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Kermit, a frizzled interacting protein, regulates frizzled 3 signaling in neural crest development

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SUMMARY

Wnts are a family of secreted glycoproteins that are important for multiple steps in early development. Accumulating evidence suggests that *frizzled* genes encode receptors for Wnts. However, the mechanism through which frizzleds transduce a signal and the immediate downstream components that convey that signal are unclear. We have identified a new protein, Kermit, that interacts specifically with the C-terminus of *Xenopus* frizzled-3 (Xfz3). Kermit is a 331 amino acid protein with a central PDZ domain. *Kermit* mRNA is expressed throughout *Xenopus* development and is localized to neural tissue in a pattern that overlaps *Xfz3* expression temporally and spatially. Co-expression of Xfz3 and Kermit results in a dramatic translocation of Kermit to the plasma membrane. Inhibition of Kermit function with morpholino antisense oligonucleotides directed against the 5′ untranslated region of *Kermit* mRNA blocks neural crest induction by Xfz3, and this is rescued by co-injection of mRNA encoding the Kermit open reading frame. These observations suggest that Kermit is required for Wnt/frizzled signaling in neural crest development. To the best of our knowledge, Kermit is the first protein identified that interacts directly with the cytoplasmic portion of frizzleds to modulate their signaling activity.

Key words: *Xenopus*, Frizzled, Wnt, Wnt1, Frizzled 3, Neural crest, PDZ domain, Dishevelled, GIPC

INTRODUCTION

Wnt1 was first identified as an oncogene activated by provirus insertion (Nusse and Varmus, 1992). Subsequently, Wnt1 was shown to be a member of a family of secreted glycoproteins (Wnts) that regulate cell fate, cell polarity, cell mobility and cell proliferation (Cadigan and Nusse, 1997; Dierick and Bejsovec, 1999; Liu et al., 1999; Moon et al., 1997; Nusse and Varmus, 1992; Parr and McMahon, 1994; Wodarz and Nusse, 1998). In vertebrates, Wnt signaling has been implicated in dorsal axis formation, patterning of the limb, organogenesis, neural crest formation and anteroposterior patterning of the central nervous system.

The current data suggest that Wnts signal through seven transmembrane receptors of the frizzled family. Although the original frizzled gene, *Drosophila frizzled* (*fz*), was identified about 100 years ago and was cloned 10 years ago (Vinson et al., 1989), frizzleds have only recently been proposed as receptors for Wnts. It has now been demonstrated that Wnts can bind to frizzled proteins (Hsieh et al., 1999) and that frizzled can mediate downstream Wnt signaling in response to Wnts in cell culture (Bhanot et al., 1996). Furthermore, frizzleds are required for Wnt signaling in vivo, as depletion of both fz and fz2 in *Drosophila* results in wingless-like phenotypes (Bhanot et al., 1999; Bhat, 1998; Chen and Struhl, 1999; Kennerdell and Carthew, 1998). Multiple fz homologs have been identified throughout the animal kingdom from worms to humans (Wang et al., 1996). Although roles for most of the vertebrate frizzled genes have not yet been established, they have been proposed to play roles in morphogenesis and dorsal axis formation (Deardorff et al., 1998; Djiane et al., 2000; Itoh et al., 1998; Nasevicius et al., 1998; Sumanas et al., 2000).

Frizzled proteins share the following structural motifs (Bhanot et al., 1996; Vinson et al., 1989; Wang et al., 1996): a putative signal sequence, a conserved cysteine rich domain (CRD), which is believed to bind Wnts, a hydrophilic and highly divergent linker region, seven conserved putative transmembrane segments, and a variable intracellular C terminus that usually contains a PDZ-binding motif.

Frizzleds appear to signal through multiple pathways, including the canonical Wnt pathway, involving arrow/LRP5,6 (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000), dishevelled (dsh), axin, glycogen synthase kinase 3β (GSK-3β), β-catenin and lymphoid enhancer factor 1 (reviewed by Cadigan and Nusse, 1997; Dierick and Bejsovec, 1999; Moon

et al., 1997; Wodarz and Nusse, 1998), and the tissue polarity pathway, which also involves dsh (Krasnow et al., 1995), but not GSK-3β and β-catenin (Boutros et al., 1998). Epistasis experiments in *Drosophila* have implicated *prickle*, *inturned*, *fuzzy*, *multiple-wing-hair* (Wong and Adler, 1993), *RhoA* (Strutt et al., 1997), Jun-N-terminal kinase (JNK) and Jun (Boutros et al., 1998) in the regulation of the tissue polarity pathway. In addition, ectopic expression of rat frizzled 2 (Fz2), but not Fz1, in zebrafish appears to function through heterotrimeric G proteins, although it is not yet clear whether this interaction is direct or indirect (Slusarski et al., 1997). Related to this, different frizzleds differ in their abilities to activate protein kinase C (PKC) (Sheldahl et al., 1999). Recent data also show that one frizzled may be able to activate multiple pathways. For example, in *Drosophila* embryos, *fz* and *fz2* serve overlapping functions, both signaling through GSK-3β/zeste white-3 and β-catenin/armadillo (Bhanot et al., 1999; Bhat, 1998; Chen and Struhl, 1999; Kennerdell and Carthew, 1998; Rulifson et al., 2000). However, fz does not appear to signal through β-catenin/armadillo to establish correct tissue polarity in the adult wing (Boutros et al., 1998). *Xenopus* fz7 (Xfz7) has also been reported to function through both canonical and noncanonical pathways (Djiane et al., 2000; Medina et al., 2000; Sheldahl et al., 1999; Sumanas et al., 2000).

The C termini of frizzleds may be essential for their functions. When tryptophan 500 in the third extracellular loop of fz is mutated to a stop codon, this mutant is phenotypically null, though it is expressed at the same level as wild type (Jones et al., 1996). The same mutation at the equivalent position in *lin-17*, a *Caenorhabditis elegans* frizzled gene, results in the disruption of asymmetric cell division (Sawa et al., 1996). Deletions of the C termini after the seventh putative transmembrane domains of frizzleds render the receptors inactive (M. A. D. and P. S. K., unpublished) and these deletion mutants sometimes behave as dominant negative receptors (Sumanas et al., 2000). These observations suggest that the C-termini of frizzleds are essential for signaling and/or interpretation of the signal and may be involved in binding of downstream targets. Umbhauer and colleagues (Umbhauer et al., 2000) suggest that a conserved motif (Lys-Thr-X-X-X-Trp) located two amino acids after the seventh transmembrane domain of frizzleds is required for activation of the Wnt/β-catenin pathway.

