E., Hammond, C. I., Ralston, R. O. and Bishop, J. M. (1987). Two yeast genes that encode unusual protein kinases. *Proc. Natl. Acad. Sci. USA* **84**, 6035-6039.
Lisovsky, M., Itoh, K. and Sokol, S. Y. (2002). Frizzled receptors activate a novel JNK-dependent pathway that may lead to apoptosis. *Curr. Biol.* **12**, 53-58.
Liu, A. and Joyner, A. L. (2001). Early anterior/posterior patterning of the midbrain and cerebellum. *Annu. Rev. Neurosci.* **24**, 869-896.
Logan, C. Y. and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* **20**, 781-810.
Marlow, F., Topczewski, J., Sepich, D. and Solnica-Krezel, L. (2002). Zebrafish Rho kinase 2 acts downstream of Wnt11 to mediate cell polarity and effective convergence and extension movements. *Curr. Biol.* **12**, 876-884.
McMahon, A. P. and Bradley, A. (1990). The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell* **62**, 1073-1085.
Millet, S., Campbell, K., Epstein, D. J., Losos, K., Harris, E. and Joyner, A. L. (1999). A role for Gbx2 in repression of Otx2 and positioning the mid/hindbrain organizer. *Nature* **401**, 161-164.
Molenaar, M., Roose, J., Peterson, J., Venanzi, S., Clevers, H. and Destree, O. (1998). Differential expression of the HMG box transcription factors XTcf-3 and XLef-1 during early *Xenopus* development. *Mech. Dev.* **75**, 151-154.
Moon, R. T. and Kimelman, D. (1998). From cortical rotation to organizer gene expression: toward a molecular explanation of axis specification in *Xenopus*. *BioEssays* **20**, 536-545.
Nieuwkoop, P. D., Faber, J. and Hubrecht-Laboratorium (Embryologisch Instituut) (1967). *Normal Table of Xenopus laevis (Daudin); A Systematical and Chronological Survey of the Development from the Fertilized Egg till the End of Metamorphosis.* Amsterdam: North-Holland.
Ossipova, O., Dhawan, S., Sokol, S. and Green, J. B. (2005). Distinct PAR-1 proteins function in different branches of Wnt signaling during vertebrate development. *Dev. Cell* **8**, 829-841.
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.
Park, M. and Moon, R. T. (2002). The planar cell-polarity gene stbm regulates cell behaviour and cell fate in vertebrate embryos. *Nat. Cell Biol.* **4**, 20-25.
Pellettieri, J. and Seydoux, G. (2002). Anterior-posterior polarity in C. elegans and Drosophila–PARallels and differences. *Science* **298**, 1946-1950.
Rothbacher, U., Laurent, M. N., Deardorff, M. A., Klein, P. S., Cho, K. W. and Fraser, S. E. (2000). Dishevelled phosphorylation, subcellular localization and multimerization regulate its role in early embryogenesis. *EMBO J.* **19**, 1010-1022.
Ruiz, J. C., Conlon, F. L. and Robertson, E. J. (1994). Identification of novel protein kinases expressed in the myocardium of the developing mouse heart. *Mech. Dev.* **48**, 153-164.
Ruzov, A. S., Mertsalov, I. B., Meehan, R., Kiselev, S. L., Buchman, V. L. and Korobko, I. V. (2004). Cloning and developmental expression of MARK/Par-1/MELK-related protein kinase xMAK-V in *Xenopus* laevis. *Dev. Genes Evol.* **214**, 139-143.
Sambrook, J., Maniatis, T. and Fritsch, E. F. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
Saneyoshi, T., Kume, S., Amasaki, Y. and Mikoshiba, K. (2002). The Wnt/calcium pathway activates NF-AT and promotes ventral cell fate in *Xenopus* embryos. *Nature* **417**, 295-299.
Sheldahl, L. C., Slusarski, D. C., Pandur, P., Miller, J. R., Kuhl, M. and Moon, R. T. (2003). Dishevelled activates Ca2+ flux, PKC, and CamKII in vertebrate embryos. *J. Cell Biol.* **161**, 769-777.
Shi, D. L., Goisset, C. and Boucaut, J. C. (1998). Expression of Xfz3, a *Xenopus* frizzled family member, is restricted to the early nervous system. *Mech. Dev.* **70**, 35-47.
Shulman, J. M., Benton, R. and St Johnston, D. (2000). The Drosophila homolog of C. elegans PAR-1 organizes the oocyte cytoskeleton and directs oskar mRNA localization to the posterior pole. *Cell* **101**, 377-388.
Slusarski, D. C., Corces, V. G. and Moon, R. T. (1997). Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling. *Nature* **390**, 410-413.
Sokol, S. (2000). A role for Wnts in morpho-genesis and tissue polarity. *Nat. Cell Biol.* **2**, E124-E125.
Sokol, S., Christian, J. L., Moon, R. T. and Melton, D. A. (1991). Injected Wnt RNA induces a complete body axis in *Xenopus* embryos. *Cell* **67**, 741-752.
Sokol, S. Y. (1996). Analysis of Dishevelled signalling pathways during *Xenopus* development. *Curr. Biol.* **6**, 1456-1467.
Sokol, S. Y. (1999). Wnt signaling and dorso-ventral axis specification in vertebrates. *Curr. Opin. Genet. Dev.* **9**, 405-410.
