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Wise, a context-dependent activator and inhibitor of Wnt signalling

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SUMMARY

We have isolated a novel secreted molecule, Wise, by a functional screen for activities that alter the anteroposterior character of neuralised *Xenopus* animal caps. *Wise* encodes a secreted protein capable of inducing posterior neural markers at a distance. Phenotypes arising from ectopic expression or depletion of *Wise* resemble those obtained when Wnt signalling is altered. In animal cap assays, posterior neural markers can be induced by Wnt family members, and induction of these markers by *Wise* requires components of the canonical Wnt pathway. This indicates that in this context *Wise* activates the Wnt signalling cascade by mimicking some of the effects of Wnt ligands. Activation of the pathway was further confirmed by nuclear accumulation of β-catenin driven by Wise. By contrast, in an assay for secondary axis induction, extracellularly *Wise* antagonises the axis-inducing ability of Wnt8. Thus, *Wise* can activate or inhibit Wnt signalling in a context-dependent manner. The Wise protein physically interacts with the Wnt co-receptor, lipoprotein receptor-related protein 6 (LRP6), and is able to compete with Wnt8 for binding to LRP6. These activities of *Wise* provide a new mechanism for integrating inputs through the Wnt co-receptor complex to modulate the balance of Wnt signalling.

Key words: Wise, Wnt signalling, *Xenopus*

INTRODUCTION

Signalling molecules play key roles in developmental events and their actions are highly regulated by endogenous modulators and antagonists in order to obtain precisely balanced outputs. The process of neural anteroposterior (AP) patterning involves integration of various signals such as retinoic acid (RA), fibroblast growth factors (FGFs) and members of the Wnt family. However, the relative roles of these cascades, the degree to which they are used at any particular axial level, and how they are integrated in organising normal AP patterning is poorly understood. Identification of factors that can modulate existing pathways or that represent novel signalling inputs will be beneficial to our understanding of how AP patterning is coordinated.

Analysis of neural patterning is complicated by the tissue interactions and dynamic morphogenetic movements that occur during gastrulation. *Xenopus* animal caps provide a simplified system for studying patterning events separate from morphogenetic movements. Animal caps alone form epidermis in culture, but when treated with antagonists of BMP signalling they can be induced to adopt an anterior neural fate (Hemmati-Brivanlou and Melton, 1997). This anterior neural tissue is capable of altering its positional identity to a more posterior character under the influence of signals from tissues surrounding the neural tube or by ectopic application of posteriorising factors, such as RA, FGF and Wnt family members (Baker et al., 1999; Blumberg et al., 1997; Domingos et al., 2001; Itoh and Sokol, 1997; Kolm et al., 1997; Lamb and Harland, 1995; McGrew et al., 1997; McGrew et al., 1995; Pownall et al., 1996).

Experiments in *Xenopus* have shown that planar signals within the neuroectoderm and vertical signals from the underlying mesoderm work in concert to control regional identity of the nervous system (Doniach, 1993). Although early AP specification of the nervous system occurs during gastrulation, the neural cells are not irreversibly committed to a particular identity. Grafting experiments in several species have revealed that plasticity in regional character is retained after gastrulation (Cox and Hemmati-Brivanlou, 1995; Gould et al., 1998; Grapin-Botton et al., 1997; Itasaki et al., 1996; Muhr et al., 1997; Trainor and Krumlauf, 2000; Woo and Fraser, 1997), suggesting that neural cells are actively receiving signals and communicating with surrounding tissues at later stages.

In this study, we performed a functional screen to search for novel factors derived from tissues surrounding the neural tube with the potential to alter the AP character of neuralised *Xenopus* animal caps. We have identified a novel gene, *Wise*, expressed in the surface ectoderm. *Wise* encodes a secreted protein that is capable of inducing posterior neural markers, and modulates the Wnt signalling pathway in a context-dependent manner. Our results provide a novel mechanism for

modulating the Wnt pathway and support a role for Wnt signalling in the neural patterning process.

MATERIALS AND METHODS

Library screening and embryo analysis

A cDNA library was made from stage 8-13 (Hamburger and Hamilton, 1951) chick embryos using tissues surrounding the neural tube (Fig. 1A) from axial levels capable of inducing *Hoxb4* expression in grafting experiments (Itasaki et al., 1996). Size-selected (>1 kb) cDNAs were directionally inserted into a modified 64T vector (Tada et al., 1998). The library contained 250,000 unamplified clones, and 50,000 of these were divided into 100 pools (500 clones per pool). For initial screening, 10 pools were mixed to prepare a single large DNA pool (5000 clones) used to synthesise capped RNA. *Xenopus* eggs were fertilised, cultured and injected as previously described (Jones and Smith, 1999). Animal caps were cut at stage 8, and incubated until siblings reached stage 25. Explants were collected and analysed for specific markers using RT-PCR (Hemmati-Brivanlou and Melton, 1994). For explant recombination assays, separate sets of embryos were injected with either *noggin* and FIDx (Molecular Probes) or *Wise* and *lacZ* RNA. FIDx and *lacZ* were used as lineage tracers. Caps were cut at stage 8, combined and cultured for assay at stage 25. To detect subcellular localisation of endogenous β-catenin, relevant RNAs were injected into the animal half of *Xenopus* embryos at the eight-cell stage. Animal caps were cut at stage 7 and fixed 10-15 minutes later when the cap edge was healed. Cryosections (16 μm) were then stained with an anti-β-catenin antibody (Sigma) and a secondary antibody conjugated with HRP.

Xenopus homologues of Wise

Xenopus Wise was isolated from stage 25 embryos by RT-PCR. The degenerate primers used were: upstream, 5′-GCTTT(C/T)AA(A/G)AA(C/T)GATGCCAC-3′; downstream, 5′-GTGAC(T/C)AC(T/G/A)GT(T/G)ATTTTGTA-3′. Two different clones were identified, presumably resulting from the pseudotetraploid *Xenopus* genome. For each clone, a longer version covering the start codon was isolated from a *Xenopus* stage 35 cDNA library.

DNA constructs

The dominant-negative Dishevelled construct DIX, specific to the canonical Wnt pathway (Axelrod et al., 1998) was made by creating a stop codon after amino acid 170 (Glutamine) by PCR and sub-cloned into pCS2+. Tagged Wise constructs were generated by PCR, and their activity was confirmed by injection into *Xenopus* embryos with *noggin* and assayed for induction of *en2* in animal caps. ΔE1-2 IgG (lacking EGF repeat 1 and 2) and ΔE3-4 IgG (lacking EGF repeat 3 and 4) of human LRP6 were generated by fusing the extracellular domains E3-4 and E1-2 of LRP6 (Mao et al., 2001) to the IgG Fc domain (Tamai et al., 2000). A FLAG tag was attached to chick Frizzled 1 extracellular domain (amino acids 1-199) by PCR. Other constructs were as previously published.