Dsh is the most proximal cytosolic component identified so far for frizzled signaling, and appears to be common to multiple frizzled pathways. Overexpression of rat Fz1 causes a dsh-GFP fusion protein to translocate from the cytoplasm to the membrane (Yang-Snyder et al., 1996). However, direct interaction between frizzleds and dsh has not been reported. Therefore, it remains of great interest to identify the molecules that interact directly with frizzleds to convey signaling.

We report here the identification of a new gene, *Kermit*, that interacts strongly with Xfz3 but not with Xfz8. *Kermit* is expressed throughout *Xenopus* development and is localized to the anterior neural tissue in a pattern highly similar to *Xfz3* temporally and spatially. In addition, co-expression of Xfz3 with Kermit recruits Kermit to the plasma membrane. Finally, depletion of Kermit using morpholino antisense oligonucleotides suggests that Kermit is required for Wnt-1/frizzled-3 signaling in neural crest development.

MATERIALS AND METHODS

Cloning of Kermit

To clone frizzled interaction proteins, the C terminus of Xfz7 (M. A. D. and P. S. K., unpublished; Djiane et al., 2000; Sumanas et al., 2000; Wheeler and Hoppler, 1999) was fused to the GAL4 DNA-binding domain and used as bait to screen a *Xenopus laevis* oocyte cDNA library fused to the GAL4 activation domain, according to standard protocols (Fields and Song, 1989; Clontech manual). Three positive clones were obtained, two of which were identical. The overlap region of these clones was used to screen *Xenopus* oocyte (gift of Alan Wolfe) and stage 30 head (gift of Richard Harland) cDNA libraries. The largest open reading frame is preceded by stops in all reading frame and encodes a protein of 331 amino acids. cDNAs containing the entire open reading frame were identified in both libraries (from the head library, two alleles were identified that are over 99% identical at the nucleotide level. These two alleles, as well as the cDNA isolated from the maternal library, encode identical polypeptides). Alignments of the deduced amino acid sequence were performed using the ClustalW alignment of MacVector 6.0 (Oxford Molecular Group). The yeast two hybrid assay was also used to study interaction of known proteins according to the protocol of Clontech except that yeast strain Y190 instead of Y187 was used.

DNA constructs, in vitro transcription and translation

The C termini of Xfz3 (amino acids 499 to 664), Xfz7 (amino acids 524 to 548) and Xfz8 (amino acids 531 to 583) were cloned into pGEX2TK (Pharmacia) to generate glutathione S-transferase (GST) fusions, and into pAS2-1 (Clontech) to generate GAL4 fusions. Kermit (amino acids 1 to 331), KΔN (amino acids 128 to 331), KΔP (amino acids 1 to 127 and 218 to 331), KΔC (amino acids 1 to 217), KN (amino acids 1 to 127), KP (amino acids 128 to 217) and KC (amino acids 218 to 331) were cloned into pCS2+ (Turner and Weintraub, 1994) and pACT2 (Clontech). A C-terminal GFP-tagged Kermit was generated by fusing the open reading frame of Kermit to GFP in pCS2+, while an N-terminal myc-tagged Kermit was generated by inserting the open reading frame of Kermit to pCS2+MT. Capped synthetic RNAs were generated using an SP6 mMessage mMachine kit from Ambion (Austin, TX). In vitro translation was carried out using rabbit reticulocyte lysate in the presence or absence of L-[^{35}S] methionine, 2 hours at 30°C.

RT-PCR, northern blot and in situ hybridization

RNAs from different stages of *Xenopus* oocytes and embryos were extracted and RT-PCR performed essentially as described (Wilson and Melton, 1994). Primer sets used in the paper were previously reported for EF1α, MyoD, NCAM, Xfz8 (Deardorff et al., 1998 and references therein), *Xtwist*, *Xslug* (LaBonne and Bronner-Fraser, 1998) and FGFR1 (Deardorff and Klein, 1999). Primers designed for this work were: Xfz3, U-TAACAATCATCCTGCTCGC, D-TTGTACCCAA-GTTGTCTCC; Xfz7, U-TCACTTACCTGGTGGACATGCG, D-TGAGAGTTTGCCTCGATAGCC; and Kermit, U-CTGCTGG-AAAGTTACATGGGAA, D-TTAATAGCGGCCAGCCTTGG.

RT-PCR analysis with these primers was performed with 25 cycles of amplification. For Northern blot, total RNA was probed using a 1.3 kb fragment of Kermit. Whole-mount in situ hybridization was performed as described (Deardorff et al., 1998). In addition, fixed embryos at the gastrula and neurula stages were bisected along the anteroposterior or left-right axes to expose inner surfaces to RNA probe and were then treated as described for intact, fixed embryos.

In vitro binding

Glutathione S-transferase (GST) fusion proteins incorporating the C-termini of Xfz3, 7 and 8 were expressed and affinity purified on glutathione agarose, washed three times in binding buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM DTT, bacterial protease inhibitor cocktail (Sigma) and 3% BSA), incubated

with ^{35}S-labeled in vitro translated Kermit for 2 hours at 4°C, and then washed with binding buffer 5 times. Proteins were eluted with 5 mM glutathione and analyzed by polyacrylamide gel electrophoresis, followed by staining with Coomassie Brilliant Blue for total protein and by autoradiography using a phosphoimager (Molecular Dynamics).

Embryos, microinjection, immunoprecipitation and immunoblotting

Fertilization and embryo culture were performed as described (Newport and Kirschner, 1982). Embryos were staged was according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). RNA in a volume of 10 nl was injected into embryos as described (Vize et al., 1991). For immunoprecipitation, *Xenopus* embryos were injected at the animal pole at the one-cell stage, developed to stage 9, and lysed in embryo lysis buffer (20 mM Tris, pH=7.5, 140 mM NaCl, 10% glycerol, 1 mM DTT, 2 mM sodium vanadate, 25 mM NaF, 1% Nonidet P-40, and protease inhibitor cocktail for mammalian cells (Sigma)). Anti-Myc monoclonal antibody (9E10, 5 μl ascites) was incubated with 450 μl of cleared lysate for 1-2 hours at room temperature, collected on anti-mouse IgG coupled protein A beads (Upstate, 50 μl) for 1 hour, washed twice with lysis buffer and twice with lysis buffer without Nonidet P-40, and analyzed by western blot using α-Xfz3 (monoclonal antibodies raised against GST-Xfz3 C terminus, amino acids 499-664) and α-Myc (9E10) antibodies. Polyclonal antibodies to neuropilin 1 interacting protein (Cai and Reed, 1999) were provided by Dr Randy Reed (Johns Hopkins School of Medicine) and to M-semF cytoplasmic domain-associated protein (Wang et al., 1999) by Dr Stephen Strittmatter (Yale University School of Medicine).