Sokol, S. Y., Klingensmith, J., Perrimon, N. and Itoh, K. (1995). Dorsalizing and neuralizing properties of Xdsh, a maternally expressed *Xenopus* homolog of dishevelled. *Development* **121**, 1637-1647.
Stennard, F., Ryan, K. and Gurdon, J. B. (1997). Markers of vertebrate mesoderm induction. *Curr. Opin. Genet. Dev.* **7**, 620-627.
Sun, T. Q., Lu, B., Feng, J. J., Reinhard, C., Jan, Y. N., Fantl, W. J. and Williams, L. T. (2001). PAR-1 is a Dishevelled-associated kinase and a positive regulator of Wnt signalling. *Nat. Cell Biol.* **3**, 628-636.
Symes, K. and Smith, J. C. (1987). Gastrulation movements provide an early marker of mesoderm induction in *Xenopus* laevis. *Development* **101**, 339-349.
Tada, M. and Smith, J. C. (2000). Xwnt11 is a target of *Xenopus* Brachyury: regulation of gastrulation movements via Dishevelled, but not through the canonical Wnt pathway. *Development* **127**, 2227-2238.
Takeuchi, M., Nakabayashi, J., Sakaguchi, T., Yamamoto, T. S., Takahashi, H., Takeda, H. and Ueno, N. (2003). The prickle-related gene in vertebrates is essential for gastrulation cell movements. *Curr. Biol.* **13**, 674-679.
Tao, Q., Yokota, C., Puck, H., Kofron, M., Birsoy, B., Yan, D., Asashima, M., Wylie, C. C., Lin, X. and Heasman, J. (2005). Maternal wnt11 activates the canonical wnt signaling pathway required for axis formation in *Xenopus* embryos. *Cell* **120**, 857-871.
Tassan, J. P. and Le Goff, X. (2004). An overview of the KIN1/PAR-1/MARK kinase family. *Biol. Cell* **96**, 193-199.
Tomancak, P., Piano, F., Riechmann, V., Gunsalus, K. C., Kemphues, K. J. and Ephrussi, A. (2000). A Drosophila melanogaster homologue of Caenorhabditis elegans par-1 acts at an early step in embryonic-axis formation. *Nat. Cell Biol.* **2**, 458-460.
Turner, D. L. and Weintraub, H. (1994). Expression of achaete-scute homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes Dev.* **8**, 1434-1447.
Unterseher, F., Hefele, J. A., Giehl, K., De Robertis, E. M., Wedlich, D. and Schambony, A. (2004). Paraxial protocadherin coordinates cell polarity during convergent extension via Rho A and JNK. *EMBO J.* **23**, 3259-3269.
Veeman, M. T., Axelrod, J. D. and Moon, R. T. (2003). A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. *Dev. Cell* **5**, 367-377.
von Bubnoff, A., Schmidt, J. E. and Kimelman, D. (1996). The *Xenopus* laevis homeobox gene Xgbx-2 is an early marker of anteroposterior patterning in the ectoderm. *Mech. Dev.* **54**, 149-160.
Wallingford, J. B. and Habas, R. (2005). The developmental biology of Dishevelled: an enigmatic protein governing cell fate and cell polarity. *Development* **132**, 4421-4436.
Wallingford, J. B., Fraser, S. E. and Harland, R. M. (2002). Convergent extension: the molecular control of polarized cell movement during embryonic development. *Dev. Cell* **2**, 695-706.
Winklbauer, R., Medina, A., Swain, R. K. and Steinbeisser, H. (2001). Frizzled-7 signalling controls tissue separation during *Xenopus* gastrulation. *Nature* **413**, 856-860.
Winter, C. G., Wang, B., Ballew, A., Royou, A., Karess, R., Axelrod, J. D. and Luo, L. (2001). Drosophila Rho-associated kinase (Drok) links Frizzled-mediated planar cell polarity signaling to the actin cytoskeleton. *Cell* **105**, 81-91.
Wu, J., Saint-Jeannet, J. P. and Klein, P. S. (2003). Wnt-frizzled signaling in neural crest formation. *Trends Neurosci.* **26**, 40-45.
Wu, J., Yang, J. and Klein, P. S. (2005). Neural crest induction by the canonical Wnt pathway can be dissociated from anterior-posterior neural patterning in *Xenopus*. *Dev. Biol.* **279**, 220-232.
Wurst, W. and Bally-Cuif, L. (2001). Neural plate patterning: upstream and downstream of the isthmic organizer. *Nat. Rev. Neurosci.* **2**, 99-108.
Yanagawa, S., van Leeuwen, F., Wodarz, A., Klingensmith, J. and Nusse, R. (1995). The dishevelled protein is modified by wingless signaling in Drosophila. *Genes Dev.* **9**, 1087-1097.
Yang-Snyder, J., Miller, J. R., Brown, J. D., Lai, C. J. and Moon, R. T. (1996). A frizzled homolog functions in a vertebrate Wnt signaling pathway. *Curr. Biol.* **6**, 1302-1306.
Zeng, X., Tamai, K., Doble, B., Li, S., Huang, H., Habas, R., Okamura, H., Woodgett, J. and He, X. (2005). A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. *Nature* **438**, 873-877.
Zohn, I. E., Chesnutt, C. R. and Niswander, L. (2003). Cell polarity pathways converge and extend to regulate neural tube closure. *Trends Cell Biol.* **13**, 451-454.