Glypican 4 modulates FGF signalling and regulates dorsoventral forebrain patterning in *Xenopus* embryos

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Summary

Heparan sulphate proteoglycans such as glypicans are essential modulators of intercellular communication during embryogenesis. In *Xenopus laevis* embryos, the temporal and spatial distribution of *Glypican 4* (*Gpc4*) transcripts during gastrulation and neurulation suggests functions in early development of the central nervous system. We have functionally analysed the role of *Xenopus Gpc4* by using antisense morpholino oligonucleotides and show that *Gpc4* is part of the signalling network that patterns the forebrain. Depletion of GPC4 protein results in a pleiotropic phenotype affecting both primary axis formation and early patterning of the anterior central nervous system. Molecular analysis shows that posterior axis elongation during gastrulation is affected in GPC4-depleted embryos, whereas head and neural induction are apparently normal. During neurulation, loss of GPC4 disrupts expression of dorsal forebrain genes, such as *Emx2*, whereas genes marking the ventral forebrain and posterior central nervous system continue to be expressed. This loss of GPC4 activity also causes apoptosis of forebrain progenitors during neural tube closure. Biochemical studies establish that GPC4 binds FGF2 and modulates FGF signal transduction. Inhibition of FGF signal transduction, by adding the chemical SU5402 to embryos from neural plate stages onwards, phenocopies the loss of gene expression and apoptosis in the forebrain. We propose that GPC4 regulates dorsoventral forebrain patterning by positive modulation of FGF signalling.

Key words: Antisense morpholino oligo, Cell survival, *Emx2*, ERK, Gastrulation, Neurulation, *Xenopus*

Introduction

The vertebrate forebrain consists of anatomically and functionally distinct domains patterned along their anteroposterior and dorsoventral axis (reviewed by Rubenstein et al., 1998). For example, the telencephalic subpallium and hypothalamus are ventral forebrain structures, whereas the telencephalic pallium and epithalamus are located dorsally. As these different forebrain structures arise from the anterior neural plate (Rubenstein et al., 1998), the identification of the mechanisms that control anterior neural plate regionalization is central to understanding morphogenesis of the forebrain. Fate mapping studies and molecular analysis of embryos from different vertebrate species have established that the anterior neural plate is regionalized through restricted activation of key transcription factors (Rubenstein et al., 1998). For example, cells of the medial anterior neural plate activate the *Nkx2.1* homeobox gene, and expression persists later in the presumptive ventral telencephalon and hypothalamus (Hollemann and Pieler, 2000; Wilson and Rubenstein, 2000). Genetic analysis has shown that *Nkx2.1* is essential for ventral forebrain identity (Wilson and Rubenstein, 2000). By contrast, the presumptive dorsal forebrain territory predominantly expresses *Emx1* and *Emx2* (Simeone et al., 1992; Pannese et al., 1998). Dorsal forebrain patterning is disrupted in *Emx*-deficient mouse embryos (Yoshida et al., 1997; Bishop et al., 2003) and mutations in the human *EMX2* gene are linked to schizencephaly, a congenital brain malformation characterized by clefts in the human cerebral cortex (Brunelli et al., 1996).

Different types of signalling molecules, their antagonists and receptors regulate regionalization of the anterior neural plate (Rubenstein et al., 1998; Wilson and Rubenstein, 2000). For example, antagonism of WNT signalling is necessary for correct subdivision of the anterior neural plate into telencephalon, diencephalon and eye territories (Wilson and Rubenstein, 2000; Houart et al., 2002). BMP7 and SHH signalling by the prechordal mesoderm induces *Nkx2.1* and directs neural plate cells towards hypothalamic fate (Wilson and Rubenstein, 2000). Other BMP family members are produced by the non-neural ectoderm adjacent to the anterior neural plate and regulate expression of anterior neural markers and dorsal forebrain development in a dose-dependent manner (Wilson and Rubenstein, 2000; Hartley et al., 2001). Accordingly, inactivation of the BMP antagonists *chordin* and *noggin* in mouse embryos causes defects in forebrain patterning (Wilson and Rubenstein, 2000). The role of FGFs during forebrain morphogenesis appears widespread as several FGFs, such as *Fgf8*, *Fgf2* and *Fgf9*, are expressed by the anterior neural plate and forebrain primordia (reviewed by Dono, 2003). For example, embryological and genetic studies have shown that FGF8, produced by the anterior neural ridge, participates in inducing the telencephalon and in

differentiation of anterior midline cells (Rubenstein et al., 1998; Eagleson and Dempewolf, 2002). Moreover, FGF8 acts in a dose-dependent manner to control cell survival in the developing forebrain in the mouse (Storm et al., 2003). In zebrafish embryos, FGFs also regulate dorsoventral forebrain patterning, as evidenced by genetic analysis (Shanmugalingam et al., 2000) and transient inhibition of FGF signal transduction by the chemical inhibitor SU5402 (Shinya et al., 2001). These latter studies showed that FGF8 and FGF3 cooperate to promote *Nkx2.1* expression and morphogenesis of the ventral telencephalon. In addition, FGF8 and FGF2 can induce dorsal forebrain genes, such as *Emx1*, in neuralized *Xenopus* animal cap explants (Lupo et al., 2002).

Cell-cell signalling interactions are modulated by cell surface proteins, including glypicans. Glypicans, like other heparan sulphate proteoglycans (HSPG), bind FGFs, WNTs and BMPs through their heparan sulphate glycosaminoglycan (HS-GAG) side-chains (Hagihara et al., 2000; Nybakken and Perrimon, 2002). It has been proposed that glypicans regulate cell signalling by either promoting or stabilizing the interactions of ligands with their cognate high affinity receptors (Nybakken and Perrimon, 2002). For example, vertebrate glypican 1 binds FGFs, thereby favouring assembly of the ligand-receptor complex (Steinfeld et al., 1996). Alternatively, glypicans such as *Drosophila* Dally-like may shape ligand gradients by restricting their diffusion within the extracellular matrix (Baeg et al., 2001). Dally, another *Drosophila* glypican regulates imaginal disc patterning and morphogenesis by positive and differential modulation of *wingless* (*wg*) and *decapentaplegic* (*dpp*) signalling (Nybakken and Perrimon, 2002). Genetic analysis of the zebrafish Knypek shows that this glypican functions to potentiate non-canonical WNT signalling. By modulating WNT11 activity, Knypek regulates the convergent-extension movements during zebrafish gastrulation (Topczewski et al., 2001). In mice, glypican 3 is required for the cellular response to BMP and FGF signalling during organogenesis (Grisaru et al., 2001). Furthermore, several glypican family members are expressed in the developing central nervous system (CNS) (reviewed by Song and Filmus, 2002). One of them, glypican 4 (Gpc4) is predominantly expressed in the presumptive forebrain territory during head-fold stages in mouse embryos (A.G. and R.D., unpublished). Subsequently, its expression persists in neuronal progenitors of the developing forebrain (Hagihara et al., 2000).

In the present study, we functionally analyse the *Xenopus Gpc4* gene by interfering with protein translation through specific antisense morpholino oligonucleotides. Such depletion of GPC4 in developing embryos results in gastrulation and axis elongation defects similar to those caused by the zebrafish *knypek* mutation. Furthermore, we identify GPC4 as a key regulator of dorsoventral forebrain patterning. In particular, loss of GPC4 activity results in downregulation of dorsal forebrain identity genes from early neural plate stages onwards, and massive cell death in the anterior CNS during neural tube closure. We show that GPC4 binds FGF2 and that inhibition of FGF signalling by SU5402 (Mohammadi et al., 1997) results in dorsal forebrain phenotypes similar to those of GPC4-depleted embryos. We conclude that establishment and patterning of the dorsal forebrain territory requires modulation of FGF signalling by GPC4.