RNA and morpholino injection

The relevant amounts of RNA injected per embryo were as follows. Initial screening: noggin (500 pg) and RNA from pools (12 ng). Fig. 1B: *noggin* (500 pg) and *Wise* (150, 300, 600, 1200 pg). Fig. 1C: *Wise* (30 ng). Fig. 1D,E: *noggin* (500 pg), *Wise* (600 pg) and *lacZ* (100 pg). Fig. 3: *Wise* (300-500 pg) and *lacZ* (50 pg); *Wise* morpholino (30 ng). Fig. 4A: *Wise* (chick, *Xenopus*) (500 pg). Fig. 4B-K: control or *Wise* morpholino (33 ng). Fig. 5A: *noggin* (500 pg), *Wise* (600 pg); Δ*Wnt8* (200 pg), Δ*LRP6* (1 ng); Δ*Dsh(dd1)* (1.2 ng), *GSK3* (500 pg) and *LEF*Δ*N* (200 pg). Fig. 5B: *noggin* (500 pg), *Wnt8* (50 pg), *Wise* (600 pg) and Δ*Fz8* (2 ng). Fig. 5C: *noggin* (500 pg), *Wnt8* (600 pg), *Wise* (800 pg), *ΔWnt8* (800 pg). Fig. 5E-G: *Tcf3* (300 pg), *Wnt8* (25 pg), *Wise* (300 pg). Fig. 6A-C: *Wnt8* (5 pg), *Wise* (200 pg) and Δ*Dsh (DIX)* (1 ng). Fig. 6D: *Wise* (1 ng), *Wnt8* (100 pg), *Dsh* (1 ng) and *β-catenin* (200 pg). Fig. 6E,F: *tBR* (900 pg) and *Wise* (50 pg). Fig. 7C: *Wise* (1 ng). Fig. 7D: *Wise* (0.5 ng, 1 ng).

Morpholino antisense oligonucleotides were designed against the beginning of the coding region of the two *Xenopus Wise* genes. The sequences were: 5′-AGCACTGGAGCCTTGAGACAACCAT-3′; 5′-AGCAGTGAAGCCTTGAGACAACCAT-3′. A 1:1 mixture of these oligonucleotides were diluted in PIPES (5 mM) buffered water and used for injection. The control morpholino was; 5′-CCTCTTACCTCAGTTACAATTTATA-3′, which is a generic control from the supplier (Gene Tools), designed against human β-globin. 30-60 ng of morpholino were injected into whole embryos, and 13-60 ng were injected into a dorsal-animal or ventral-animal blastomere to target the surface ectoderm.

Protein analysis

To test the secretion of Wise protein, 15 oocytes were injected with RNA encoding HA-tagged Wise and incubated in a 96-well dish with 150 μl of OR2 medium (Wallace et al., 1973) + 0.01% BSA for 2 days. Oocytes and the conditioned medium were collected separately and used for western blotting with an anti-HA antibody (Roche).

For immunoprecipitation of conditioned medium, 293 cells were transfected with DNA and the conditioned medium (opti-MEM, Gibco) was concentrated 20- to 40-fold by ultrafiltration. Wnt8-myc medium was collected from S2 cells as described (Hsieh et al., 1999). The medium was mixed and incubated overnight at 4°C. Immunoprecipitation was performed using protein A beads (Amersham) and wash-buffer 150 mM NaCl, 50 mM Tris-HCl (pH 7.5) and 0.1% Triton X-100 as previously described (Hsieh et al., 1999; Tamai et al., 2000).

RESULTS

Isolation of β-catenin and Wise

The functional screen is based on the ability of *noggin* to induce neural tissue in *Xenopus* animal caps with an anterior character that in the presence of other factors can be converted to a more posterior character (Lamb and Harland, 1995; Smith and Harland, 1992). As a source of putative factors, we prepared a cDNA library from dissected tissue (somites, surface ectoderm and endoderm) from stages 8 to 13 (Hamburger and Hamilton, 1951) chick embryos (Fig. 1A), which is known to alter AP character when grafted to an ectopic location (Itasaki et al., 1996). After co-injection of *noggin* RNA with pools of RNA from the library, the induction of posterior character was monitored by RT-PCR on animal caps (Fig. 1A) by assaying for expression of *en2*, *Krox20* and *Hoxb9*, which mark the midbrain, hindbrain and spinal cord, respectively. Myosin was used as a marker for mesoderm induction to allow us to focus on pools that influence neural patterning in the absence of mesoderm. Successive rounds of subdivision and sib selection identified two clones with this inducing activity. One encoded a 5′-truncated version of β-catenin, consistent with data that the canonical Wnt pathway induces posterior neural markers (Domingos et al., 2001; McGrew et al., 1995). The second clone encoded a novel protein, which we have designated *Wise* (Wnt modulator in surface ectoderm) based on this study. Injection of *noggin* with increasing amounts of *Wise* induced progressively more posterior markers (*en2* and *Krox20*) in the absence of mesoderm (myosin) (Fig. 1B). Wise alone exhibited no neural- or mesoderm-inducing activity, as confirmed using NCAM, *myosin* (Fig. 1B), *brachyury*, *wnt8* and *Xhox3* (data not

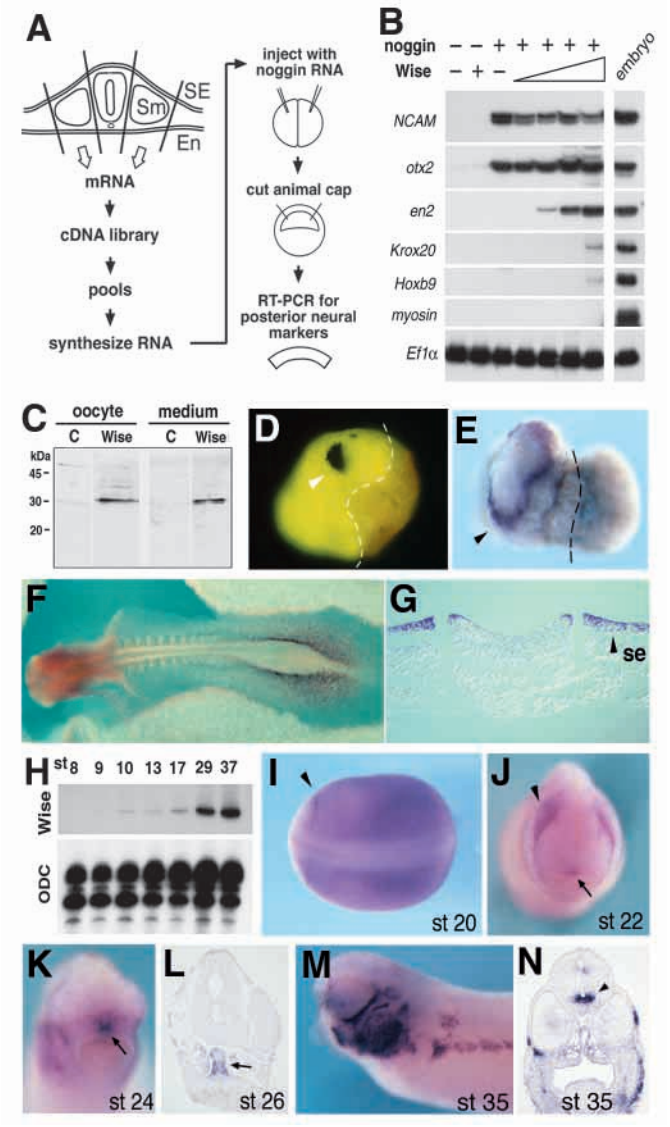

Fig. 1. Isolation, characterisation and expression of Wise. (A) Outline of the screen. (B) RT-PCR analysis using *Wise* RNA. *Wise* alone (600 pg) does not induce pan-neural (*NCAM*) or mesodermal (*myosin*) markers. In the presence of noggin, increasing amounts of *Wise* RNA (150, 300, 600 and 1200 pg) induce progressively more posterior neural markers (*en2*, *krox20*, *Hoxb9*). (C) Western blot detecting HA-tagged Wise protein secreted into the medium after RNA injection into oocytes. C, control uninjected oocytes. (D,E) Recombination of noggin-expressing and Wise-expressing animal caps assayed for induction of *Krox20* (D) or *en2* (E). In D, the *noggin* RNA injected cap was marked with FIDx (to the left of the broken line) and in E the Wise cap with *lacZ* (to the right of the broken line). Wise induces patches of *Krox20* or a ring of *en2* expression (arrowheads) in a non-cell-autonomous manner in the *noggin*-injected cap. (F,G) In situ hybridisation of chick embryo at stage 10. *Wise* is expressed in the surface ectoderm (se in G) from the level of presomitic mesoderm to the posterior. Expression is also seen faintly in the head surface ectoderm. (H) RNase protection of *Xenopus* embryos with stages noted above each lane. *Wise* is first detected at an early gastrula stage (st. 10) and the expression persists into tadpole stages. ODC is a loading control. (I-N) In situ hybridisation of *Xenopus* embryos at indicated stages. At the neurula stages (I-L), Wise is expressed in the surface ectoderm broadly at anteroposterior levels, strongest at the edge of the neural tube and the posterior edge of the eye (arrowheads in I,J). The expression is also seen in the stomodeal-hypophyseal anlage (arrow in J, front view; arrow in L, transverse section). At the tailbud stage (M,N) the expression appears to be localised in cranial placodes, lateral line placodes and the ventral neural tube at the diencephalon level (arrowhead in N).

shown) as markers.

