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RESEARCH ARTICLE

Reorganization of Actin Cytoskeleton by FRIED, a Frizzled-8 Associated Protein Tyrosine Phosphatase

Keiji Itoh,† Mikhail Lisovsky,† Hiroki Hikasa, and Sergei Y. Sokol*

Frizzled receptors transduce signals from the extracellular Wnt ligands through multiple signaling pathways that affect cytoskeletal organization and regulate gene expression. Direct intracellular mediators of Frizzled signaling are largely unknown. We identified FRIED (Frizzled interaction and ectoderm defects) by its association with the C-terminal PDZ-binding motif of *Xenopus* Frizzled 8. FRIED contains an N-terminal KIND domain, a FERM domain, six PDZ domains, and a tyrosine phosphatase domain, being similar in structure to the protein tyrosine phosphatase PTP-BAS/PTP-BL. We report that FRIED proteins with the FERM domain localize to the apical cortex and can inhibit Wnt8-mediated, but not β-catenin-mediated, secondary axis induction in *Xenopus* embryos, suggesting a specific interaction with Wnt signaling. A FRIED construct containing the FERM domain induced reorganization of pigment granules and cortical actin in *Xenopus* ectoderm. Wnt5a suppressed the depigmentation of ectoderm triggered by FRIED, demonstrating that Wnt5a and FRIED functionally interact to regulate the cytoskeletal organization. Our data are consistent with the possibility that FRIED functions by modulating Rac1 activity. We propose that FRIED is an adaptor protein that serves as a molecular link between Wnt signaling and actin cytoskeleton. *Developmental Dynamics 234:90–101, 2005.* © 2005 Wiley-Liss, Inc.

Key words: Xenopus; ectoderm; Wnt; PTP-BAS; Frizzled; FERM; apical

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INTRODUCTION

The Wnt family of extracellular factors controls cell fate, polarity, and proliferation in embryonic development (Cadigan and Nusse, 1997; Sokol, 1999). Wnt signals are transmitted from the Frizzled (Fz) and LRP transmembrane receptors to Dishevelled (Dsh), the downstream cytoplasmic component of signaling (Boutros and Mlodzik, 1999). In the canonical pathway, Dsh leads to stabilization of the cytoplasmic β-catenin, which is destroyed by a complex of Axin (Zeng et al., 1997; Willert et al., 1999; Kishida et al., 1999; Itoh et al., 2000; Salic et al., 2000), the adenomatous polyposis coli protein (Rubinfeld et al., 1996), and glycogen synthase kinase 3 in the absence of a Wnt signal (Yost et al., 1996; Ikeda et al., 1998; Itoh et al., 1998a; Hart et al., 1998). Stabilized β-catenin associates with members of the T-cell factor family of transcription factors (Molenaar et al., 1996; Behrens et al., 1996) and stimulates transcription of the target genes, such as *Siamois, Cyclin D1*, and *c-Myc* (Sokol, 1999; Behrens, 2000).

In *Drosophila*, another Fz- and Dsh-dependent signaling pathway is involved in the establishment of planar cell polarity (PCP) that is reflected in the asymmetric cortical distribution of several signaling proteins in the plane of epithelial tissues (Adler, 1992; Shulman et al., 1998; Strutt, 2001; Axelrod, 2001). Among molecular components of this pathway are small

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GTPases of the Rho family and c-Jun N-terminal kinase (JNK, Strutt et al., 1997; Boutros et al., 1998; Fanto et al., 2000). In vertebrates, a pathway related to PCP is thought to regulate convergent extension movements of mesoderm and neuroectoderm (Sokol, 1996; Tada and Smith, 2000; Heisenberg et al., 2000; Wallingford et al., 2000; Habas et al., 2001, 2003). These movements consisting of mediolateral cell polarization and intercalation lead to body axis elongation during gastrulation and neurulation (Keller, 2002). Among other proteins regulating convergent extension are Daam1 (Habas et al., 2001), Strabismus (Park and Moon, 2002; Darken et al., 2002), and Prickle (Veeman et al., 2003; Takeuchi et al., 2003). Besides β-catenin and PCP pathways, Fz receptors can activate G-protein-dependent intracellular Ca^{2+} release and protein kinase C (PKC, Slusarski et al., 1997; Sheldahl et al., 1999). Fz7 has been shown to act through protein kinase C to regulate germ layer separation during gastrulation (Winklbauer et al., 2001). A separate pathway from Fz8 to JNK has been reported to utilize a novel mechanism that involves the receptor carboxy-terminus (Lisovsky et al., 2002).