Materials and methods

Identification of the *Xenopus Gpc4* gene and generation of antisense morpholino oligonucleotides

A *Xenopus laevis Gpc4* cDNA clone was identified by a BLAST search of the GenBank EST database, using the mouse *Gpc4* sequence (Watanabe et al., 1995). The corresponding clone (RZPD clone ID:IMAGE998F078241Q2; see www.rzpd.de) was obtained from the RZPD Consortium. The entire EST (2569 bases) was sequenced to show that it contains the complete ORF and part of the 5′ and 3′ UTRs. The *Xenopus Gpc4* DNA sequence is 100% identical to the sequence available from the NCBI database (Accession number ABO82534). The 5′UTR of an additional *Xenopus Gpc4* allele was isolated by 5′RACE PCR (GeneRacer kit, Invitrogen). Based on the sequence of the two alleles, a 25-nucleotide antisense morpholino oligo against the 5′UTR of *Gpc4* (Gpc4Mo) was designed to inhibit translation from both alleles (Gene Tools, USA). The Gpc4Mo is complementary to a sequence 70 bases upstream of the ATG start codon (5′-TGCA-AAGTGCTGAGAATCCCCTAGT-3′). An antisense morpholino oligo against the human β-globin gene (CoMo) was used as standard control and was injected at the same concentration as the Gpc4Mo. Injection of 60 to 80 ng Gpc4Mo per embryo gave rise to the phenotypes described in this study. Injection of a second independent morpholino complementary to the *Gpc4* RNA sequence surrounding the ATG start codon resulted in similar phenotypes (data not shown) (see also Ohkawara et al., 2003). For in vitro translation of capped *Xenopus Gpc4* mRNA, transcripts were synthesized using SP6 RNA polymerase (Ambion). 50 ng of the capped mRNA (Ambion) was translated by using rabbit reticulocyte lysate (Promega) and [^{35}S]methionine in the presence of increasing amounts of Gpc4Mo (0.1, 0.4, 1.6 and 4 μg) or equal amounts of CoMo.

Embryo manipulations

Xenopus laevis eggs were fertilized and cultured following standard protocols (Sive et al., 2000). For the functional analysis of GPC4, two-cell stage embryos were injected with 30-40 ng antisense morpholino oligo per blastomere at the animal pole. To test the efficiency of the Gpc4Mo in vivo, 600 pg capped *Gpc4GFP* mRNA was injected into two-cell stage embryos. Subsequently, a total of 100 ng Gpc4Mo or CoMo was injected in either one or both blastomeres. To rescue the molecular and morphological defects of Gpc4Mo-injected embryos, a total of 60 ng Gpc4Mo (or CoMo) was injected into both blastomeres of two-cell stage embryos. After completion of the second division, a total of 800 pg mouse *Gpc4* capped mRNA was injected into the two dorsal blastomeres. For the inhibition of FGF signalling by SU5402 (Calbiochem) treatment of embryos, embryos were cultured in normal medium (MBS) (Sive et al., 2000) until the onset of neurulation (stage 13). From stage 13 onwards, embryos were cultured in MBS supplemented with SU5402 (0.1 mg/ml final concentration; dissolved in DMSO) or DMSO (same final concentration) until harvesting them between stages 15 and 21-22 for analysis.

Whole-mount in situ hybridisation and detection of apoptotic cells

Whole-mount in situ hybridisation was performed as previously described (Sive et al., 2000), and pigment granules were bleached as described (Song and Slack, 1994). Apoptotic cells were detected by using the in situ cell death detection kit (sections, fluorescein; whole mounts, POD, Roche) according to the manufacturer instructions with only minor modifications.

Proteins binding assays and immunoblot analysis

For binding assays, NIH3T3 cells were transfected with 10 μg mouse Gpc4-Myc plasmid. Cells were lysed 36 hours after transfection in PBS containing 0.5% NP40. After sonication, GST-FGF2 binding assays were performed as described (Fumagalli et al., 1994). Proteins

were separated by 8% SDS-PAGE and Myc epitope-tagged GPC4 was detected by anti-Myc antibodies. For analysis of ERK and SMAD1 phosphorylation levels embryos were lysed and proteins separated on a 15% gel. Proteins were immunoblotted using anti-pSMAD1 (Persson et al., 1998), anti-pERK (Cell Signalling) and anti-αtubulin antibodies (Sigma).

Results

Distribution of *Gpc4* transcripts in *Xenopus* embryos

We identified the *Xenopus Gpc4* gene by searching an expressed sequence tag (EST) database with mouse *Gpc4* cDNA (see Materials and methods). The predicted *Xenopus* GPC4 protein core is encoded by 556 amino acids and is orthologous to mouse *Gpc4* [71.4% identity, 81% similarity (Watanabe et al., 1995)], and most likely to zebrafish *knypek* also [57.4% identity, 71% similarity (Topczewski et al., 2001)].

Xenopus Gpc4 is a maternally expressed gene as transcripts are detected in the animal hemisphere from the two-cell stage up to blastula stages (Fig. 1A; data not shown). At the onset of gastrulation, expression expands to the marginal zone (Fig. 1B). During progression of gastrulation (Fig. 1C,D), *Gpc4* transcripts become progressively localized to the dorsal side of the embryo. In particular, high levels of *Gpc4* transcripts are detected in the area of Spemann's organizer during gastrulation (Fig. 1C,D). At this stage, the *Gpc4* transcript domain encompasses those of *Noggin* (compare Fig. 1D and E) (Smith and Harland, 1992) and *Chordin* (data not shown), which indicates that *Gpc4* is expressed by the prechordal endomesoderm and chordamesoderm (see also Ohkawara et al., 2003). In addition, the *Gpc4* expression domain also encompasses that of *Sox2* (Mizuseki et al., 1998), an early marker for neural fates (compare Fig. 1D and F). This latter result shows that presumptive neuroectodermal cells express *Gpc4* during neural cell fate specification.

During neurulation, *Gpc4* expression is high in presomitic mesoderm and the developing CNS (Fig. 1G,I,J,L). In the posterior neural plate, *Gpc4*-expressing neuroectodermal cells form two longitudinal stripes spanning the presumptive spinal cord (white arrow in Fig. 1G). In the anterior neural plate, *Gpc4*-expressing cells form a single arch, which crosses the midline (black arrow in Fig. 1G) and borders the *Fgf8*-expressing anterior neural ridge (data not shown). This anterior *Gpc4* expression domain overlaps with that of *Bf1* (compare Fig. 1G and H), which is the earliest known marker for telencephalic cell fates (Bourguignon et al., 1998). *Gpc4* transcripts are present in both the epithelial and sensory layers of the neuroectoderm (Fig. 1I), whereas expression in the underlying prechordal plate fades away (white arrowhead in Fig. 1I).

By mid-neurulation (Fig. 1J), the anterior *Gpc4* expression resolves into two distinct domains. The posterior domain overlaps with that of *Emx2* (compare Fig. 1J and K), one of the earliest genes expressed in presumptive dorsal forebrain territories (Pannese et al., 1998). In the developing dorsal forebrain, *Gpc4* transcripts persist up to early neural tube stages (Fig.1L; data not shown). From tailbud stages onwards, other predominant sites of *Gpc4* expression include the developing branchial arches, somites and pronephric ducts (data not shown).