Structural and functional properties of Wise

The predicted Wise protein consists of 206 amino acids and contains a cysteine knot-like domain found in a number of growth factors, as well as in Slit, mucin and CCN (Cef10/Cyr61, CTGF and Nov) family members (Bork, 1993) (Fig. 2A). Among these, the C-terminal domain of the CCN family members showed the highest homology to Wise, but other motifs conserved within the CCN family were absent in Wise (Fig. 2B). Hence, Wise is related to but not a member of the CCN family. A homology search revealed that Wise showed the highest amino acid identity (38%) to Sclerostin (SOST), identified by positional cloning of the gene mutated in sclerosteosis (Brunkow et al., 2001). There are a number of EST sequences homologous to Wise in zebrafish, mouse and human databases (Fig. 2A), but none was found in the *Drosophila* or *C. elegans* genomes.

A signal sequence motif is present at the N terminus of Wise, and its secretion was confirmed by western blotting after expression of an HA-tagged version of the protein in *Xenopus* oocytes (Fig. 1C) and COS cells (data not shown). We tested the ability of Wise to posteriorise neural tissue in a cell non-autonomous manner by using a tissue recombination assay in which a Wise-expressing animal cap was combined with a noggin-expressing animal cap. We found that both *en2* and *Krox20* were induced in discrete domains in the noggin caps (Fig. 1D,E). Hence, Wise has the ability to induce posterior markers at a distance.

Wise expression

In situ hybridisation analyses revealed that *Wise* is highly expressed in the surface ectoderm of the embryo in a dynamic pattern. In chick, expression was first detectable broadly at stage 9, and then localised in the posterior surface ectoderm overlying the presomitic mesoderm by stage 10-11 (Fig. 1F,G). This expression resolved into a small posterior domain in the tail bud by stage 13 (data not shown). At later stages, *Wise* was expressed in other tissues such as branchial arches, limbs and feather buds (data not shown). By cloning the *Xenopus* homologue, we found that transcripts were first detected at the gastrula stage (Fig. 1H), and in comparison to chick more extensively along the AP axis in the surface ectoderm at the neurula stage (Fig. 1I). Other localised expression at neurula and tailbud stages was seen in the stomodeal-hypophyseal anlage (Fig. 1J-L), the ventral diencephalon (Fig. 1N) and presumably cranial and lateral line placodes (Fig. 1J,M,N). Some of the corresponding patterns were seen in chick embryos as well (data not shown).

Functional analysis of Wise in *Xenopus* embryos

The function of Wise was first studied in *Xenopus* embryos using overexpression approaches. Injection of *Wise* RNA in

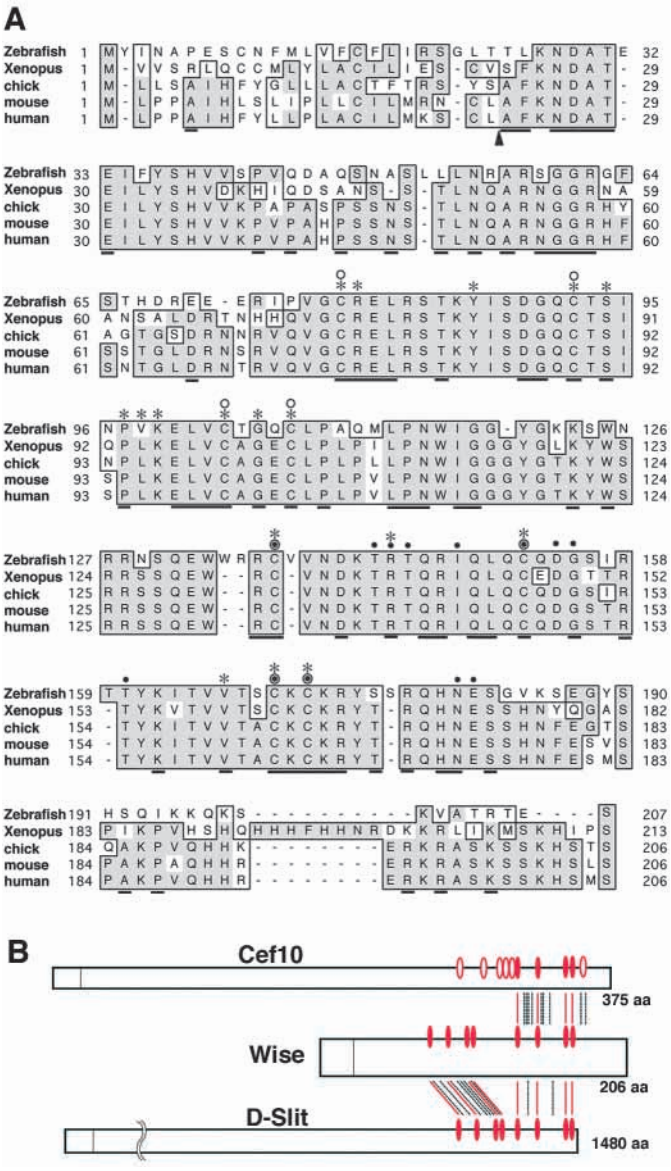

Fig. 2. Molecular structure of Wise. (A) Alignment of the predicted amino acid sequence of Zebrafish, *Xenopus*, chick, mouse and human Wise proteins. Shaded boxes represent identical amino acids between species; thick line underneath shows conserved amino acids in SOST, asterisks indicate residues conserved in *Drosophila* Slit and dots identify residues conserved in Cef10 (chick homologue of Cyr61 in CCN family). Circles mark conserved cysteine residues. The arrowhead delineates the site of signal peptide cleavage predicted in the chick protein. (B) Diagram showing alignment of conserved amino acids between Wise, Cef10 and *Drosophila* Slit. Red filled ovals and lines indicate cysteine residues conserved between Wise, Cef10 and Slit. Open ovals indicate additional cysteines in Cef10 conserved in the CT domain of CCN family members but not in Wise or Slit. Black dotted lines show other conserved amino acids.