How Fz receptors transmit signals to different intracellular targets remains to be elucidated. One way to address this problem is to identify Fz-associated proteins. A known example is Kermit, a novel cytoplasmic protein that binds Fz receptors and is necessary for neural crest development (Tan et al., 2001). In this study, we carried out a yeast two-hybrid screen for proteins that bind the cytoplasmic tail of *Xenopus* Fz8 (Xfz8). This screen resulted in the identification of a multi-domain protein that we named FRIED (Frizzled interaction and ectoderm defects). FRIED has several protein-protein interaction domains including a FERM (band four-point-one, ezrin, radixin, moesin) domain (Hamada et al., 2000; Pearson et al., 2000), six PDZ (PSD95, Dishevelled and ZO1) domains (Hung and Sheng, 2002), and a protein tyrosine phosphatase domain. The structure of FRIED is similar to that of the mammalian protein tyrosine phosphatase PTP-BAS/FAP1/PTP-BL (Maekawa et al., 1994; Sato et al., 1995), suggesting that FRIED may correspond to its frog homologue. FRIED specifically associates with the cytoplasmic tail of Xfz8 and induces animal pole cell depigmentation that is accompanied by the loss of cortical actin and downregulation of Rac1. The depigmentation effect of FRIED was suppressed by Wnt5a, suggesting that FRIED links Wnt signaling to actin cytoskeleton.

RESULTS

FRIED Is a Frizzled 8 Associating Protein

In a yeast two-hybrid screen for proteins that interact with the cytoplasmic tail of Xfz8, we identified a fragment of a multi-domain protein that we named FRIED (see Experimental Procedures section). Isolated full-length FRIED cDNA is predicted to encode a protein of 2,500 aa (Fig. 1A) that contains a kinase noncatalytic C-lobe domain (KIND, aa 1–239; Ciccarelli et al., 2003), a FERM domain (aa 575–868), six PDZ domains (aa 1,083–1,169, 1,347–1,429, 1,478–1,566, 1,752–1,835, 1,849–1,932, and 1,973–2,038, Fig. 1A,B), and a tyrosine phosphatase domain at the carboxy terminus. The recently identified KIND domain is present in *Drosophila* Spir protein, mammalian PAK kinases, the PTP-BAS phosphatase, and several other hypothetical proteins (Ciccarelli et al., 2003). FERM domain, also known as the band 4.1 domain, is found in adaptor proteins that function at the interface of the plasma membrane and cytoskeleton (Hamada et al., 2000; Pearson et al., 2000; Bretscher et al., 2002). Similarly, PDZ domains participate in signal transduction and mediate protein-protein interactions at the cell membrane (Hung and Sheng, 2002; Fanning and Anderson, 1999). FRIED is closely related to the human non-receptor protein tyrosine phosphatase PTP-BAS (Maekawa et al., 1994; Fig. 1B), also known as FAP1 (Sato et al., 1995), hPTP1E (Banville et al., 1994), PTPL1 (Saras et al., 1994), and related to the mouse phosphatase PTP-BL (Hendricks et al., 1995). FRIED has the same domain structure as PTB-BAS, except for the additional sixth PDZ domain. Thus, FRIED is a possible *Xenopus* homologue of PTP-BAS/FAP1. Extensive searches of *Drosophila* and *C. elegans* databases have not revealed proteins related to FRIED/PTP-BAS, suggesting that these proteins are conserved only in chordates. Overall, the structural analysis of FRIED indicates that it may organize multiple components of signal transduction at the cell membrane.

We wished to confirm the association of Xfz8 and FRIED immunochemically. We expressed myc-tagged FRIED fragment isolated in the two-hybrid screen (FRIED-443, Fig. 2A), and glutathione-S-transferase-tagged cytoplasmic tail of Xfz8 (GST-CT) in *Xenopus* embryos and analyzed their interaction by binding to glutathione-agarose beads. Additionally, we wanted to test whether the binding is mediated by the C-terminal SQV sequence of Xfz8, a variant of the consensus C-terminal S/T-X-V/I motif typically involved in interactions with PDZ domains (Songyang et al., 1997). For that purpose, the C-terminal SQV residues were mutated to ARD to yield GST-CT-ARD. FRIED-443 associated with GST-CT, but not with GST-CT-ARD (Fig. 2B). This result verified the association of FRIED with Xfz8 in embryonic cells and established the requirement for the SQV motif in this interaction.