GPC4 is required for gastrulation and nervous system patterning in *Xenopus* embryos

An antisense morpholino oligonucleotide directed against the 5′ leader of the *Xenopus Gpc4* mRNA was used to block GPC4 protein translation. Initially, we assessed the efficiency of two candidate oligos (see Materials and methods). One of these, Gpc4Mo, blocks translation of *Gpc4* mRNA very efficiently both in vitro (Fig. 2A, upper panel) and in vivo (Fig. 2C,D). Therefore, Gpc4Mo and an unrelated control antisense morpholino oligo (CoMo; Fig. 2A lower panel, Fig. 2B) were used for all studies shown.

Injection of Gpc4Mo into both blastomeres of two-cell stage

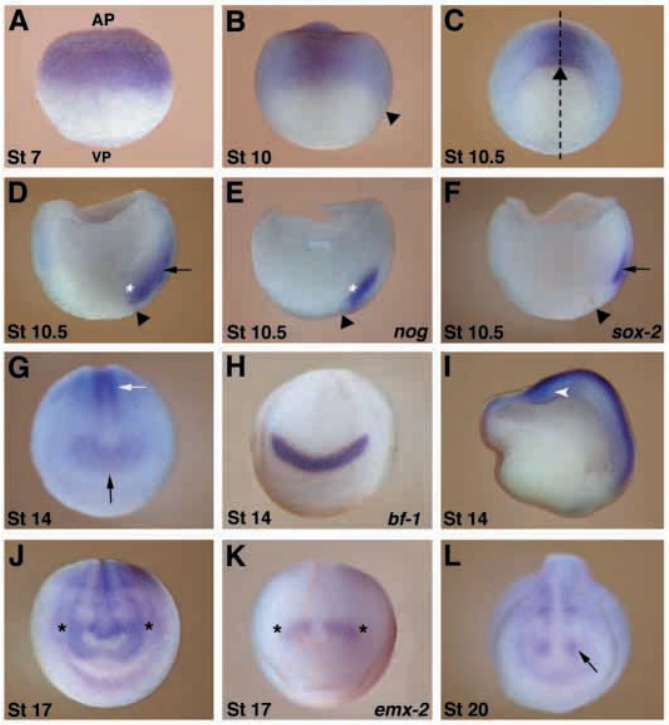

Fig. 1. *Gpc4* expression during early development of *Xenopus* embryos. Arrowheads (B-F) point to the dorsal blastopore lip. (A) Blastula (stage 7) showing localization of *Gpc4* transcripts in the animal hemisphere. AP, animal pole; VP, vegetal pole. (B) Expression of *Gpc4* at the onset of gastrulation (stage 10). (C) Dorso-vegetal view of an early gastrula stage embryo (stage 10.5). The broken line indicates the plane of the hemi-sections shown in panels D-F and I. (D-F) Hemi-sections of embryos cut along the dorsoventral axis (stage 10.5). (D) *Gpc4* transcripts in prechordal endomesoderm and chordamesoderm (asterisk) and in the neuroectodermal cell layer (arrow). (E) Distribution of *Noggin* transcripts in the prechordal endomesoderm and chordamesoderm (asterisk). (F) *Sox2* in the neuroectodermal cell layer (arrow). (G) Frontal view of an early neural plate embryo (stage 14). Note *Gpc4* transcripts in the anterior neural plate (black arrow) and presumptive spinal cord (white arrow). (H) Frontal view of a stage 14 embryo showing *Bf1* expression in the anterior forebrain. (I) Expression of *Gpc4* in a hemi-sectioned embryo (stage 14). Anterior is to the left. The white arrowhead points to decreasing expression in the prechordal plate. (J) Frontal view of a mid-neurula (stage 17). Asterisks point to *Gpc4* transcripts in the presumptive dorsal forebrain. (K) *Emx2* expression in the presumptive dorsal forebrain (stage 17; asterisks). (L) Expression of *Gpc4* following closure of the anterior neural tube (stage 20). Arrow points to transcripts in the forebrain.

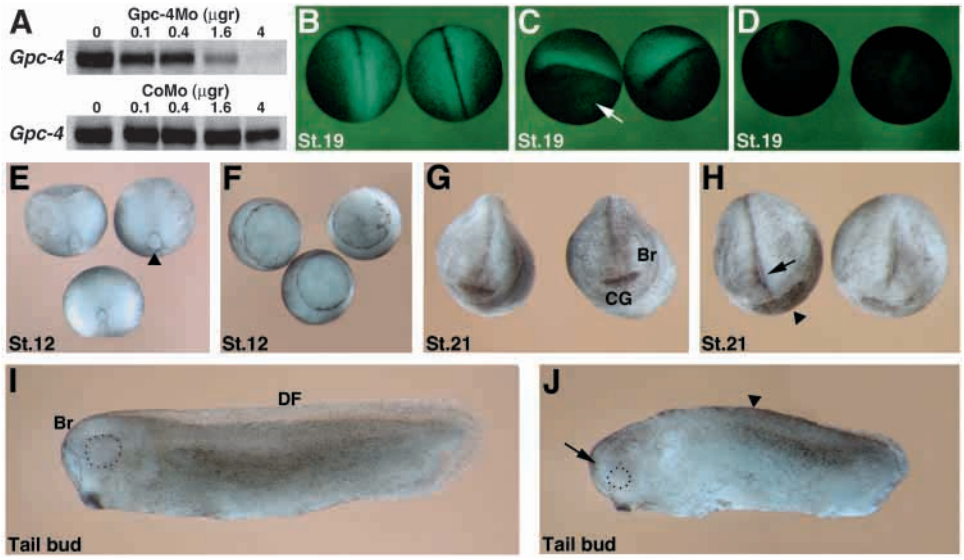

Fig. 2. GPC4 is required for early embryonic development. (A) The Gpc4Mo inhibits translation of *Gpc4* mRNA in vitro. Capped mRNA was in vitro translated in the presence of increasing amounts (indicated in μg) of Gpc4Mo (top panel) or CoMo (bottom panel). (B-D) Gpc4Mo specifically inhibits translation of *Gpc4* transcripts in vivo. (B) Embryos injected with chimeric *Gpc4GFP* transcripts and CoMo. (C,D) Embryos injected with *Gpc4GFP* mRNA and Gpc4Mo in one blastomere (arrow in C) or both blastomeres (D). Injection of Gpc4Mo inhibits *Gpc4GFP* mRNA translation as evidenced by lack of GFP activity. (E-J) Two-cell embryos were injected with CoMo (E,G,I) or Gpc4Mo (F,H,J) and analysed at different developmental stages. (E,F) GPC4 functions during gastrulation. Dorso-vegetal view of stage 12 embryos. (E) Blastopore has closed in embryos injected with CoMo (stage 12). (F) Blastopore remains open in embryos injected with Gpc4Mo (stage 12). (G,H) GPC4 is required for anterior CNS development. Frontal view of stage 21 embryos. Embryos injected with Gpc4Mo (H) retain an open anterior neural tube (arrow) but develop a cement gland (arrowhead). (I,J) Side view of tailbud stage embryos. In contrast to control embryos (I), embryos injected with Gpc4Mo (J) are shorter, lack the dorsal fin and have small heads. Arrowhead in J points to the missing dorsal fin; the arrow indicates microcephaly. The developing eyes are encircled. CG, cement gland; Br, brain; DF, dorsal fin.

embryos severely alters embryogenesis (Fig. 2F,H,J; 86%, *n*=193), whereas CoMo-injected embryos develop normally (Fig. 2E,G,I; 91%, *n*=107). Gpc4Mo-injected embryos develop normally up to gastrulation (data not shown) but gross-morphological defects appear from gastrulation onwards (Fig. 2F,H,J). Initially, a delay in blastopore closure becomes apparent as a large open blastopore remains at a stage by which gastrulation is almost complete in control embryos (compare Fig. 2E and F). At the end of neurulation, the anterior neural tube remains open in GPC4-depleted embryos and the brain vesicles are less pronounced (compare Fig. 2G and H). By the tailbud stage, GPC4-depleted embryos are shorter with a kinked axis, and their dorsal fin and head structures are reduced (compare Fig. 2I and J). Both eye fields are present but are significantly reduced in size (indicated by circles in Fig. 2I,J), whereas the cement gland appears normal (compare Fig. 2I and J). GPC4-depleted embryos fail to reach the swimming tadpole stage (data not shown). These phenotypes are less severe than those recently described by Ohkawara et al. (Ohkawara et al., 2003). However, injections of higher amounts of Gpc4Mo resulted in embryos with spina bifida (data not shown) as described by Ohkawara et al. (Ohkawara et al., 2003).