Membrane translocation

Kermit-GFP membrane translocation was carried out in similar to dsh-GFP membrane translocation (Yang-Snyder et al., 1996). Briefly, *Xenopus* embryos were injected into the animal pole at the one-cell stage with either GFP mRNA (1 ng) or Kermit-GFP mRNA (1 ng), in the presence or absence of Xfz3 or Xfz8 mRNA (1 ng). Animal caps were dissected and fixed in 4% paraformaldehyde-phosphate-buffered saline (PBS) for 1 hour at room temperature. After four washes with PBST (PBS with 0.1% Tween-20), the caps were mounted upside down in Vectashield (Vector Laboratories). Confocal images were taken with Leica TCS NT confocal microscope using the fluoroscein filter and a 40× objective lens. The thickness of the images showed is 1 μm *z* steps.

Neural crest induction assay

Neural crest induction in ectodermal explants was assayed essentially as described previously (Saint-Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998) using 100 pg of chordin, 0.1 pg of Xwnt1, 130 pg of Xfz3 and 40-1000 pg of Kermit (as indicated in Figs 7, 8). To deplete Kermit, 2-4 ng of morpholino antisense oligonucleotide (MO; purchased from Gene Tools, LLC in Corvallis, OR) directed against the Kermit 5′ untranslated region (5′-CCACGGACAGCAAA-TCTCACACAG-3′) was injected together with chordin and Xwnt-1 or Xfz3 mRNAs. The control MO was also purchased from Gene Tools, LLC. For rescue, 10 pg of Kermit mRNA lacking the 5′UTR was co-injected with the above mRNAs and Kermit MO into the animal pole at the one-cell stage.

RESULTS

Cloning of Kermit

To identify genes that interact with the frizzled C-termini, a yeast two hybrid assay was used to screen a *Xenopus* oocyte cDNA library using the Xfz7 C terminus as bait. Three overlapping clones were identified and used to screen *Xenopus* maternal and stage 30 head cDNA libraries, yielding two clones that encode the entire open reading frame. The largest open reading frame preceded by stop codons in all reading frames encodes a novel protein with 331 amino acids (Fig. 1). We named this gene Kermit.

Kermit contains a C-terminal acyl carrier domain (amino acid 262-318) and a central PDZ domain (amino acid 128-217) (Fig. 1, underlined), a proposed protein-protein interacting domain. This PDZ domain, is most similar to the first PDZ domain of rGRIP (glutamate receptor interacting protein; 38% identical and 63% similar) (Dong et al., 1997), and the fourth PDZ domain of multi PDZ domain protein (40% identical and 57% similar) (Ullmer et al., 1998).

Although Kermit is not homologous to any genes of known function, several genes within the GenBank database are similar to Kermit, including a gene identified in mammals as GIPC (RGS-GAIP interacting protein), TIP2 (TAX interaction protein 2), M-semF cytoplasmic domain-associated protein, and neuropilin 1 interacting protein (De Vries et al., 1998; Rousset et al., 1998; Cai and Reed, 1999; Wang et al., 1999). The predicted Kermit sequence is also similar to uncharacterized genes from *Drosophila* and *C. elegans* (*C35 D10.2* and *F44D12*), as well as two additional genes identified by searching the human genome database. An EST from *Xenopus* was also identified in GenBank that is approximately 66% similar to Kermit. The amino acid sequence alignment of Kermit with its *C. elegans* and human homologs is shown in Fig. 1. At the amino acid level, Kermit is 74% identical to human GIPC, 48% identical to the *Drosophila* Kermit-like gene, and 35% identical to *C35D10.2*. The high degree of similarity of these genes to Kermit suggests that they may represent homologs of Kermit or closely related family members. As these genes are from organisms as diverse as human and *C. elegans*, it appears that Kermit is highly conserved during evolution.

Expression of Kermit in *Xenopus*

To characterize the temporal pattern of Kermit expression, we extracted RNA from multiple stages of embryogenesis and performed northern blots as well as RT-PCR. Thus, Kermit is encoded by a 4.7 kb mRNA that was detected maternally (oocyte RNA) and in early embryos (blastula stage) as detected by northern blot (Fig. 2A). To study the time course of Kermit expression in more detail, total RNA was isolated at different stages from oocytes to tadpole stages of development and analyzed by RT-PCR using Kermit-specific primers (Fig. 2B). This analysis confirmed that Kermit is a maternal gene and is expressed throughout development similar to Xfz3 and Xfz7, but distinct from Xfz8 (Fig. 2B).

Kermit is first clearly detected by whole-mount in situ hybridization at the gastrula stage, with high expression in the dorsal marginal zone at stage 10+ (Fig. 3A). In the neurula, Kermit expression persists in dorsal mesoderm, including notochord and somites (Fig. 3B), and is now also expressed throughout the central nervous system, with highest expression in anterior neural tissue (Fig. 3B,C). At tadpole stages, Kermit mRNA expression remains high in the anterior neural tube, but is also present in the spinal cord, otic vesicle, the eyes, branchial arches (Fig. 3D-F) and the pronephric duct (not shown). The expression pattern of Kermit in the anterior neural