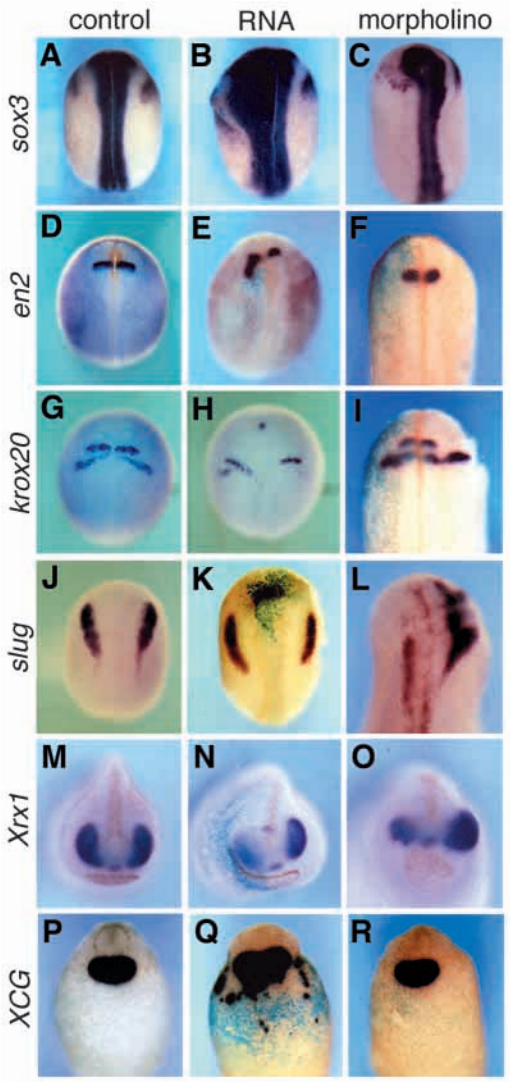

Fig. 3. Phenotypes after blastomere injection at four to eight cell stage of *Wise* RNA (B,E,H,K,N,Q) or antisense morpholino oligonucleotides (C,F,I,L,O,R) in comparison with control embryos (A,D,G,J,M,P) assayed by whole-mount in situ hybridisation at neurula stages. Marker is noted on the left of the panel. In most embryos, *lacZ* RNA was co-injected as a lineage tracer (blue staining). Injected side is to the left of each panel.

amounts >200 pg into the whole embryo lead to gastrulation defects and loss of eyes (data not shown). At lower amounts (<100 pg), gastrulation proceeded normally, but neural tube closure was abnormal and the neural plate appeared thicker and shorter than controls (data not shown). To evaluate further the effects of *Wise* on development of the neural tube, RNA or DNA was injected into specific blastomeres at 4-16 cell stages. When *Wise* RNA injections were targeted to presumptive neural regions, lateral expansion of the neural plate on the injected side was observed (Fig. 3A,B). AP specific markers (*en2* and *Krox20*) were generally displaced laterally and posteriorly (Fig. 3D,E,G,H). When *Wise* injection was targeted to the forebrain region, ectopic expression of *Krox20* and *slug* was observed (Fig. 3H,K). This indicates that forebrain cells acquired a more posterior character in response to Wise. Other defects included a failure in eye and cement gland formation (Fig. 3M,N, and data not shown). Conversely, when *Wise* RNA was injected ventrally, ectopic cement glands were induced (Fig. 3P,Q). Identical results were obtained using DNA constructs for injection, where *Wise* expression commenced at mid-blastula

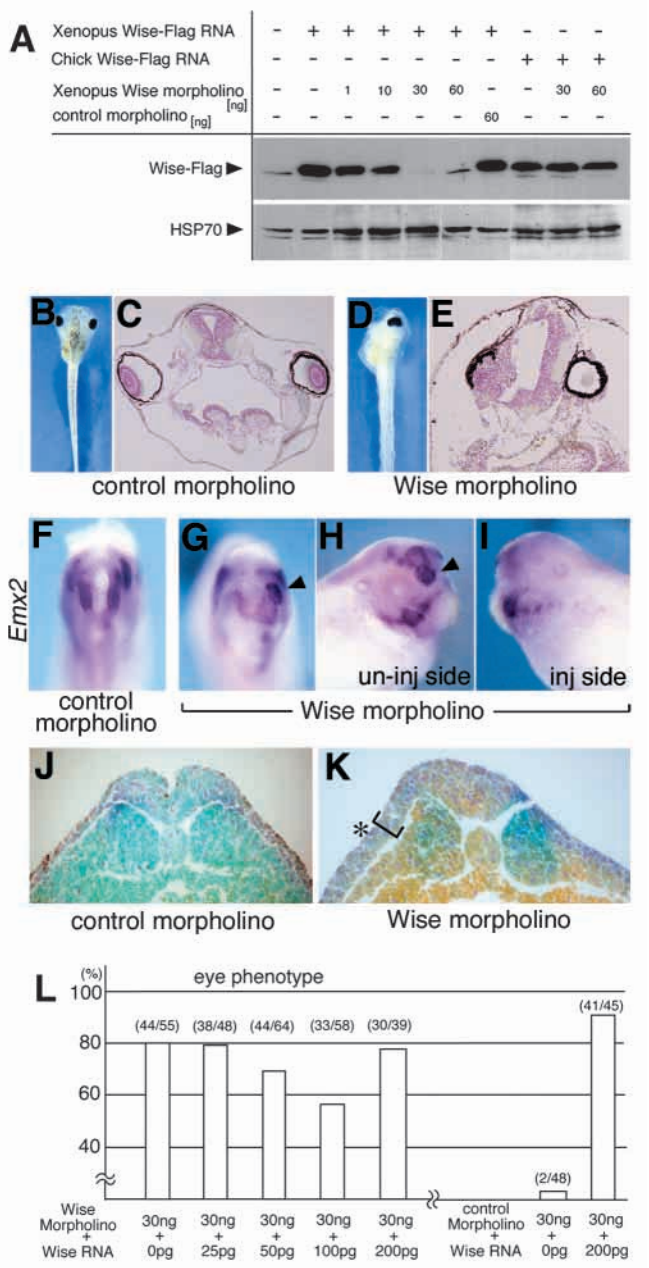

Fig. 4. Analysis with antisense morpholino oligonucleotides against *Wise*. (A) Antisense morpholino oligonucleotides against *Xenopus Wise* specifically blocks translation of *Xenopus Wise* RNA. Embryos were injected as indicated, animal caps were cut at stage 8, and the caps were collected at stage 9. Cell extracts were analysed by western blotting using anti-Flag and anti-HSP70 antibodies. (B-E) Phenotypes arising from whole-embryo injection of control (B,C) or *Wise* antisense (D,E) morpholino oligonucleotides at stage 43. (B,D) Whole-mount dorsal view; (C,E) transverse sections at the eye level. (F-I) In situ hybridisation analysis of *Emx2* at stage 35 after injection of control (F) or *Wise* (G-I) morpholino oligonucleotides into a dorsal-animal blastomere at four- to eight-cell stages. (F,G) Dorsal view, anterior towards the top. (H,I) Lateral view. *Wise* morpholino oligonucleotides injection causes loss of olfactory placode. Arrowheads in G and H indicate unaffected placode on the uninjected side. (J,K) Injection of *Wise* antisense morpholino oligonucleotides causes thick surface ectoderm (* in K) in comparison with the un-injected side or control embryo (J). (L) The eye defect caused by antisense morpholino oligos can be partially rescued by co-injection of chick *Wise* RNA.

stages under the control of a cytoskeletal actin promoter (data not shown). Thus, ectopic expression of *Wise* altered aspects of AP patterning in embryos, as well as in animal caps.