To investigate the molecular and cellular defects underlying the gross-morphological alterations of GPC4-depleted embryos (Fig. 2), we analysed the expression of genes regulating gastrulation and neurulation. The expression of *Goosecoid* (*Gsc*) (Cho et al., 1991) appears initially normal, indicating that GPC4 does not affect establishment of Spemann's organizer (compare Fig. 3A and B; *n*=3/3). During gastrulation, *Gsc*-expressing cells ingress and move toward the anterior of the embryo. Because of this anterior expansion, the *Gsc* expression domain narrows and elongates in control embryos (Fig. 3C), whereas it remains broad in Gpc4Mo-injected embryos (Fig. 3D; *n*=8/8). Changes in the spatial distribution of mesodermal and neuroectodermal genes become more apparent towards the end of gastrulation. For example, *Xenopus Brachyury* (*Xbra*) (Smith et al., 1991) is detected in the developing mesoderm around the blastopore and in the presumptive notochord in control embryos (Fig. 3E). In Gpc4Mo-injected embryos, the length of the presumptive notochord is very much reduced (arrow in Fig. 3F; *n*=9/10) and *Xbra* expression remains predominantly around the enlarged blastopore. Accordingly, analysis of *Noggin* expression in the prospective notochord (Smith and Harland, 1992) shows that the posterior extension of its expression domain is shorter and remains wider in comparison with control embryos (compare Fig. 3G and H; *n*=13/17). By contrast, the anterior *Noggin* (asterisks in Fig. 3G,H; *n*=13/17) and *Dkk1* expression domains (data not shown), which mark the anterior endoderm and prechordal endomesoderm, seem normal. Neural induction is also not affected, as expression levels of the pan-neural marker *Sox2* (Mizuseki et al., 1998) are normal (compare Fig. 3I and J). However, the posterior neuroectoderm lacks the characteristic neural plate morphology (asterisk in Fig. 3J; *n*=9/10) apparent in control embryos (asterisk, Fig. 3I), which is in agreement with the altered *Xbra* and *Noggin* expression in the notochord (compare Fig. 3F and H). Finally, analysis of *Et* expression (Li et al., 1997) in GPC4-depleted embryos

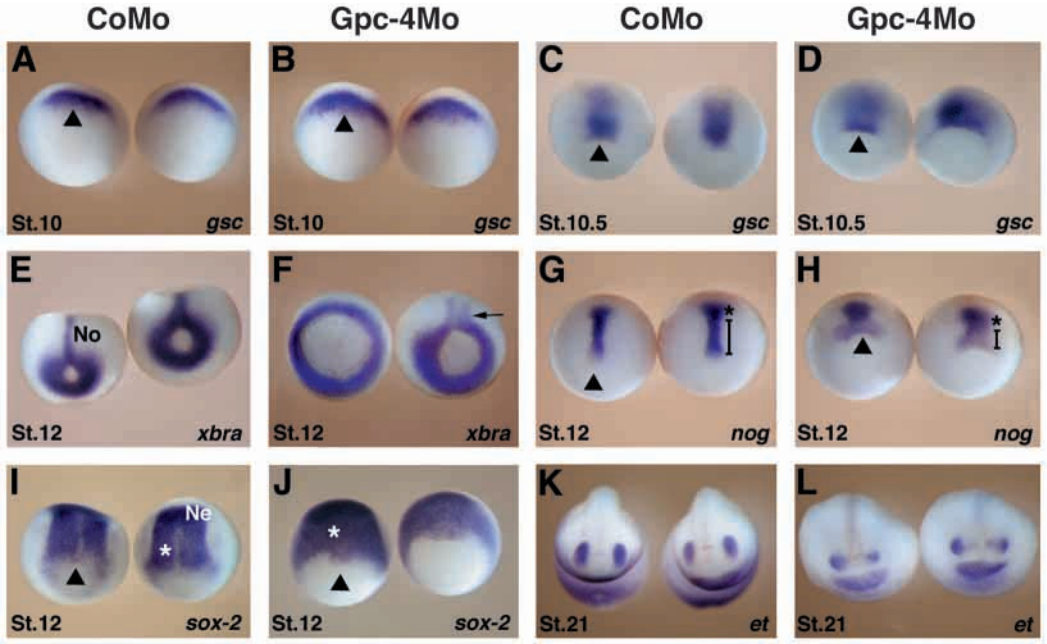

Fig. 3. Changes in gene expression become apparent during gastrulation of GPC4-depleted embryos. Dorso-vegetal view of embryos injected with CoMo (A,C,E,G,I) and Gpc4Mo (B,D,F,H,J). Arrowheads in panels A-D and G-J indicate the blastopore lip. Anterior is to the top. (A-D) *Gsc* expression during gastrulation (stage 10 to 10.5). (E,F) *Xbra* expression during late gastrulation (stage 12). (E) Expression in the developing notochord (No) in control embryos. Arrow in F indicates reduced length of notochord expression in GPC4-depleted embryos. (G,H) *Noggin* transcripts at stage 12. Asterisk indicates anterior-most expression. Bar indicates length of expression domain in the presumptive notochord. *Noggin* transcripts are normal in the anterior mesendoderm (asterisk), but the length of the presumptive notochord is reduced in GPC4-depleted embryos (compare G with H). (I,J) *Sox2* expression in neuroectoderm (Ne) at stage 12. *Sox2* is not expressed in the posterior midline of control embryos (asterisk in I), and *Sox2* expression is not excluded from posterior midline in Gpc4Mo-injected embryos (asterisk in J). (K,L) Frontal view of *Et* expression (stage 21) to show that two retina fields form in CoMo- (K) and Gpc4Mo-injected embryos (L).

shows that two retinal and eye primordia develop (compare Fig. 3K and L). These findings are in agreement with normal *Shh* expression in the ventral midline (data not shown). Taken together, these results show that inhibition of GPC4 function during gastrulation affects anteroposterior axis elongation, whereas the head organizer, specification of the anterior neuroectoderm and ventral midline formation seem normal.

Gpc4 (Fig. 1) and other family members are expressed in the developing neural tube (Song and Filmus, 2002), but their functions during CNS morphogenesis remain to be identified. To gain an insight into the roles of glypicans in this process, we further investigated the brain defects observed in GPC4-depleted *Xenopus* embryos. Analysis of *Sox2* distribution after neural tube closure (Fig. 4A) reveals the phenotypic alterations of the neural tube morphology (Fig. 4E; *n*=10/11). Histological sections of the embryonic CNS demonstrate that patterning of the forebrain and midbrain are predominantly affected (compare Fig. 4B and F, and Fig. 4C and G). In particular, the size of the forebrain is reduced, the mesencephalon and eye vesicles are less pronounced, and neural tube closure has not occurred correctly (white arrowheads in Fig. 4F,G). These results show that anterior CNS structures are severely affected in GPC4-depleted *Xenopus* embryos, although the spinal cord appears rather normal (compare Fig. 4D and H).