FRIED Is Expressed in a Dynamic Pattern During Early Development

To characterize the temporal pattern of *FRIED* expression in *Xenopus* embryogenesis, we isolated total embryo RNA at different developmental stages. Northern analysis using *FRIED* antisense probe revealed a transcript of approximately 8 kb in 32-cell embryos, late blastula, gastrula, early neurula, tailbud, and tadpole stages at constant levels (Fig. 2C). Since the majority of zygotic genes start to be transcribed at stage 8.5, we conclude that *FRIED* is present as both maternal and zygotic mRNA.

We next studied the spatial expression patterns of *FRIED* by whole mount in situ hybridization (Fig. 3). In the beginning of gastrulation (stage 10+), *FRIED* RNA is visible in dorsal blastopore lip region (Fig. 3B,C). At the early neurula stages, *FRIED* is expressed in anterior ectoderm area

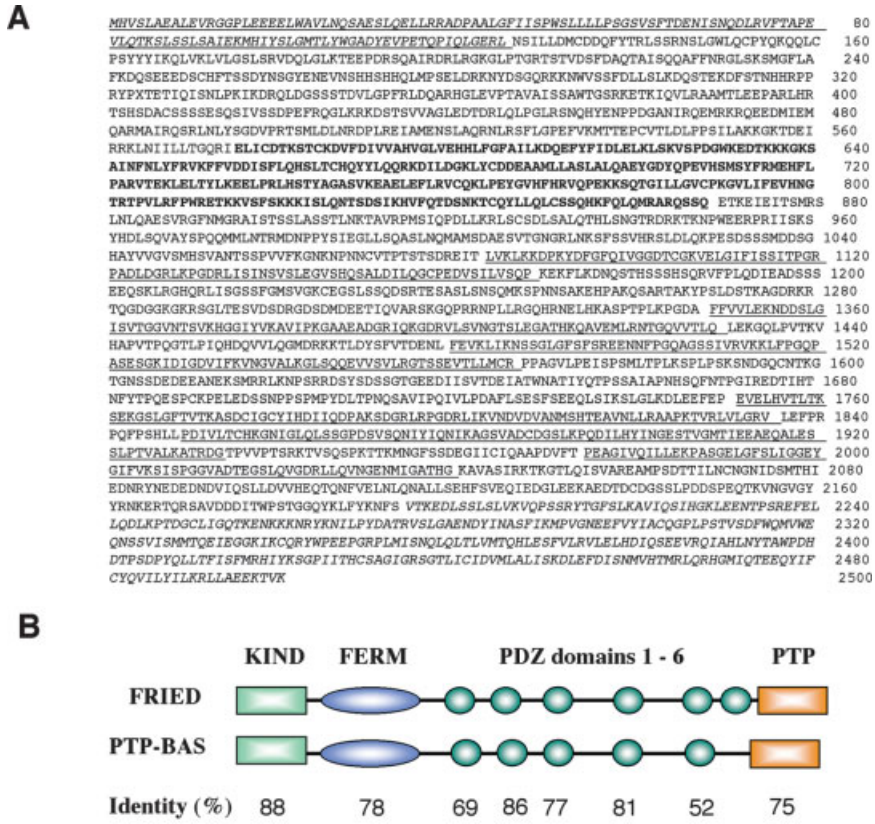


Fig. 1. Primary sequence and domain structure of FRIED. **A:** Deduced amino acid sequence of FRIED. PDZ domains are underlined; KIND is underlined and italicized; FERM is bolded; the phosphatase domain is italicized. **B:** Structure and sequence comparison of FRIED and PTP-BAS showing percent sequence identity of the corresponding conserved domains of FRIED and PTP-BAS. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