To analyse the role of *Wise* in normal *Xenopus* development, we injected antisense morpholino oligonucleotides that specifically interfere with translation of *Wise* (Fig. 4A). By injection of morpholino oligonucleotides into both blastomeres at the two-cell stage, embryos became cyclopic (Fig. 4B-E). Localised injection of the morpholino into one of the dorsal animal blastomeres at four to eight cell stages resulted in a reduction of neural tissue (Fig. 3C), decreased amount of *slug*-positive neural crest cells (Fig. 3L), and the formation of a smaller eye (Fig. 3O) on the injected side. AP neural markers such as *en2* and *Krox20* and a cement gland marker *XCG* were not obviously affected (Fig. 3F,I,R). In the forebrain, Wise morpholino oligonucleotides caused loss of the olfactory placode revealed by *Emx2* staining (Fig. 4F-I). Histological sections at the trunk level of neurula stage embryos showed that Wise morpholino injection caused thicker surface ectoderm (Fig. 4J,K). These results indicated that *Wise* has endogenous roles in controlling eye, olfactory placode and surface ectoderm formation. The strong expression of *Wise* in cranial placodes at the tailbud stage (Fig. 1M,N) further suggests a key role for *Wise* in placode formation. Wise is likely to play a permissive role in the eye development, as the eye tissue shows only a small domain of expression at the tailbud stage (Fig. 1M), and depletion of Wise by morpholino injection affects only growth of the eye and not the initial formation of the eye primordium, as marked by *Xrx1* (Fig. 3O).

The fact that ectopic expression of *Wise* RNA and depletion of endogenous translation by the morpholino oligonucleotides both result in similar defects in eye formation (Fig. 3N,O; Fig. 4L) suggests that this process requires a precise level of signalling mediated by Wise. Defects in eye formation are also observed following injection of molecules that activate Wnt signalling (*Wnt8* DNA, *Frizzled3*) (Christian and Moon, 1993; Rasmussen et al., 2001) as well as molecules that inhibit the pathway (*Frzb1, Crescent*) (Pera and De Robertis, 2000). Other aspects of the phenotypes observed by altering *Wise* expression are also reminiscent of those seen when the Wnt signalling pathway is perturbed. For example, ectopic induction of cement gland is observed following injection of GSK3β (Itoh et al., 1995), and proper gastrulation and convergent extension movements require Wnt11 and Dishevelled (Heisenberg et al., 2000; Tada and Smith, 2000; Wallingford et al., 2000). Wnts/β-catenin function as posteriorising factors in animal cap assays (Domingos et al., 2001; McGrew et al., 1995), and also regulate induction of neural crest cells (Saint-Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998; Garcia-Castro et al., 2002). These similarities prompted us to test whether *Wise* acts through or modulates the Wnt signalling pathway.

Wise activates the canonical Wnt pathway in animal caps

As Wnts and Wise both induce *en2* expression in *noggin*-injected animal caps, we investigated whether the ability of Wise to induce *en2* requires Wnt signalling. To test this, *Wise*

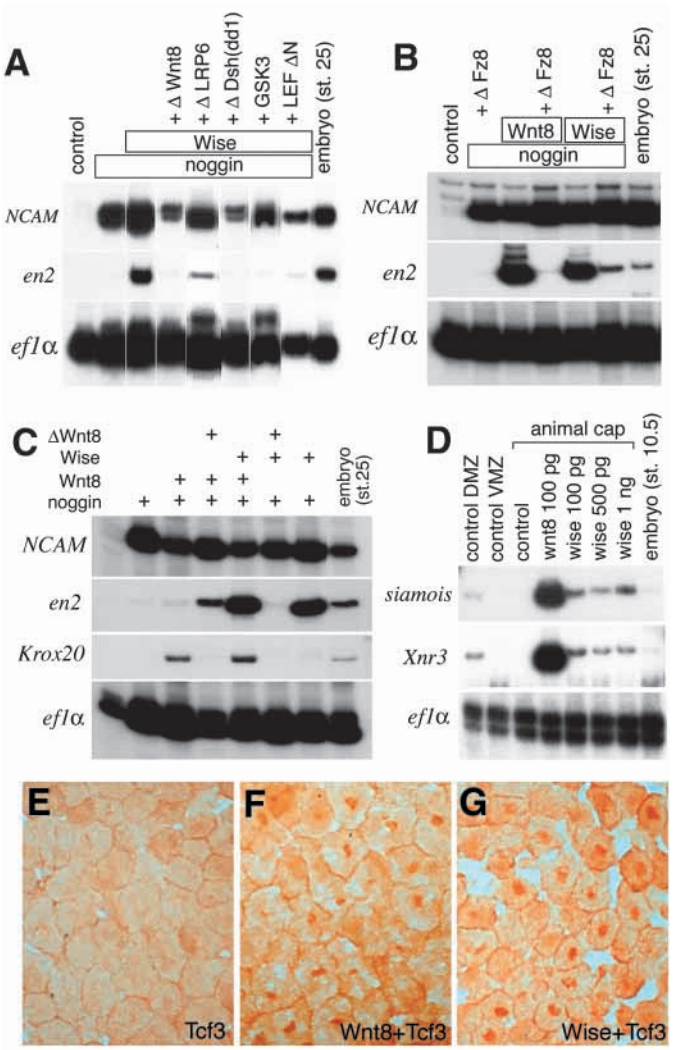

Fig. 5. Wise requires components of the Wnt pathway for *en2* induction and activates the canonical Wnt pathway. (A,B) RT-PCR of *noggin*-injected animal caps assayed for *en2* induction. Induction of *en2* is attenuated or blocked by dominant-negative (dn)-Wnt8 (ΔWnt8), dn-LRP6 (ΔLRP6), dn-Dishevelled [ΔDsh (dd1)], GSK3-β (GSK3), dn-Lef1 (LEFΔN) (A) and dn-Frizzled8 (ΔFz8) (B). (C) Wise does not interfere with the ability of Wint8 to induce *Krox20* in *noggin*-injected animal caps. *Wnt8*-induced *Krox20* expression is blocked by ΔWnt8, but not by Wise. (D) Long-exposure of RT-PCR result showing that *Wise*-injected animal caps express *siamois* and *Xnr3*, although very weakly in comparison with the induction by Wnt8. (E-G) Staining for subcellular localisation of endogenous β-catenin detected immunocytochemically in *Xenopus* animal caps after RNA injection of (E) Tcf3, (F) Wnt8+Tcf3 and (G) Wise+Tcf3. Both Wnt8 and Wise promote nuclear accumulation of β-catenin.

RNA was co-injected with blocking reagents of the Wnt pathway such as wild type GSK3β (Dominguez et al., 1995) and dominant-negative (dn) versions of Wnt8 (Hoppler et al., 1996), Frizzled8 (Itoh and Sokol, 1999), LRP6 (Tamai et al., 2000), Dishevelled (Sokol, 1996) or Lef1 (Vleminckx et al., 1999). These reagents either eliminated or attenuated the ability of Wise to induce *en2* in neuralised animal caps (Fig. 5A,B). With respect to the intracellular component Dishevelled, only dominant-negative constructs affecting the canonical Wnt pathway abolished *en2* induction (data not shown). Wnt8 and Wise showed an additive effect in induction of *en2* (Fig. 5C). To confirm the activation of the canonical Wnt pathway by Wise, we examined its effects in other assays. First, in animal cap explants in the absence of *noggin*, Wise showed a weak dorsalising activity by inducing *siamois* and *Xnr3*, two direct targets of the Wnt signalling pathway (Brannon et al., 1997) (Fig. 5D). Second, Injection of Wise RNA increased nuclear accumulation of β-catenin in animal caps, in a manner similar to that of Wnt8. This phenomenon was enhanced by co-injection of Tcf3, a co-factor of β-catenin for transcriptional activation (Fig. 5E-G). As animal caps were assayed before mid-blastula transition, we believe the observed effect is not due to secondarily induced transcription. These data suggest that Wise activates Wnt signalling and requires components of the canonical pathway to induce the signal in animal caps.