GPC4 regulates expression of transcription factors required for dorsal forebrain development

The *Otx2* gene is expressed by the fore- and midbrain during CNS patterning (Pannese et al., 1995). Depletion of GPC4 eliminates most of the *Otx2* expression in the forebrain (arrowheads in Fig. 5A,B; *n*=13/16), whereas its midbrain expression domain is less affected (asterisks in Fig. 5A,B). Similarly, *Bf1* expression is reduced in the developing telencephalon (arrows in Fig. 5C,D; *n*=6/8). By contrast, *Hoxb9* expression in the spinal cord (arrowheads in Fig. 5E,F; *n*=10/10) (Cho et al., 1988), *Krox20* expression in the hindbrain (brackets in Fig. 5E,F; *n*=16/16) (Bradley et al., 1993), and *Fgf8* expression in the isthmus and anterior neural ridge (asterisk in Fig. 5G,H; *n*=9/9) (Eagleson and Dempewolf, 2002) appear normal.

Following neural induction, the vertebrate forebrain is also regionalized along its dorsoventral axis. One hallmark of these early patterning events is the expression of *Emx2* in the dorsal, and *Nkx2.1* in the ventral, forebrain territories (Rubenstein et al., 1998). In GPC4-depleted embryos, *Emx2* expression is drastically reduced or absent following neural tube closure (compare Fig. 5K and L; *n*=31/37), whereas *Nkx2.1* continues to be expressed (compare Fig. 5I and J; *n*=12/12). Similar to *Emx2*, the expression of other dorsal forebrain genes, such as *Emx1* and *Eomesodermin*, is also downregulated (data not shown).

Rescue of forebrain patterning defects by co-injection of *mouse Gpc4* mRNA

The following rescue experiment was performed to assess whether the molecular and morphological defects in forebrain

patterning are specifically caused by the interference of Gpc4Mo with GPC4 function. *Xenopus* embryos were co-injected with Gpc4Mo and mouse *Gpc4* mRNA, which lacks the Gpc4Mo target sequence (data not shown). Such co-injection, rescues *Emx2* expression in 69% of all embryos (Fig. 5N; Table 1). Furthermore, forehead morphology and *Emx2*

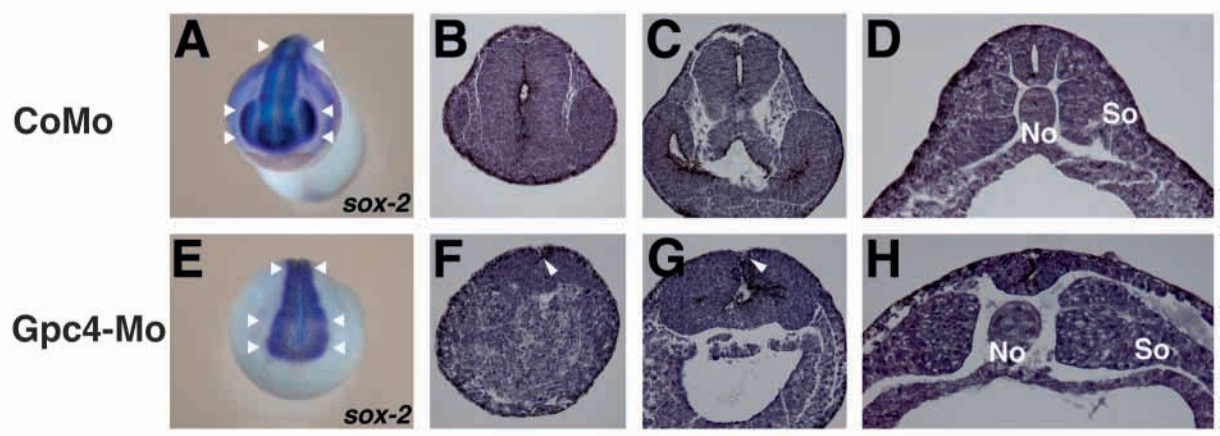

Fig. 4. Forebrain defects in GPC4-depleted embryos. (A-D) Embryos injected with CoMo. (E-H) Embryos injected with Gpc4Mo. (A,E) Frontal view of *Sox2* distribution at stage 21. White arrowheads indicate the level of the transverse sections shown in panels B-D and F-H. (B-D,F-H) Histological sections are at the level of the forebrain in panels B and F, the midbrain in panels C and G, and the spinal cord in panels D and H. Arrowhead in panels F and G points to defects in dorsal neural tube closure. No, notochord; So, somites.