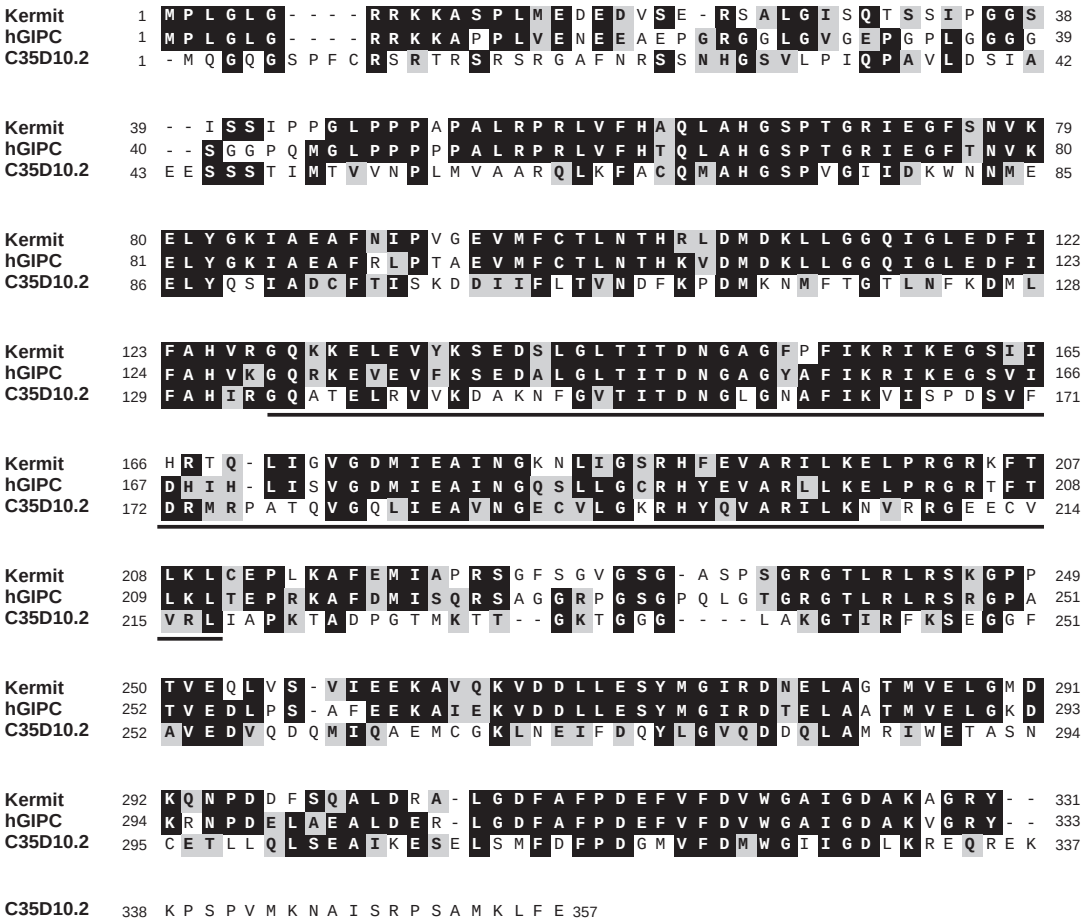

Fig. 1. Sequence of Kermit. Deduced amino acid sequences from Kermit, human GIPC and *C. elegans* gene *C35D10.2* were aligned using MacVector 6.0 ClustalW alignment. Identical amino acids are in black boxes and similar amino acids are in gray. The PDZ domain is underlined. Kermit sequence has been deposited into the GenBank database under Accession Number AF215838.

tube is similar to Xfz3 (compare Fig. 3F with 3G; Shi et al., 1998) but also overlaps with Xfz9, which is highly expressed in the dorsal midbrain and hindbrain at late neurula and tadpole stages. Kermit expression partially overlaps with Xfz7, which is broadly expressed in anterior structures (Wheeler and Hoppler, 1999), and overlaps to a more limited extent with Xfz2, Xfz4 and Xfz8 (Deardorff and Klein, 1999; Shi and Boucaut, 2000; Deardorff et al., 1998; Itoh et al., 1998). The parallel in expression patterns suggests that frizzled-3/Kermit interactions may be functionally relevant.

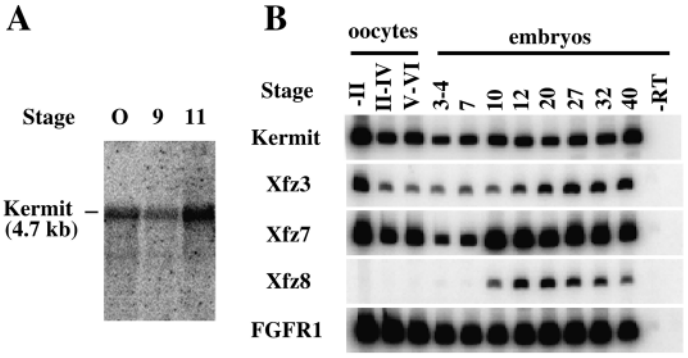

Fig. 2. Temporal pattern of Kermit expression. (A) Northern blot of Kermit expression. Kermit was detected as a single band at 4.7 kb. Lanes 1 to 3: total RNA (20 μg) from oocytes, stage 9, and stage 11 embryos. (B) RT-PCR analyses of Kermit, Xfz3, Xfz7 and Xfz8 expression. Lanes 1 to 3: stage II, III-IV and V-VI oocytes. Lanes 4 to 11: RNA from stage 3-4, 7, 10, 12, 20, 27, 32 and 40 embryos. Lane 12: negative control without reverse transcriptase. FGFR1 was used as a loading control.

Interaction of Kermit with Xfzs

Although Kermit was initially identified through its interaction with Xfz7, the parallel in expression between Kermit and Xfz3 suggested they might also interact. Therefore we analyzed Kermit interaction with the C terminus of Xfz3 as well as Xfz7 and Xfz8, using the two hybrid assay. Colonies containing Kermit and Xfz3 grew faster in selective medium and hydrolyzed X-Gal faster than those with Kermit and Xfz7, while colonies with Kermit and Xfz8 did not grow on selective medium and did not hydrolyze X-Gal. This suggests that Kermit interacts more strongly with the Xfz3 C terminus than with Xfz7 C terminus, and not at all with Xfz8 C terminus (Fig. 4A). In other words, Kermit distinguishes between frizzled C-termini, suggesting that the interaction is specific.