It is important to note that, although Wise and Wnts both activate the canonical pathway, there are distinct differences in their outputs. *Wnt8* RNA (50 pg) is sufficient to induce both *en2* and *Krox20* (Domingos et al., 2001), and a higher amount (600 pg) induces only *Krox20* (Fig. 5C). By comparison, it takes much higher amounts of *Wise* RNA (300-600 pg) just to induce *en2*, and 1.2 ng of Wise RNA is only sufficient to weakly induce *Krox20* (Fig. 1B). Similarly, Wnt8 robustly induces *siamois* and *Xnr3* at a low amount of RNA (100 pg), although Wise only induces these genes in a relatively weak manner, even at the highest levels of RNA (100-1000 pg) (Fig. 5D). These results show that Wise has weaker posteriorising and dorsalising activities in comparison with Wnt8, raising the possibility that there are both quantitative and qualitative differences in the outputs of the Wnt pathway when activated by these two proteins. The fact that Wnt8 induces dorsal mesoderm in animal caps in the presence of *noggin* (Domingos et al., 2001) although Wise does not (Fig. 1B), further supports their qualitative difference.

Wise can interfere with Wnt signals

Although induction of *en2* can be explained by activation of Wnt signalling, the effects of injected *Wise* RNA on cement gland induction (Fig. 3Q) resemble those observed when the Wnt pathway is inhibited (Itoh et al., 1995). Thus, it is possible that in some contexts Wise blocks Wnt signalling. When *Wnt8* RNA is injected into a ventral vegetal blastomere at the four to eight cell stage, it induces an ectopic secondary axis (Smith and Harland, 1991; Sokol et al., 1991) (Fig. 6A). Based on the ability of Wise to induce *siamois* and *Xnr3*, we expected that Wise on its own might induce a secondary axis or work in synergy with Wnt8 in this process. However, injection of *Wise* RNA did not exhibit secondary axis formation (data not shown). Rather, co-injection of *Wise* RNA completely blocked Wnt8-induced secondary axis formation (Fig. 6B), as did a dominant negative form of Dishevelled (Fig. 6C). This inhibitory activity was confirmed at the molecular level in ventral marginal zone explants by demonstrating that the Wnt-dependent induction of *siamois* and *Xnr3* is greatly reduced by co-injection of *Wise* (Fig. 6D). These results suggest that in the presence of both Wnt8 and Wise, Wise interferes with the level of activity of Wnt8. *Wise* had no effect on the ability of injected intracellular components such as Dishevelled and β-catenin to

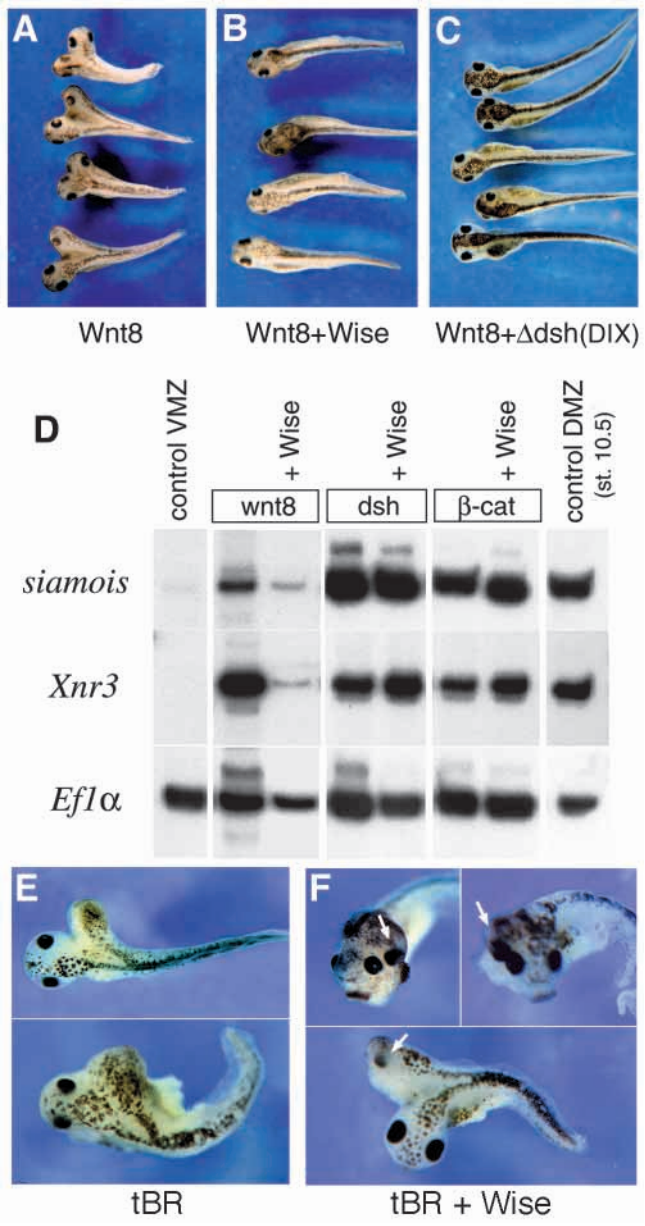

Fig. 6. Wise interferes with Wnt signalling. (A-C) Wise blocks secondary axes induced by Wnt8. Injection of *Wnt8* RNA into a ventral vegetal blastomere of four- to eight-cell stage embryos induces complete secondary axis formation (A). Co-injection of *Wise* blocks formation of Wnt8-induced secondary axes (B), as does co-injection of a dominant-negative dishevelled, ΔDsh (DIX) (C). (D) Wise functions extracellularly to block induction of *siamois* and *Xnr3* by the Wnt pathway in ventral marginal zone (VMZ) explants. Wise blocks the ability of Wnt8 to induce *siamois* and *Xnr3*, but does not interfere with the ability of dishevelled (dsh) or β-catenin (β-cat) to induce these markers. DMZ, dorsal marginal zone explant. (E,F) Wise acts as a Wnt inhibitor and complements a truncated BMP receptor (tBR) to induce head structures. When BMP signalling is blocked in the ventral marginal zone by injection of *tBR*, an incomplete secondary axis is formed (E). Co-injection of *tBR* and *Wise* induces a complete secondary axis with eyes (arrows) and cement gland (F).

induce *Xnr3* and *siamois* (Fig.6D), suggesting that Wise functions extracellularly to interfere with the canonical Wnt pathway. The inhibitory effect of Wise on Wnt signalling was further examined by assaying secondary head induction, which can be induced by simultaneous inhibition of both BMP and Wnt signalling (Glinka et al., 1997) (Fig. 6E). Co-injection of Wise and a dominant-negative BMP receptor (Suzuki et al., 1994) induced a complete secondary axis with eyes and cement gland (Fig. 6F), demonstrating that Wise functions as a Wnt inhibitor in this context. This contrasts with our analysis in animal caps, which reveals that Wise does not interfere with the action of Wnt in the induction of *Krox20* (Fig. 5C). Therefore, modulation of the Wnt pathway by Wise (activation or inhibition) varies with respect to both target genes and cellular contexts.

Wise might affect the planar cell polarity pathway of Wnt signalling

Although the activating and inhibiting properties of Wise in animal caps and embryos described above are dependent upon the canonical Wnt pathway, it is possible that Wise also influences the planar cell polarity (PCP) pathway that branches at Dishevelled. Wnt11 is required for proper convergent extension movements of mesoderm during gastrulation in *Xenopus* and Zebrafish, and this has been shown to be dependent upon the PCP pathway of Wnt signalling (Heisenberg et al., 2000; Tada and Smith, 2000; Wallingford et al., 2000). Animal caps cultured in the presence of activin form mesoderm, and undergo convergent extension movements that can be blocked by reagents that either elevate or decrease Wnt signalling (Tada and Smith, 2000). This implies that precise levels of Wnt signalling through the PCP pathway are essential for coordinated cell movements.