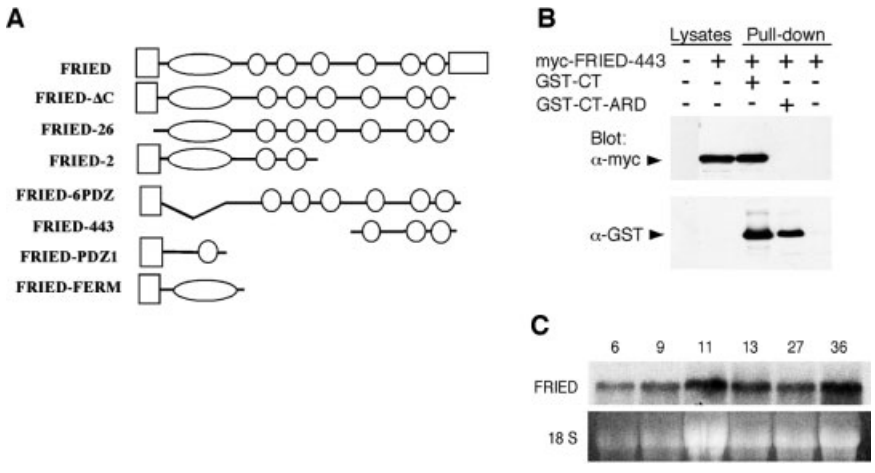


Fig. 2. FRIED associates with Frizzled 8 and is expressed in *Xenopus* early embryos. **A:** Constructs of FRIED used in this study (all are myc-tagged). **B:** FRIED associates with the C-terminal region of Xfz8. Myc-tagged FRIED-443 and GST-CT or GST-CT-ARD RNAs (1 ng each) were co-injected into all blastomeres of four-cell *Xenopus* embryos. Embryo lysates were prepared at gastrula stage 11, incubated with glutathione-agarose beads, washed and analyzed by SDS-PAGE. Western analysis with anti-myc and anti-GST antibodies demonstrates that FRIED-443 binds GST-CT, but not GST-CT-ARD. **C:** Northern analysis of FRIED. Total RNA was isolated at different developmental stages and hybridized with a FRIED antisense RNA probe. The amount of total RNA per lane corresponds to two embryos. FRIED RNA is detectable as a unique 8-kb band. Loading is controlled by ethidium bromide-stained 18 S rRNA. Stages: 32-cell stage (st. 6), late blastula (st. 9), gastrula (st. 11 and 13), tailbud and tadpole stages (st. 27 and 36).

and around closed blastopore (stage 14). At the tailbud stage, *FRIED* transcripts are clearly detectable in the brain, spinal cord, eyes, otic vesicles, and lateral plate mesoderm adjacent to the developing pronephros. This pattern significantly overlaps with the expression of Xfz8 RNA in early development (Itoh et al., 1998b; Deardorff et al., 1998), consistent with the idea that FRIED and Xfz8 interact in vivo.

FRIED Interferes With Xwnt8, But Not β-Catenin Signaling

To test if FRIED can influence Wnt signaling, we generated several FRIED constructs differing in composition (Fig. 2A) and compared their effects in secondary axis induction in *Xenopus* embryos. One ventral vegetal blastomere of 8-cell embryos was injected with Xwnt8 or β-catenin RNA, either separately or together with FRIED-2 or FRIED-ΔC RNA. We observed that FRIED-2, but not FRIED-ΔC, inhibited secondary axis induction by Xwnt8 (Fig. 4A–E). In contrast, axis-inducing ability of β-catenin RNA was not affected (Fig. 4F), consistent with the possibility that FRIED modulates canonical Wnt signaling at a level upstream or parallel to β-catenin.

The Effect of FRIED on Embryonic Ectoderm

In our initial experiments, expression of the full-length mouse phosphatase PTP-BL in the early embryo did not have noticeable effects on embryonic development, even though the protein was well expressed (data not shown). This was not unexpected, since intramolecular interactions in ERM proteins were reported to render them inactive (Gary and Bretscher, 1995; Cooper et al., 2003). To gain insight into the function of FRIED, we assessed its activity by ectopically expressing the FRIED-ΔC construct that lacks the phosphatase domain (Fig. 2A). While control animal pole cells had centrally located pigment and hardly visible cell borders, cells containing FRIED-ΔC RNA lost most of their pigment in the area of injection. A small amount of pigment relocated to the cell periphery, making cell