To identify the regions of Kermit required for interaction, multiple deletions in Kermit were generated (Fig. 4B) and used in the yeast two hybrid assay together with *Xenopus* frizzled C termini. Deletion of the N terminus of Kermit maintained the interaction between Kermit and Xfz3; in fact, deletion of the N terminus appeared to increase binding. All other deletions abolished the interaction. These results suggest that the N terminus is not required for binding, while the PDZ domain and the C terminus are required for interaction with

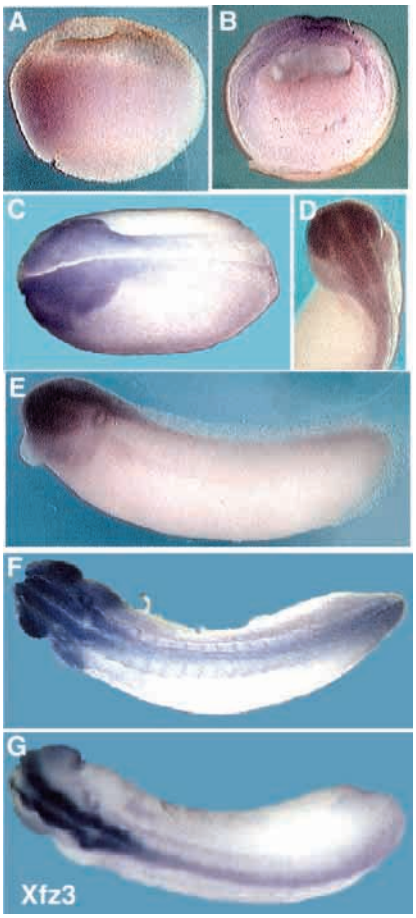

Fig. 3. Spatial pattern of Kermit expression. In situ hybridization was performed at early gastrula, early and late neurula, and tadpoles stages. (A) A stage 10+ gastrula, with the dorsal lip visible in the lower left. (B) A hemisection of a stage 14 neurulae, with dorsal oriented upwards; diffuse staining is visible in the neural plate, notochord, and paraxial mesoderm. (C) Strong staining in the anterior neural plate of a stage 18 neurula (anterior is towards the left). (D,E) Stage 26 and (F) stage 32 tadpoles, with kermit expression in the neural tube, eye, otic vesicle and branchial arches. (G) Expression of Xfz3 in a stage 32 tadpole.

A

	growth in -trp, -leu	growth in -trp, -leu, -his	X-gal assay
Xfz3	+++	+++	blue/30 min
Xfz7	+++	++	blue/1~2 h
Xfz8	+++	-	-

B

structure	growth in -trp, -leu	growth in -trp, -leu, -his	X-gal assay
Kermit (PDZ)	++	+++	blue
KΔN	++	++++	blue
KΔPDZ	++	-	-
KΔC	++	-	-
KN	++	-	-
KPDZ	++	-	-
KC	++	-	-

Fig. 4. Interaction of Kermit with the C termini of Xfzs in yeast two hybrid assays. Yeast strain Y190 was transformed with Kermit or Kermit mutants and Xfz3, Xfz7 or Xfz8, plated on either nonselective medium (-trp, -leu) or selective medium (-trp, -leu, -his). Growth was checked 3 to 4 days later, and the hydrolysis of X-gal was monitored on filter lifts from all plates. Plus signs indicate size of colonies; minus sign indicates no growth (in left and middle columns) or no X-gal hydrolysis (right column). (A) Interaction of Kermit with the C termini of Xfz3, Xfz7 and Xfz8. (B) Domain requirement of Kermit in interaction with Xfz3. Blue, N terminus (amino acids 1-127); green, PDZ domain (amino acids 128-217); purple, C terminus (amino acid 218-331).

the Xfz3 C terminus. In addition, neither the PDZ domain alone nor the C-terminal fragment is sufficient for Xfz3 C terminus interaction. The PDZ domain also does not interact with Xfz8.

To confirm the interaction in yeast, in vitro binding assays were performed using the C-termini of Xfz3, Xfz7 and Xfz8 purified as GST-fusion proteins (N-terminal GST), and in vitro translated [^{35}S]-methionine-labeled Kermit. Kermit bound the C termini of both Xfz3 and Xfz7, although the binding between Kermit and Xfz3 was apparently stronger (Fig. 5A). Kermit did not bind appreciably to the C terminus of Xfz8 (Fig. 5A), consistent with the lack of interaction in the yeast two-hybrid assay (Fig. 4). Full-length Xfz3 also co-immunoprecipitated with Kermit (Fig. 5B) when both were co-expressed in *Xenopus* embryos, further confirming the interaction.

Membrane translocation of Kermit

As frizzleds are integral membrane proteins, frizzled-interacting proteins might be expected to localize to the membrane as well. To examine the subcellular localization of Kermit protein, Kermit was tagged with GFP and expressed in the animal pole of *Xenopus* embryos with or without full-length Xfz3 or Xfz8. Embryos were cultured until the blastula stage and animal pole explants (animal caps) were examined by confocal microscopy. Kermit-GFP by itself was predominantly localized to both cytosol and nucleus (Fig. 6A), similar to GFP alone (Fig. 6C), except that a small amount of Kermit-GFP was also detected at the plasma membrane. Expression of Xfz3 caused a marked translocation of Kermit-GFP to the membrane (Fig. 6B), with little remaining in the nucleus or cytoplasm. By contrast, Xfz8 had only a small effect on Kermit subcellular distribution (compare Fig. 6E with 6A), consistent with the lack of Xfz8/Kermit interaction by GST-pull down and by two hybrid assay. Xfz3 had no effect on the distribution of GFP alone (compare Fig. 6C with 6D).

Overexpression of Kermit inhibits neural crest induction by both Xwnt1 and Xfz3

The data above show that Kermit interacts strongly with the C-terminus of Xfz3 and is expressed in a pattern highly similar to Xfz3. To test whether this interaction is functionally relevant, we have examined the effect of Kermit on Xfz3 signaling.

In recent work, we have found that Xfz3, which is expressed in the dorsal neural tube, mediates Wnt1-dependent induction of neural crest; overexpression of Xfz3 induces neural crest and inhibition or depletion of Xfz3 blocks neural crest induction in *Xenopus*, suggesting that Xfz3 is required for neural crest induction in *Xenopus* (Deardorff et al., 2001). We have therefore tested the effect of Kermit on Wnt1/Xfz3-mediated neural crest induction using a previously established