In this animal cap assay, injection of *Wise* RNA blocked cell movements preventing elongation of animal caps, but had no affect on activin-induced mesoderm formation (Fig. 7). This suggests that Wise might influence the Wnt-dependent PCP pathway, but whether this is mediated by its ability to activate or inhibit the pathway cannot be distinguished. This effect on cell behaviour in animal caps is consistent with and may explain the phenotypic effects observed in Wise-injected whole embryos. Wise perturbed the morphogenesis of the neural tube, which failed to close. It was thickened and shortened, and there was a lateral expansion, broadening or posterior-shift of AP markers. Many of these defects appear to relate to abnormal convergent extension movements during gastrulation. However, the fact that morpholino antisense oligonucleotides do not interfere with gastrulation (Fig. 4D) and neural AP patterns (Fig. 3F,I), and that *Wise* is not predominantly expressed at gastrula stage (Fig. 1H), both suggest that endogenous Wise is unlikely to be involved in normal gastrulation movements. This assay suggests that Wise has a potential to interfere with Wnt-mediated PCP as well as the canonical pathway.

Wise interacts with Wnt co-receptor LRP6

Wise encodes a secreted protein and interacts with the Wnt pathway extracellularly (Fig. 5A,B; Fig. 6D). Therefore, to begin to approach the mechanisms of action, we investigated

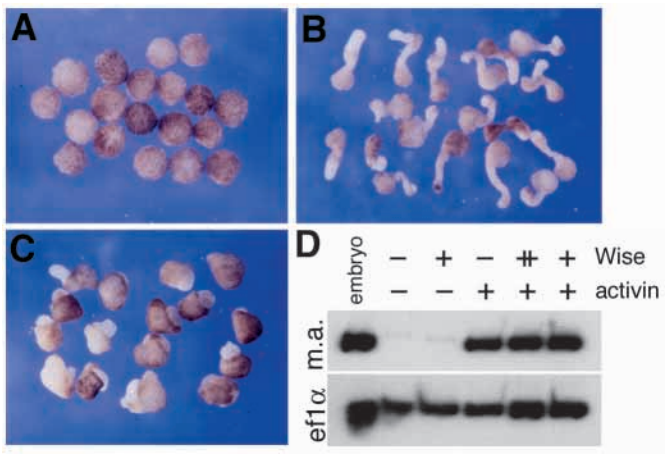

Fig. 7. Wise blocks cell movements in activin-treated animal caps. (A) Control animal caps at stage 15. (B) Control animal caps treated with activin (8 units/ml). They undergo gastrulation-like movements in response to activin and become elongated. (C) Animal caps from *Wise* RNA (1 ng) injected embryos, treated with activin. Elongation is blocked. (D) RT-PCR of animal caps assayed for muscle-actin (m.a.) and ef1α. All caps were assayed at stage 15. Induction of muscle actin by activin is not interfered by preceded injection of 500 pg (+) or 1 ng (++) of Wise RNA, showing that failure in elongation in response to activin in C is not due to failure in muscle induction.

potential physical interactions of Wise with Wnt family members or their putative co-receptors Frizzled8 (Hsieh et al., 1999) and LRP6 (Tamai et al., 2000) (Fig. 8). We mixed conditioned medium of 293 or S2 cells containing a secreted form of LRP6 (Tamai et al., 2000) or Frizzled8 (Hsieh et al., 1999) with Wise conditioned medium, and assayed for interactions by immunoprecipitation (IP). In this assay, Wise bound to LRP6 but not to Frizzled8 (Fig. 8A) or Wnt8 (Fig. 8B). Recent studies have shown that individual members of the Dickkopf (Dkk) family of secreted proteins can either antagonise or stimulate Wnt signalling through interaction with LRP6 (Brott and Sokol, 2002; Mao et al., 2001; Wu et al., 2000). Therefore we performed IP experiments to determine if Wise shares common binding sites with Dkk1 or Wnt on LRP6. The extracellular domain of LRP6 contains four EGF repeats and Dkk1 interacts with repeats 3-4, while Wnt interactions seem to involve mainly repeats 1-2 (Mao et al., 2001). We found that Wise binds to LRP6 and a variant where EGF repeats 3 and 4 are deleted (ΔE3-4), but not to one in which EGF repeats 1 and 2 are removed (ΔE1-2) (Fig. 8A). Conversely, Dkk1 binds to LRP6 and ΔE1-2, but not to ΔE3-4 (Fig. 8A). These results show that Wise shares the domain on LRP6 essential for interaction with Wnts and that Wise and Dkk1 modulate LRP6 activity by interacting through different domains. We also tested whether Wise and Wnt8 can bind to LRP6 at the same time or whether they compete for binding. As shown in Fig. 8C, Wise interferes with the binding of Wnt8 to LRP6. This suggests a mechanism whereby Wise inhibits Wnt signalling by competing with Wnt8 for binding to LRP6 (Fig. 8D).

DISCUSSION

In this study, we have identified and characterised a novel secreted protein, Wise, that modulates the