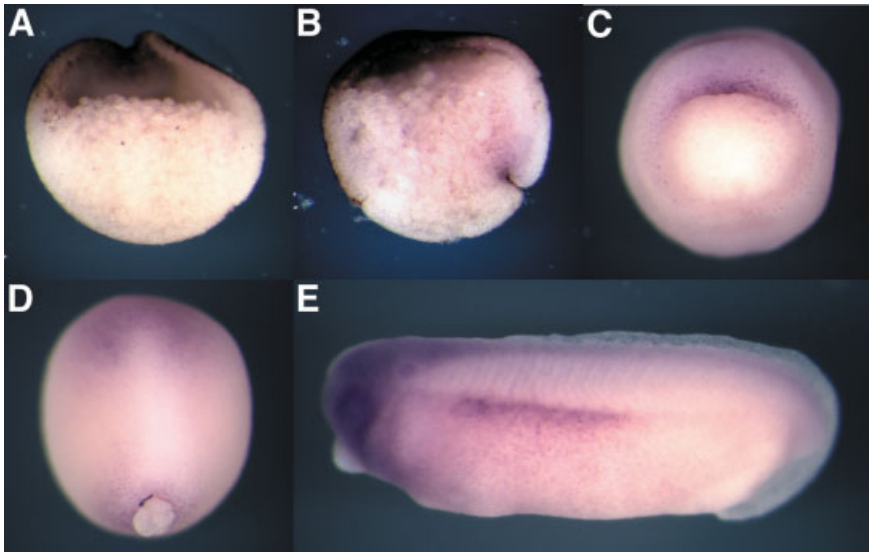
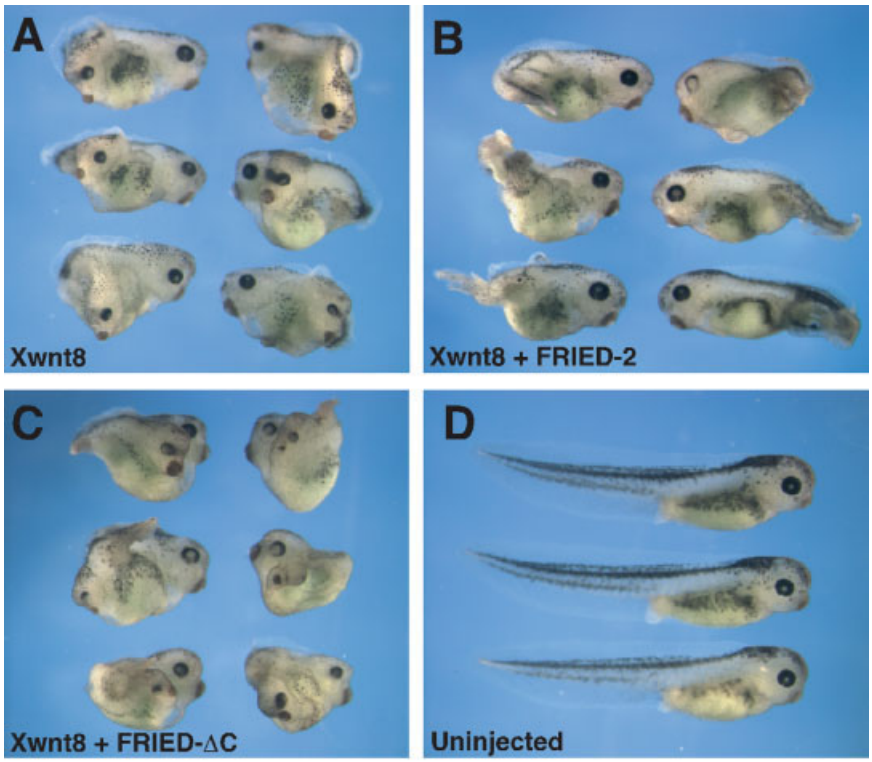


Fig. 3. Spatial distribution of *FRIED* transcripts in *Xenopus* embryos. Wholemount in situ hybridization analysis has been carried out with digoxygenin-labeled *FRIED* anti-sense RNA probes with embryos at different developmental stages. **A:** Stage 10, animal pole is at the top, dorsal is to the right. *FRIED* RNA is detectable in animal pole ectoderm of a bisected embryo. **B,C:** At stage 10+, *FRIED* transcripts are visible in the dorsal blastopore lip. A,B: Saggital view. C: Vegetal pole view. **D:** Stage 14, dorsal view, anterior is at the top. *FRIED* is expressed in anterior ectoderm area and around closed blastopore at the beginning of neurulation. **E:** Tailbud stage, anterior is to the left. *FRIED* transcripts are clearly detectable in the brain and spinal cord, eyes and otic vesicles, and in lateral plate mesoderm. A control RNA probe did not produce similar staining patterns (data not shown).