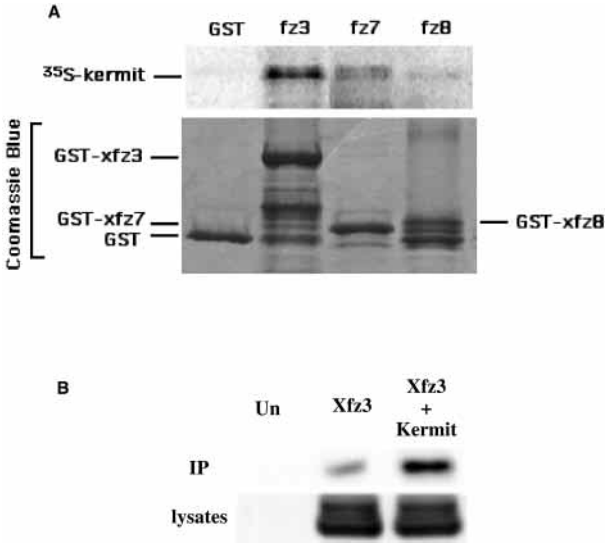

Fig. 5. Kermit interacts with Xfzs in vitro and in *Xenopus* embryos. (A) In vitro binding: Xfz3, Xfz7 and Xfz8 C-termini purified as GST-fusion proteins were incubated with ^{35}S-labeled Kermit (in vitro translated). Autoradiography (top panel) shows that Kermit bound strongly to the Xfz3 C terminus and weakly to Xfz7, but not to Xfz8. The lower panel shows Coomassie Brilliant Blue staining of the GST fusion proteins. (B) Co-immunoprecipitation of Kermit and Xfz3. Embryos were injected at the one-cell stage with mRNAs encoding Xfz3 and Myc-tagged Kermit, and cultured until the late blastula stage. Xfz3/Kermit complexes were immunoprecipitated from embryo lysates with anti-Myc antibody and Xfz3 was visualized by Western blotting.

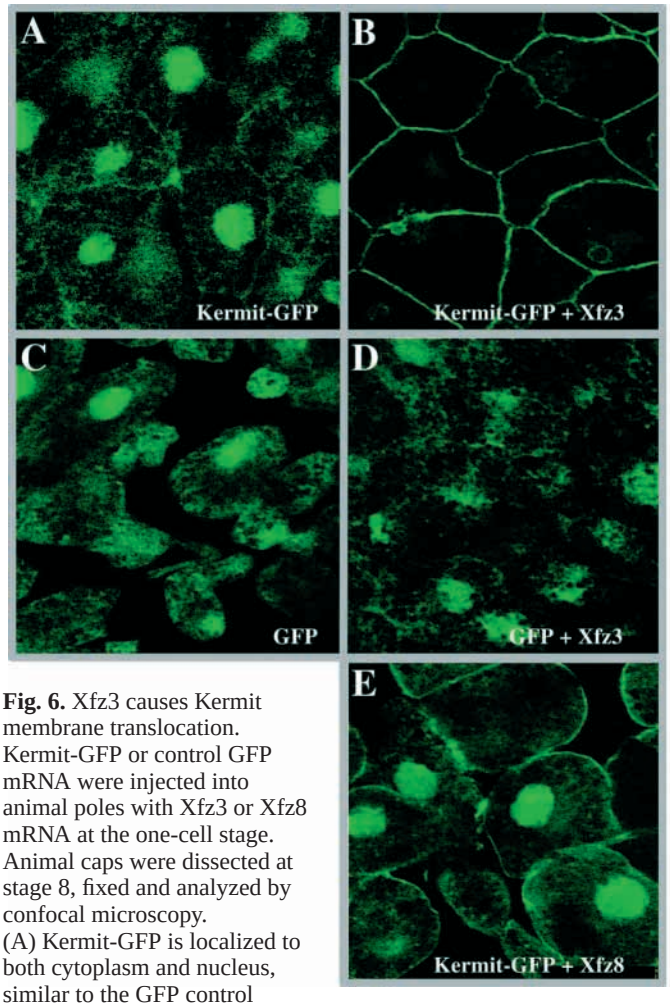

Fig. 6. Xfz3 causes Kermit membrane translocation. Kermit-GFP or control GFP mRNA were injected into animal poles with Xfz3 or Xfz8 mRNA at the one-cell stage. Animal caps were dissected at stage 8, fixed and analyzed by confocal microscopy. (A) Kermit-GFP is localized to both cytoplasm and nucleus, similar to the GFP control (compare A with C). (B) Expression of Xfz3 causes a marked translocation of Kermit to the plasma membrane, with little remaining in the nucleus or cytoplasm. (C) Control expression of GFP, with localization in cytoplasm and nucleus. (D) Xfz3 expression has no effect on the distribution of GFP alone. (E) Xfz8 has only a small effect on Kermit subcellular distribution (compare A with E).

assay in which Wnt-1 (Saint-Jeannet et al., 1997) or Xfz3 (Deardorff et al., 2001) is co-expressed with a neuralizing agent such as chordin or noggin in an ectodermal explant. Expression of neural crest-specific markers such as *Xtwist* and *Xslug* is then measured by RT-PCR (Fig. 7A,B). Over-expression of Kermit inhibited *Xtwist* and *Xslug* induction by Wnt1 or Xfz3 in a dose-dependent manner (Fig. 7A, lanes 4 to 6; Fig. 7B lanes 6 to 10). Kermit had no effect on induction of the neural markers NCAM, Xnrp-1, or the anterior neural marker Xotx2 by chordin (Fig. 7A-C); epidermal keratin was also unaffected by Kermit overexpression (Fig. 7C). Wnt-1 was more sensitive than Xfz3 to overexpressed Kermit, perhaps because Xwnt-1 functions through endogenous Xfz3 (Deardorff et al., 2001), which is present at lower levels than overexpressed Xfz3.

Kermit is required for Xfz3 mediated neural crest induction

The overexpression experiment could indicate that endogenous Kermit is an inhibitor of Xfz3, but it is equally possible that Kermit is required to couple Xfz3 to a downstream effector. In this latter case, overexpression of Kermit might inhibit signaling by binding Xfz3 and the unknown downstream component independently, to prevent coupling between these two components. To distinguish these two possibilities, we blocked the function of Kermit using morpholino antisense oligonucleotides, which inhibit protein translation (Summerton and Weller, 1997) and have recently been demonstrated to be highly effective in depleting β-catenin in *Xenopus* embryos (Heasman et al., 2000). Thus, a morpholino antisense oligo (MO) directed against the 5′ untranslated region of Kermit blocks translation of full-length Kermit mRNA in a dose-dependent manner but has no effect on Kermit mRNA that lacked the 5′UTR (Fig. 8A). The kermit-MO reduced or eliminated neural crest induction by Xfz3 (Fig. 8B, compare lanes 4 and 6) but did not block expression of the pan-neural marker NCAM; a control MO had no effect in *Xslug* expression (Fig. 8B, lane 5). Co-injection of Kermit mRNA that lacked the 5′UTR reversed the inhibition (Fig. 8C, compare lane 3 with lane 4), suggesting that the kermit-MO specifically blocked the translation and function of Kermit. These data suggest that Kermit acts as an adaptor to link Xfz3 to a downstream effector. In support of this hypothesis, low level overexpression of Kermit modestly but reproducibly enhanced neural crest induction by Xfz3 (Fig. 7B, compare lanes 5 and 6, and data not shown).