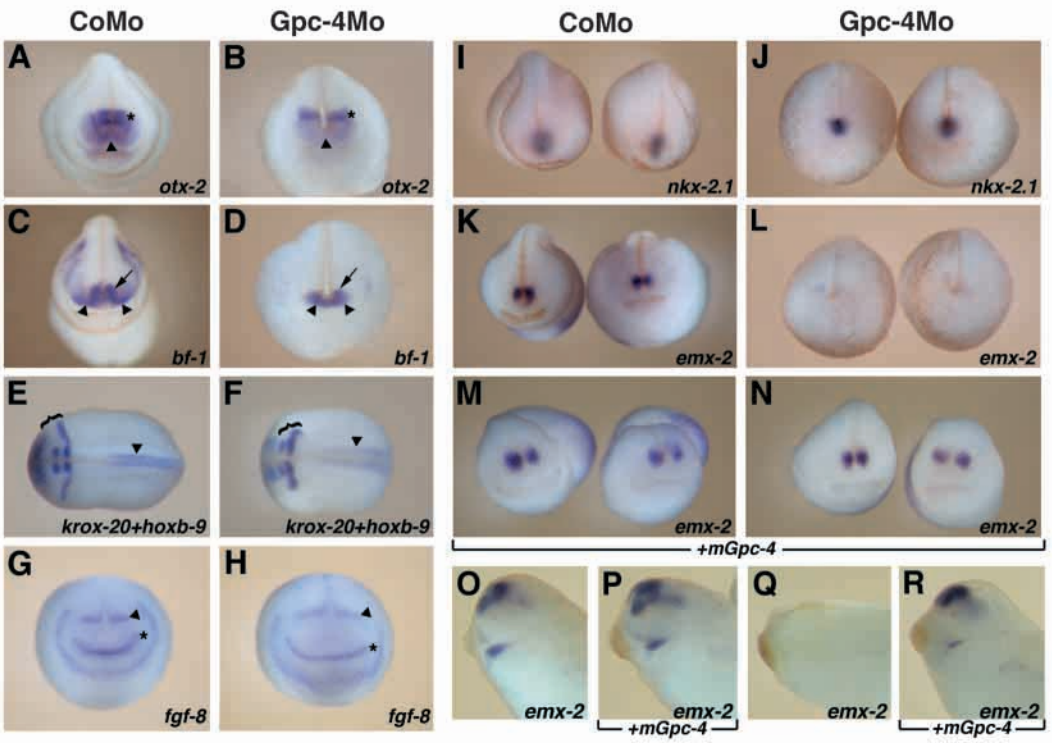

Fig. 5. GPC4 regulates expression of dorsal forebrain markers. Molecular analysis of neural markers in CoMo- and Gpc4Mo-injected embryos. (A-D,G-N) Frontal views; (E,F) dorsal views; (O-R) side view. Anterior is to the left. (A,B) *Otx2* expression (stage 21). Arrowhead indicates forebrain expression; asterisk indicates midbrain expression. (C,D) *Bf1* expression (stage 21). Arrows indicate expression in the developing telencephalon; arrowhead indicates expression in the olfactory placodes. (E,F) Expression of the posterior neural markers *Krox20* (bracket) and *Hoxb9* (arrowhead) in stage 21 embryos. (G,H) *Fgf8* expression (stage 17). Asterisk indicates anterior neural ridge; arrowhead indicates isthmus. (I,J) *Nkx2.1* expression in the ventral forebrain (stage 21); note that *Nkx2.1* expression persists in GPC4-depleted embryos (J). (K,L) *Emx2* expression in the dorsal forebrain of developing embryos (stage 21); note that *Emx2* expression is drastically reduced in GPC4-depleted embryos (L). (M) *Emx2* expression in embryos co-injected with CoMo and mouse *Gpc4* (m*Gpc4*) mRNA; note that overexpression of mouse *Gpc4* does not affect *Emx2* expression (compare with K). (N) Rescue of *Emx2* expression in embryos co-injected with Gpc4Mo and mouse *Gpc4* mRNA (compare with L). (O) *Emx2* expression in a tailbud embryo injected with CoMo. (P) *Emx2* expression in a tailbud embryo co-injected with CoMo and mouse *Gpc4* mRNA. (Q) Loss of *Emx2* expression in a tailbud embryo injected with Gpc4Mo. (R) Rescue of *Emx2* expression and forehead morphology in a tailbud embryo co-injected with Gpc4Mo and mouse *Gpc4* mRNA.

distribution in the dorsal forebrain of rescued tailbud embryos (Fig. 5R) are similar to control embryos (Fig. 5O). By contrast, mouse *Gpc4* mRNA does not significantly alter *Emx2* expression and dorsal forebrain patterning upon co-injection with CoMo (Fig. 5M,P). Taken together, these results demonstrate that GPC4 function is required to regulate expression of dorsal forebrain identity genes.

GPC4 is required for establishment of the *Emx2* expression domain and survival of forebrain cells

As *Emx2* is one of the earliest known genes expressed in the presumptive dorsal forebrain territory, we determined whether GPC4 is required to establish *Emx2* expression or only to maintain its expression during neural tube closure (Fig. 5K-N; Table 1). Analysis of *Xenopus* embryos prior to neural tube closure (from stage 14 to 17) shows that GPC4 is required for *Emx2* expression in the dorsal forebrain, from early neural plate stages onwards (compare Fig. 6A and B; absent, *n*=17/28; low, *n*=11/28; data not shown). The reduced forebrain vesicles (Fig. 2J, Fig. 4F, Fig. 5Q) of Gpc4Mo-injected embryos prompted us to analyse possible effects of GPC4 depletion on cell survival. No differences in the level of apoptotic cells are detected when comparing control embryos (Fig. 6C) and GPC4-depleted embryos prior to anterior neural tube closure (stage 17; Fig. 6D; *n*=3/4). By contrast, massive apoptosis is observed in the CNS of GPC4-depleted embryos during closure of the anterior neural tube (stage 20; compare Fig. 6E and F; *n*=6/8). In particular, cell death is abundant in the anterior brain, encompassing the dorsal forebrain (compare Fig. 6G and H). This cell death is rescued in Gpc4Mo embryos co-injected with mouse *Gpc4* mRNA (Fig. 6I). These results show that downregulation of *Emx2* (Fig. 6B) long precedes the onset of apoptosis (Fig. 6H), and that GPC4 functions are required for the survival of neural progenitors in the developing forebrain (Fig. 6I).

Evidence for a role of GPC4 in modulating FGF signalling during dorsal forebrain development

Members of the BMP and FGF signalling families have been implicated in the regulation of vertebrate forebrain morphogenesis (Wilson and Rubenstein, 2000). In particular, during early *Xenopus* forebrain development, the FGF2 protein is distributed in a pattern similar to *Gpc4* transcripts [compare Fig. 1G,I,J to Song and Slack (Song and Slack, 1994)], raising the possibility of a direct interaction. Biochemical analysis reveals that a glutathione S-transferase (GST)-FGF2 fusion protein retains the fully heparan-sulphated GPC4 protein of about 200 kDa (arrow in Fig. 7A), but not the unmodified 60 kDa protein (asterisk in Fig. 7A). Furthermore, the two proteins can be co-immunoprecipitated from chicken embryonic fibroblast protein extracts (data not shown), indicating that GPC4 complexes with FGF2 in vivo. These biochemical studies suggest that GPC4, like other glypicans (Grisaru et al., 2001), modulates FGF signalling. ERK protein kinases are targets of FGF signalling in neurulating *Xenopus* embryos (Christen and Slack, 1999). Therefore, their phosphorylation levels serve as an intracellular indicator of FGF signal transduction (Fig. 7B). Biochemical analysis of *Xenopus* embryos shows that ERK phosphorylation levels are reduced about two- to threefold when injected with Gpc4Mo at the two-cell stage (pERK; Fig. 7B; compare lane 'CoMo' with 'Gpc4Mo'; data not shown). This downregulation of ERK phosphorylation in Gpc4Mo-injected embryos is rescued

Table 1. Mouse *Gpc4* mRNA rescues *Emx2* expression in Gpc4Mo-injected embryos

		Emx2 expression		
Injection	Number of embryos	Normal	Reduced	Absent
CoMo	14	100%		
Gpc4Mo	15	20%	20%	60%
Gpc4Mo+mouse *Gpc4*	39	69%	18%	13%
CoMo+mouse *Gpc4*	17	95%	5%	

The table summarizes the results of analysing *Emx2* expression in two independent experiments.

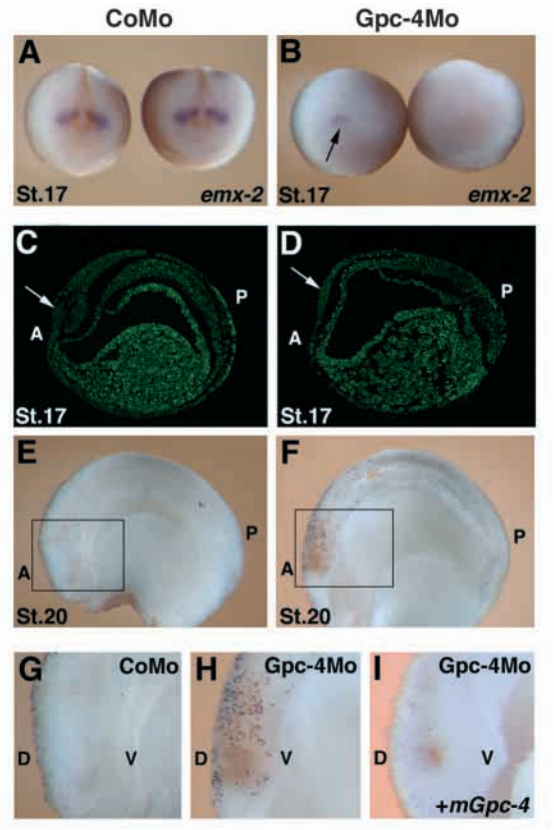

Fig. 6. GPC4 is required for establishment of *Emx2* expression and survival of anterior CNS cells. (A,B) *Emx2* transcript distribution during neurulation (stage 17) in CoMo- (A) and Gpc4Mo-injected (B) embryos; frontal views are shown. *Emx2* expression in GPC4-depleted embryos is either very low (arrow) or absent. (C-I) TUNEL assays to detect apoptotic cells in neurulating embryos. (C,E,G) CoMo-injected embryos. (D,F,H) Gpc4Mo-injected embryos. Anterior is to the left. (C,D) Fluorescence analysis of cell death on sagittal sections of a stage 17 embryo. Fluorescence was used as it is more sensitive for detection of low numbers of apoptotic cells. Arrows in C and D point to the presumptive forebrain. (E-I) Detection of apoptotic cells by whole-mount analysis of hemi-sectioned embryos (stage 20). Massive cell death is apparent in the brain of Gpc4Mo-injected embryos (F) in contrast to CoMo-injected embryos (E). Boxed areas in panels E and F indicate the enlargements shown in panels G and H. (I) Cell death is rescued in embryos co-injected with Gpc4Mo and mouse *Gpc4* mRNA. A, anterior; P, posterior; D, dorsal; V, ventral.