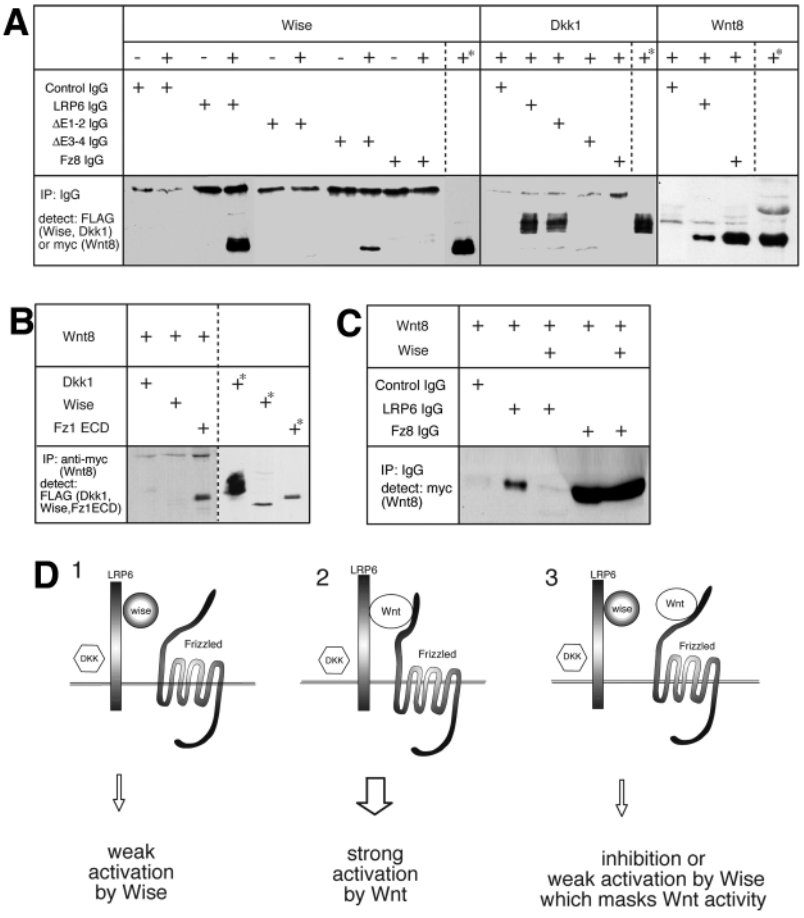

Fig. 8. Wise interacts with the extracellular domain of LRP6. (A-C) All components were provided as concentrated conditioned medium for the immunoprecipitation study. Asterisks show samples of input loaded directly on to the gel. Extracellular domains of LRP6 and Frizzled8 are fused to human IgG Fc domain. ΔE1-2 and ΔE3-4 are deletion constructs of LRP6 in which the first two or last two EGF repeats respectively are missing (Mao et al., 2001). (A) Wise binds to LRP6 and a deletion construct of LRP6 (ΔE3-4), but not to Frizzled8 or LRP6 ΔE1-2. Dkk1 binds to LRP6 and ΔE1-2 LRP6, but not to ΔE3-4 LRP6 or Frizzled8. Wnt8 binds to LRP6 and Frizzled8. (B) Wise does not bind to Wnt8. Dkk1 and extracellular domain of Frizzled1 (Fz1 ECD) are used as a negative and a positive control for binding to Wnt8, respectively. (C) Binding of Wnt8 and LRP6 is attenuated in the presence of Wise, but binding of Wnt8 and Frizzled8 is not. (D) A possible model of Wise action on Wnt signalling. Open arrows in each panel show weak (1,3) and strong (2) activation of the downstream pathway. (1,2) Wise and Wnt share the same binding domain of LRP6, while Dkk1 interacts with a different domain on LRP6. Wise induction of downstream targets of the canonical Wnt pathway is weaker compared with Wnt. (3) Wise competes with Wnt for binding to LRP6, resulting in attenuation of the Wnt action (compare 2 with 3).

Wnt signalling pathway. *Wise* is conserved in vertebrates and contains a cysteine knot-like domain, present in members of the CCN family and other growth factors. *Wise* is expressed in the surface ectoderm, in which Wnt signalling plays multiple roles. The ability of Wise to affect gastrulation, neural tube morphogenesis and AP patterning in *Xenopus* explant and embryonic assays are consistent with its functioning to alter Wnt signalling. The novel aspect of Wise compared with other extracellular Wnt modulators is that it both activates and inhibits Wnt signalling in different contexts. In some contexts, Wise stimulates the canonical Wnt pathway, whereby it acts through Dishevelled and β-catenin. The ability of Wise to interact physically with LRP6 by sharing the same binding domain with Wnt8 suggests that it might function as an alternative ligand for the receptor. In other contexts, Wise antagonises Wnt signalling through the canonical pathways presumably by blocking accessibility of certain Wnts to their receptors. The ability of Wise to both activate and inhibit Wnt signalling provides a new mechanism for modulating Wnt signalling and adds a new level of complexity to how the array of ligands and inhibitors are integrated to control the balance of Wnt signalling in different developmental contexts.

Role for Wise in surface ectoderm

The ability of Wise to interact with the Wnt pathway and the fact that it is normally expressed in a transient manner in the non-neural surface ectoderm, suggest that it might have a role in modulating Wnt signalling in this tissue. Morpholino antisense oligonucleotide against Wise caused thick surface ectoderm (Fig. 4K), and overexpression of Wise by RNA injection caused expanded neural plate (Fig. 3B). These results suggest that endogenous expression of *Wise* at the edge of the neural plate might regulate the balance of neural and non-neural ectoderm transition. It is known that a balance between Wnt and BMP signalling in the surface ectoderm and dorsal neural tube is important in modulating dorsal fates and the generation of neural crest cells (Dickinson et al., 1995; Garcia-Castro et al., 2002; Liem et al., 1995; Trainor and Krumlauf, 2002). Furthermore, Wnts in the surface ectoderm influence patterning of the underlying somites and their derivatives (Capdevila et al., 1998; Munsterberg et al., 1995). The distribution and timing of Wise expression in the surface ectoderm together with the result of morpholino experiments suggest that it promotes precise levels of Wnt signalling to control some of these interactions.

Wise, Wnts and patterning

Wise was isolated on the basis of its ability to alter AP neural patterning, an activity consistent with its interaction with the Wnt cascade. Recent studies provide us with evidence on involvement of Wnt pathway in AP patterning (Davidson et al., 2002; Domingos et al., 2001; Kiecker and Niehrs, 2001). Furthermore, an increasing number of extracellular and intracellular inhibitors of Wnt signalling have been found, highlighting the considerable complexity in the nature of regulating this cascade. The roles for Wise in Wnt signalling raises the possibility that other members of this class of cysteine-knot proteins may also exert some of their functions by modulating Wnt activity. Indeed, the CCN family member Cyr61 is also capable of regulating Wnt signalling, although its mode of action is unknown (Latinkic et al., 2003). Wise is distinguished from other Wnt modulators as it seems to have multiple roles in modulating and integrating the readout of Wnt signalling depending upon the local context.

Even though Wise requires the canonical Wnt pathway to posteriorise noggin-treated animal caps, there are differences in the patterns of induction compared with stimulation by Wnt ligands such as Wnt8. *Wise* induces *en2* at low levels of injected RNA, and *en2* plus *Krox20* only at high levels (Fig. 1B). By contrast, *en2* and *Krox20* are simultaneously induced by Wnt8, even with very low amounts of RNA, and Wnt8 can induce more posterior Hox genes such as *Hoxb9* (Domingos et al., 2001). Another difference is that although Wnts or β-catenin downregulate forebrain markers (*Otx2*, *BF1*) at the same time as inducing posterior genes (McGrew et al., 1997; McGrew et al., 1995) *Wise* does not (Fig. 1B). The basis of these differences is not clear. It has been suggested that *en2* is under the direct regulation of Tcf3 (McGrew et al., 1999), which seems to reflect processes in the isthmic region, where Wnt1 is required for expression of *en2* (Danielian and McMahon, 1996; McMahon et al., 1992). However, the activation of other downstream targets, such as *Krox20* and *Hoxb9*, could be indirect and involve multiple steps downstream of the direct action of β-catenin and Lef/Tcf. For example, Wise does not induce mesoderm in the presence or absence of *noggin*, whereas Wnts or β-catenin do (Sokol, 1993). As mesoderm can influence AP patterning, the differences associated with induction by Wnt might in part be mediated indirectly through mesoderm. Hence, even though Wise and Wnts stimulate the same pathway, there are differences in the nature of their outputs and Wise appears to be a much weaker inducer of posterior genes.

Dual roles for Wise: a context dependent agonist and antagonist

Recent studies on secreted proteins that affect Wnt signalling suggest complex mechanisms modulating the canonical Wnt pathway. Different Frizzled-related protein and Dkk family members exhibit opposite effects in a variety of in vivo and in vitro assays (Bradley et al., 2000; Brott and Sokol, 2002; Li et al., 2002; Mao and Niehrs, 2003; Wu et al., 2000). The activation of the Wnt pathway by Wise is either weaker or different than that seen using Wnts because it takes higher concentrations of Wise to induce *en2* and *Krox20* and the relative levels of induction of *siamois* and *Xnr3* are much lower. With respect to inhibition, in the presence of both Wnt and Wise, Wise competes with Wnts for the binding to LRP6. This could result in either a less efficient activation of the receptors, which masks Wnt dependent activity, or a complete block of receptor activity (Fig. 8D). It is also possible that Wise could affect Wnt signalling through additional mechanisms. The interaction of Wise with LRP6 may also interfere with the function of Dkks, which could result in either activation or inhibition of the Wnt pathway depending upon which Dkk family member is present. There remains the possibility that Wise interacts with other receptors or modulators that work through intracellular Wnt signalling components. We observed that Wise interferes with cell movements in activin-treated animal caps (Fig. 7), consistent with the gastrulation defects observed in *Wise*-injected whole embryos. As the pathway involved in cell movements does not appear to require LRP6 (Semenov et al., 2001), this result implies that Wise could

interact with other proteins for its function. The studies presented here reveal new mechanisms through which a fine balance in Wnt signalling is regulated in various developmental processes.

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