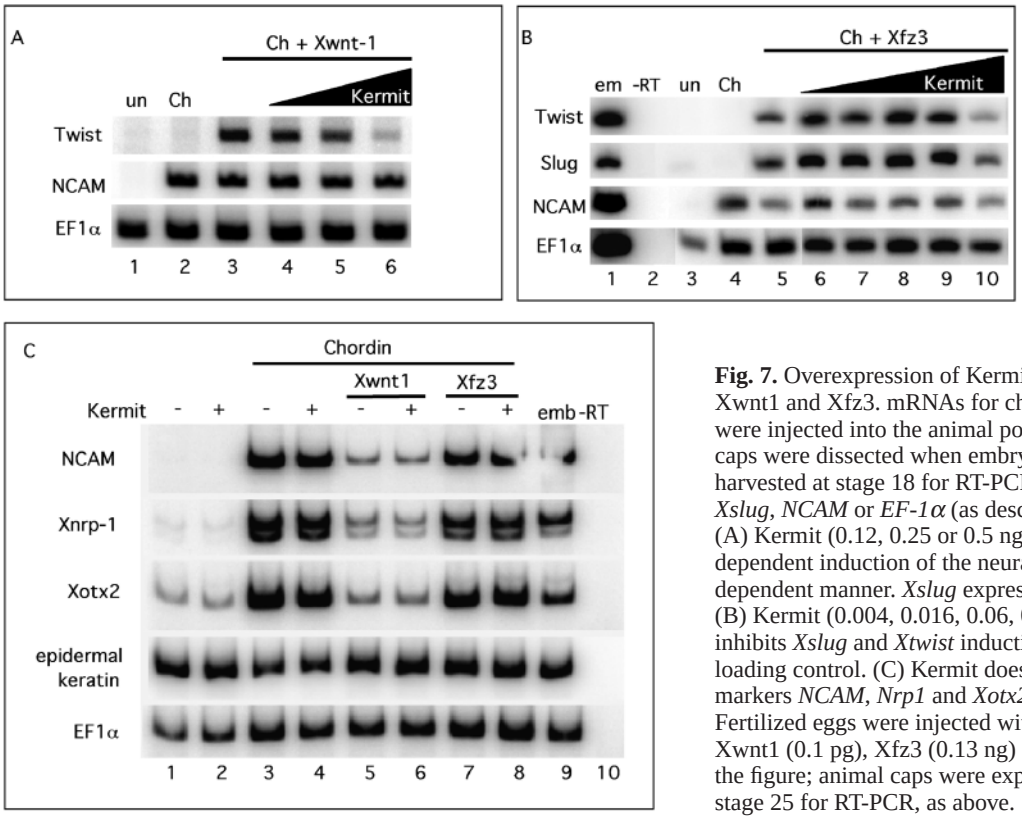

Fig. 7. Overexpression of Kermit inhibits neural crest induction by Xwnt1 and Xfz3. mRNAs for chordin, Xwnt1 or Xfz3, and Kermit were injected into the animal pole of fertilized eggs. (A,B) Animal caps were dissected when embryos reached stage 8-9 and RNA was harvested at stage 18 for RT-PCR analysis using primers for *Xtwist*, *Xslug*, *NCAM* or *EF-1α* (as described in the Materials and Methods). (A) Kermit (0.12, 0.25 or 0.5 ng of Kermit mRNA) inhibits Xwnt1-dependent induction of the neural crest marker *Xtwist* in a dose-dependent manner. *Xslug* expression was also inhibited (not shown). (B) Kermit (0.004, 0.016, 0.06, 0.25 or 1.0 ng of Kermit mRNA) inhibits *Xslug* and *Xtwist* induction by Xfz3. *EF-1α* was used as a loading control. (C) Kermit does not affect expression of neural markers *NCAM*, *Nrp1* and *Xotx2*, or of the epidermal marker *keratin*. Fertilized eggs were injected with mRNA for chordin (0.1 ng), Xwnt1 (0.1 pg), Xfz3 (0.13 ng) and kermit (1.0 ng), as indicated in the figure; animal caps were explanted at stage 8-9 and harvested at stage 25 for RT-PCR, as above.

DISCUSSION

We have identified a frizzled interacting gene named Kermit that interacts specifically with Xfz3 and 7 but not with Xfz8. Kermit expression in *Xenopus* embryos parallels the expression of Xfz3 and co-expression of Xfz3 and Kermit results in a dramatic translocation of Kermit to the cell surface. Furthermore, inhibition of Kermit with morpholino antisense oligos inhibits Xfz3-mediated neural crest induction. These observations suggest that Kermit is required for Xfz3 signaling in neural crest formation.

Co-expression of frizzleds can also recruit Disheveled (dsh) to the plasma membrane (Yang-Snyder et al., 1996). However, unlike Kermit, dsh is not able to distinguish between Xfz3 and Xfz8; in fact, Xfz2 (Deardorff and Klein, unpublished observation), Xfz3, Xfz7, Xfz8 and rat Fz1 all recruit dsh to the plasma membrane (Rothbacher et al., 2000). This suggests that dsh is a common component for