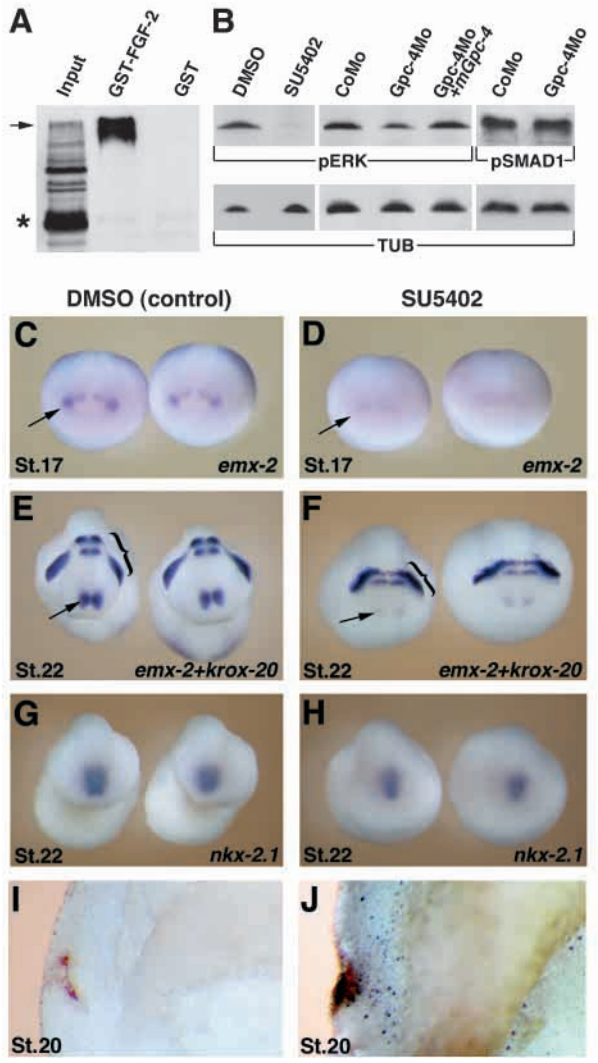

Fig. 7. GPC4 modulates FGF signalling during neurulation. (A) Immunoblot analysis of GPC4/FGF2 complexes, as detected by anti-Myc antibodies. The 'Input' lane contains NIH3T3 cells transfected with the Myc epitope-tagged mouse *Gpc4* cDNA. The HS-GAG modified mouse GPC4 proteins have an apparent M_r of around 200 kDa (arrow), whereas the unmodified proteins run at 60 kDa (asterisk). In the 'GST-FGF-2' lane only the modified 200 kDa GPC4 protein (arrow) binds to FGF2. Mouse GPC4 does not bind to GST (control; 'GST' lane). (B) Immunoblot analysis of phosphorylated ERK (pERK) and SMAD1 (pSMAD1) proteins in *Xenopus* embryos (stage 15). Levels of phosphorylated proteins were determined in embryos that were: cultured in the presence of the FGF inhibitor SU5402 (0.1 mg/ml; lane 'SU5402'); cultured with DMSO as a control (lane 'DMSO'); injected with CoMo (lane 'CoMo'); injected with Gpc4Mo (lane 'Gpc4Mo'); or co-injected with Gpc4Mo and mouse *Gpc4* mRNA (lane 'Gpc4Mo + *mGpc4*'). TUB, α-Tubulin levels in the extracts were determined to normalize samples. (C-H) Molecular analysis of embryos cultured with DMSO (panels C,E,G) and with SU5402 (0.1 mg/ml; panels D,F,H). Arrows in panels C-F indicate *Emx2* expression. (C) *Emx2* expression in control embryos cultured with DMSO (stage 17). (D) Downregulation of *Emx2* in embryos cultured with SU5402 (stage 17). (E) *Emx2* and *Krox20* (bracket) expression in control embryos cultured with DMSO (stage 22). (F) Downregulation of *Emx2*, but not *Krox20* (bracket), in embryos cultured with SU5402 (stage 22). (G) *Nkx2.1* expression in embryos cultured with DMSO (stage 22). (H) *Nkx2.1* expression in embryos cultured with SU5402 (stage 22). (I) Lack of cell death in the forebrain region of an embryo cultured with DMSO (stage 20). (J) Apoptotic cells detected in the forebrain region of an embryo cultured with SU5402 (stage 20).

following co-injection of mouse *Gpc4* mRNA (Fig. 7B; lane 'Gpc4Mo + mGpc4'). By contrast, phosphorylation of the SMAD1 protein, indicative of BMP signal transduction, is not altered (pSMAD1; Fig. 7B) (Persson et al., 1998). These studies show that GPC4 interacts with FGF ligands and that, although it is not essential for FGF signalling, it is required to enhance FGF signal transduction during neurulation of *Xenopus* embryos.

The potential roles of FGFs during forebrain patterning were further investigated by blocking FGF signal transduction using SU5402 (Mohammadi et al., 1997) (see also Fig. 7B; compare lane DMSO to SU5402). To avoid perturbing gastrulation, SU5402 was added to *Xenopus* embryos from early neural plate stages onwards (stage 13; see Materials and methods). Analysis of SU5402-treated embryos shows that *Emx2* expression is downregulated from stage 17 (compare Fig. 7C and D; *n*=5/5) onwards (compare Fig. 7E and F; *n*=11/11). By contrast, expression of *Nkx2.1* (Fig. 7G,H; *n*=5/5) and *Krox20* (bracket in Fig. 7E,F; *n*=7/7) is only slightly affected. Similar to Gpc4Mo-injected embryos, inhibition of FGF signalling by SU5402 results in death of forebrain cells at the onset of anterior neural tube closure (Fig. 7J). In summary, inhibition of either GPC4 function or FGF signal transduction affects *Emx2* expression similarly (compare Fig. 6B with Fig. 7D, and Fig. 5L with Fig. 7F). These findings indicate that GPC4 regulates *Emx2* expression and, thereby, dorsal forebrain development by positive modulation of FGF signalling.

Discussion

We have functionally analysed the *Glypican 4* gene in developing *Xenopus* embryos using Gpc4Mo antisense morpholino oligonucleotides. Gpc4Mo specifically blocks GPC4 protein translation, as evidenced by biochemical analysis and phenotypic rescue by mouse *Gpc4* transcripts. The short body axis of GPC4 depleted *Xenopus* embryos is reminiscent of the phenotype of *knypek*-deficient zebrafish embryos (Topczewski et al., 2001). The Glypican encoded by *knypek* is highly homologous to the product of the *Gpc4* and *Gpc6* genes, and regulates cell polarity during convergent-extension movements. Similar to *knypek*, *Gpc4* is expressed in tissues undergoing extensive movements during gastrulation (reviewed by Wallingford et al., 2002). These tissues include the involuting mesoderm and the posterior neuroectoderm. Injection of Gpc4Mo into *Xenopus* embryos causes defects in axial elongation of mesoderm and neuroectodermal tissues during gastrulation, similar to those seen in *knypek*-mutant zebrafish embryos. Analysis of these embryos shows that *knypek* promotes non-canonical WNT signalling (WNT11), which is required for convergent-extension movements during zebrafish gastrulation. Indeed, Ohkawara et al. (Ohkawara et al., 2003) recently showed that GPC4, like Knypek in zebrafish, regulates convergent extension movements during *Xenopus* gastrulation by modulation of the non-canonical WNT pathway. Therefore, the present study focuses on

analysing key GPC4 functions during early forebrain patterning and provides evidence that GPC4 is required to enhance FGF signalling.

GPC4 is required for forebrain patterning in *Xenopus* embryos

It is unlikely that GPC4 acts during head and anterior neural plate induction, as the cement gland, ventral forebrain, two eye primordia and olfactory placodes form. The latter two structures derive from the most anterior neural plate (Rubenstein et al., 1998), which indicates that the most anterior brain structures are present in GPC4-depleted *Xenopus* embryos. In agreement with this, *Otx2*, the earliest anterior neural plate marker (Rubenstein et al., 1998), is expressed during gastrulation and is only downregulated during neurulation. In contrast to abrogation of GPC4, inhibition of *Dkk1* and *Igf*, which regulate head- and anterior neural plate induction, results in severe microcephaly and a complete loss of the cement gland and eyes (Glinka et al., 1998; Pera et al., 2001). Moreover, abrogation of *Tlc* and *Axin*, two inhibitors of WNT signalling, disrupts anteroposterior regionalization of the forebrain, causing loss of both ventral and dorsal forebrain and eye fields (Wilson and Rubenstein, 2000; Houart et al., 2002). These phenotypes are much more severe, and their appearance significantly precedes the ones observed in GPC4-depleted *Xenopus* embryos.

Subsequently, inductive signals emanating from the prechordal plate (e.g. SHH) and anterior neural ridge (e.g. FGF8) act on anterior neural plate cells to establish regional differences, such as specification of dorsal and ventral forebrain identities (Rubenstein et al., 1998). *Gpc4* is expressed by the prechordal endomesoderm during gastrulation and by the anterior neural plate at the time when these signalling centers are active. However, the *Shh* and *Fgf8* expression domains are established correctly in Gpc4Mo-injected *Xenopus* embryos. Inactivation of *Shh* and *Fgf8* causes ventral forebrain defects (Rubenstein et al., 1998) in contrast to interfering with GPC4 activity (this study). Therefore, the dorsal forebrain defects observed in Gpc4Mo-injected embryos most likely arise by altering the reception of signals targeted to dorsal neuroectodermal cells prior to closure of the anterior neural tube (see below).

In *Xenopus* and mouse embryos, cells of the presumptive forebrain begin to express Gpc4 during neurulation (this study) (A.G. and R.D., unpublished), and in the embryonic mouse brain expression persists in telencephalic neural precursors (Hagihara et al., 2000). Mutations in human *GPC3* and *GPC4* genes, which are next to one another on the X-chromosome, have been linked to the Simpson-Golabi-Behmel syndrome (SGBS). The SGBS syndrome is characterized by general pre- and postnatal overgrowth (reviewed by DeBaun et al., 2001). A fraction of SGBS patients also show mental retardation, seizures and a high risk for neuroblastoma (DeBaun et al., 2001). In the present study, we show that abrogation of GPC4 activity in *Xenopus* embryos disrupts forebrain patterning and cell survival, and causes microcephaly. Therefore, our findings raise the possibility that some of the CNS abnormalities affecting SGBS patients may arise as a consequence of disrupting *Gpc4* gene function during neurulation. In GPC4-depleted *Xenopus* embryos, the expression of dorsal forebrain identity genes, such as *Emx2* and *Emx1*, is disrupted already during neurulation. Previous genetic analysis of Emx genes in mice has established that they regulate regionalization and expansion of the dorsal forebrain compartment and subsequent cerebral cortex morphogenesis (Yoshida et al., 1997; Mallamaci et al., 2000). In particular, *Emx1* and *Emx2* compound-mutant embryos have greatly reduced telencephalic vesicles prior to initiation of cerebral cortex development (Bishop et al., 2003). Therefore, the dorsal forebrain defects observed in GPC4-depleted *Xenopus* embryos could be a consequence of mainly disrupting expression of the EMX genes during neurulation.

GPC4 modulates FGF signalling in the developing dorsal forebrain

Patterning of the vertebrate CNS depends to a large extent on extracellular regulation of signals (Rubenstein et al., 1998; Wilson and Rubenstein, 2000). Glypicans regulate signalling by modulating the formation of receptor-ligand complexes (Nybakken and Perrimon, 2002). In agreement with this, abrogation of GPC4 function in neurulating *Xenopus* embryos reduces phosphorylation of ERK protein kinases, which are specific targets of FGF signalling (Christen and Slack, 1999). This result shows that GPC4 participates in enhancing FGF signal transduction during embryogenesis. Similarly, genetic studies in *Drosophila* show that formation of an active FGF receptor-ligand complex depends on the presence of HSPGs (Lin et al., 1999). Inhibition of FGF signalling by SU5402 in *Xenopus* embryos phenocopies aspects of depleting GPC4 function, such as loss-of *Emx2* expression and increased apoptosis of forebrain progenitors. Several FGF ligands and their cognate receptors are expressed during patterning of the vertebrate CNS (Dono, 2003). Genetic and functional analysis established that two of these ligands, FGF8 and FGF3, function during formation of mid-hindbrain and rhombomere boundaries, respectively, in vertebrate embryos. Moreover, both FGF ligands participate in patterning of the anterior telencephalic midline and the anterior and post-optic commissure (Wilson and Rubenstein, 2000; Shinya et al., 2001). The present study establishes that FGF signalling also regulates dorsal forebrain development, but the involved FGF ligand(s) remains to be identified. Candidates are FGF9 (Song and Slack, 1996) and, in particular, FGF2, as this FGF ligand is present throughout the brain during *Xenopus* neurulation (Song and Slack, 1994) and binds GPC4 (this study). FGF2-deficient mice display defects in dorsal telencephalon patterning, albeit only much later during cerebral cortex layer formation (Dono, 2003). Therefore, further functional and genetic analysis is necessary to identify and study the FGF ligands interacting with GPC4 in embryos.

Comparative analysis of GPC4-depleted and SU5402-treated *Xenopus* embryos suggests that modulation of BMP and/or WNT signalling does not significantly contribute to *Emx2* regulation in the dorsal forebrain. By contrast, the similarities in the axis defects between GPC4-depleted *Xenopus* (Ohkawara et al., 2003) and *knypek*-deficient zebrafish embryos points to possible effects on non-canonical WNT signalling during gastrulation (see before). Therefore, glypicans may control the activity of different ligands in a stage- and/or tissue-specific manner as shown for *Drosophila* Dally, which regulates *wg* during embryonic development and *dpp* signalling during post-embryonic development

(Nybakken and Perrimon, 2002). Modifications of proteins by HS-GAG side chains are not uniform and changes in the distribution of sulphate groups affect ligand-binding properties. Enzymes involved in HSPG biosynthesis modify the HS-GAG side chains of Glypicans and regulate their ability to bind signal peptides during *Drosophila* embryogenesis (Giraldez et al., 2002). It will be important to determine if, and to what extent, alterations of HS-GAG side-chains of GPC4 can confer it with the ability to bind WNT during gastrulation (Ohkawara et al., 2003) and FGF ligands during neurulation (this study). Such alterations may explain cell-type and developmental-stage specific modulation of ligand-receptor interactions by glypicans during vertebrate embryogenesis.

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