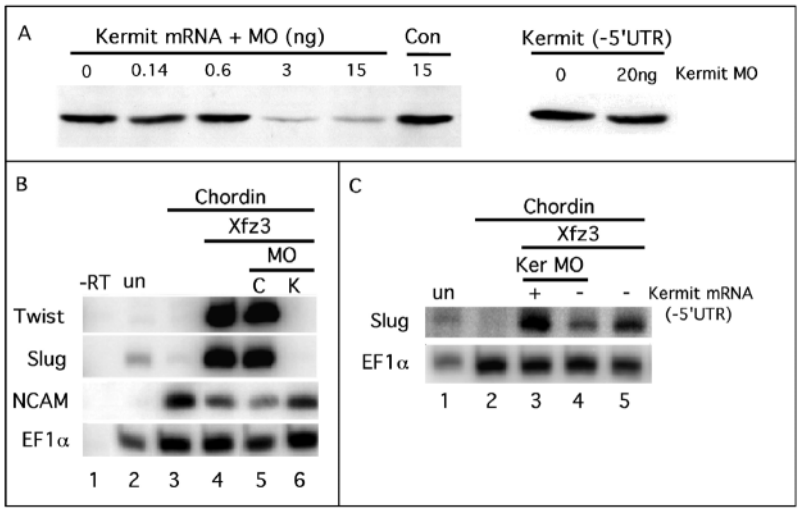

Fig. 8. Kermit is required for neural crest induction by Xfz3. (A) Kermit morpholino antisense oligonucleotide (MO) directed against the 5′UTR of Kermit mRNA blocks translation of Kermit protein. Kermit mRNA was injected into embryos with increasing concentrations of Kermit MO or control MO and embryo lysates were analyzed by western blotting with anti-NIP antibodies (which crossreact with overexpressed Kermit). Translation of Kermit mRNA lacking the 5′UTR was not inhibited by the Kermit MO (20 ng MO; right panel). (B) Depletion of Kermit with antisense MO (4 ng; lane 6) reduces or eliminates neural crest induction by Xfz3 (lanes 4 and 6). Neural crest induction and RT-PCR were performed as in figure 7. Lane 1 is a no reverse transcriptase control; lane 2 shows uninjected control caps (un); lane 3 shows samples expressing chordin alone; lane 4 shows induction of neural crest makers by Xfz3 + chordin; lane 5 shows Xfz3 + chordin + control MO (C); and lane 6 shows Xfz3 + chordin + Kermit-MO (K). (C) Kermit mRNA lacking the 5′UTR rescues inhibition by Kermit MO. Neural crest induction assay was performed as above. Kermit MO (4 ng) was injected (lanes 3, 4) with (+) or without (–) Kermit mRNA (10 pg) lacking the 5′UTR.

multiple frizzled pathways, while Kermit may be pathway specific.

Kermit expression parallels that of Xfz3, consistent with a role in Xfz3 signaling. Although this is most evident in the developing neural tube and neural crest, Kermit and Xfz3 are also expressed maternally and throughout early development. The role of Kermit and Xfz3 at these earlier stages is not known. Whole-mount in situ hybridization analysis does not show localization of Kermit before the gastrula stage, and overexpression has no clear effect on development before the induction of neural crest (data not shown). However, Kermit and Xfz3 are also both expressed in the developing eye. Recently, Xfz3 has been shown to induce ectopic eye formation, and overexpression of Kermit can interfere with endogenous eye formation, consistent with a potential role for Kermit in Xfz3 signaling in the developing eye (Rasmussen et al., 2001).

Several genes have been identified with sequences similar to Kermit. Although a functional role for these genes has not yet been defined, they have been proposed to play roles in vesicular (De Vries et al., 1998) and membrane trafficking (Cai and Reed, 1999), and may regulate the subcellular distribution of M-SemF in brain (Wang et al., 1999). The sequence similarity between Kermit and these other genes suggests that they could be homologues of Kermit, but it is not known yet whether they can interact with Xfz3 or have activities similar to Kermit. Furthermore, the requirement for the C-terminus in addition to the PDZ domain for binding to Xfz3 distinguishes Kermit-frizzled interactions from the mammalian Kermit-related molecules, for which the PDZ domain alone appears to be sufficient for binding to target molecules (De Vries et al., 1998; Rousset et al., 1998). It is also unclear why these Kermit-like proteins interact with molecules as diverse as GLUT1, syndecan, RGS-GAIP, TAX, M-SemF and neuropilin 1. These related PDZ domain-containing proteins may have multiple functions, which could explain why Kermit has not shown up in genetic screens for wnt/frizzled pathway components. Two other Kermit-like genes are present in the recently released human genome database, but the roles of these related genes are also not known. In addition, we have identified a Kermit-related gene (Kermit 2) expressed in early *Xenopus* embryos (Aaron Gitler, C. T. and P. S. K., unpublished); this Kermit 2 could represent a redundant gene or may interact with other frizzleds, such as Xfz8, that do not bind to Kermit.

Our data suggest that Kermit is required for Xfz3 induction of neural crest in neuralized explants and that Kermit may function as an adaptor between Xfz3 and a downstream component of the pathway. Overexpression of Kermit inhibits Xfz3 signaling in neural crest formation, but this could be through binding of Kermit independently to both Xfz3 and this downstream component, interfering with the normal function of Kermit. Thus, high levels of Kermit prevent coupling, while low levels of Kermit could facilitate coupling. A candidate for this postulated effector downstream of Kermit has not been identified. So far, attempts to show direct interaction between Kermit and dishevelled have not been successful. An interesting but speculative possibility is that Kermit could regulate G-protein-mediated signaling, as proposed for GIPC and M-SemF (Wang et al., 1999). In support of this idea, mouse fz3 has been shown to activate protein kinase C, which has been suggested to be through G proteins (Sheldahl et al., 1999). However, Wnts appear to function through the canonical Wnt pathway in neural crest induction, as overexpression of GSK-3β blocks neural crest induction by Wnts (Saint-Jeannet et al., 1997). We would propose that fz3 can activate both canonical and noncanonical pathways, depending on cellular context, as observed for *Drosophila* fz (Bhanot et al., 1999; Bhat, 1998; Chen and Struhl, 1999; Kennerdell and Carthew, 1998; Rulifson et al., 2000) and Xfz7 (Djiane et al., 2000; Medina et al., 2000; Sheldahl et al., 1999; Sumanas et al., 2000). Whether Kermit plays a role in coupling to both pathways or in specifying the downstream pathway used by Xfz3 is a topic for future study.

An important caveat to our observations is that we have so far been unable to observe inhibition of neural crest formation in whole embryos after depletion of Kermit with the morpholino antisense oligo; this negative result could indicate the presence of a compensating or redundant activity in whole embryos that is absent in the animal cap explants. In support of this, we have recently identified a second kermit gene (67% amino acid similarity with Kermit 1) that is expressed in early *Xenopus* embryos. Additional work will be required to test whether Kermit 2 is redundant with Kermit 1 in embryos.

In conclusion, we have identified a frizzled interacting gene named Kermit, which interacts specifically with Xfz3 (and more weakly with Xfz7). Xfz3, but not Xfz8, strongly recruits Kermit to the plasma membrane in *Xenopus*. At neurula and tailbud stages, Kermit is highly localized in the anterior neural tissue, similar to Xfz3, and depletion of Kermit blocks neural crest induction by Xfz3. Based on these observations, we propose that Kermit is a frizzled 3 interacting protein that directly mediates endogenous frizzled 3 signaling in the dorsal neural tube.

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