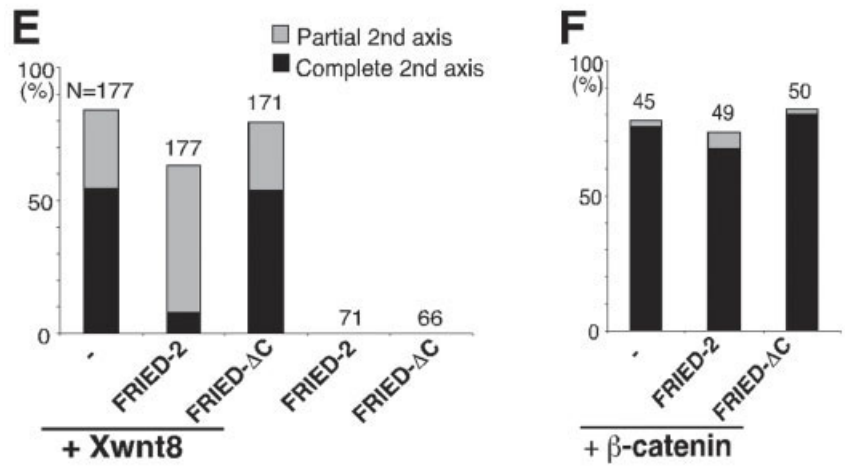


Fig. 4. FRIED-2 inhibits secondary axis induction by Xwnt8, but not β-catenin. **A–D:** One ventral vegetal blastomere of 8-cell embryos was injected with Xwnt8 RNA (6 pg) or β-catenin RNA (0.2 ng) alone or together with 1 ng of FRIED-2 or FRIED-ΔC RNA as indicated. **E,F:** Combined data for three experiments are shown. Secondary axis induction was scored when sibling embryos reached stage 40. Complete secondary axes include head structures with eyes and cement glands, while partial secondary axes are induced up to the hindbrain level and lack eyes. FRIED-2, but not FRIEDΔC, RNA inhibits secondary axis induction by Xwnt8, but neither RNA affects β-catenin-induced secondary axes.

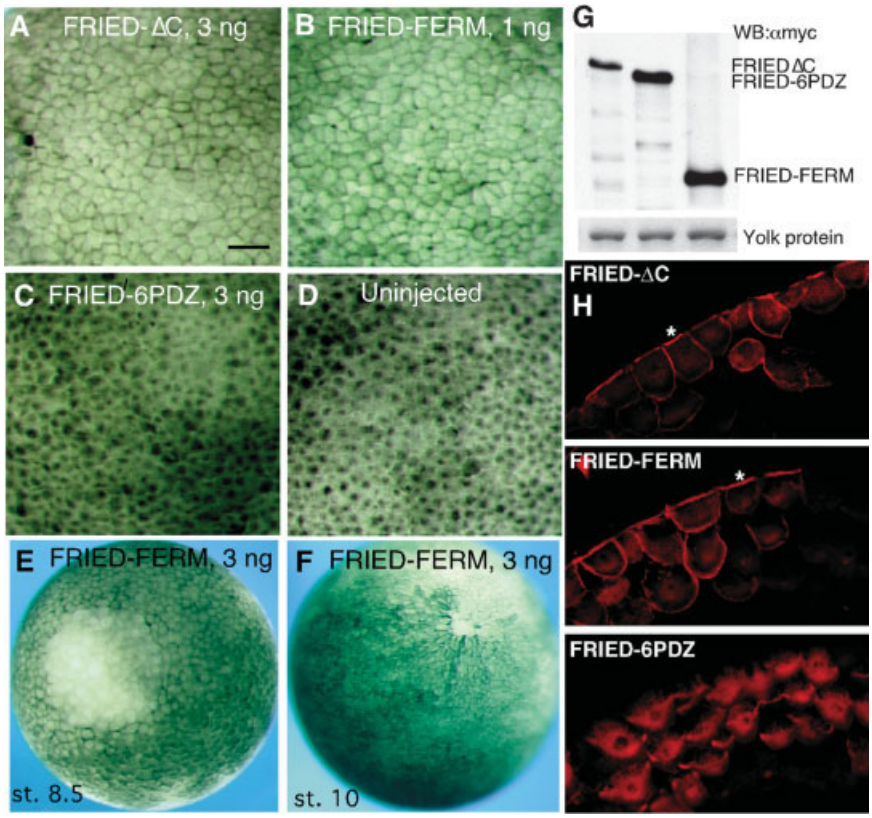
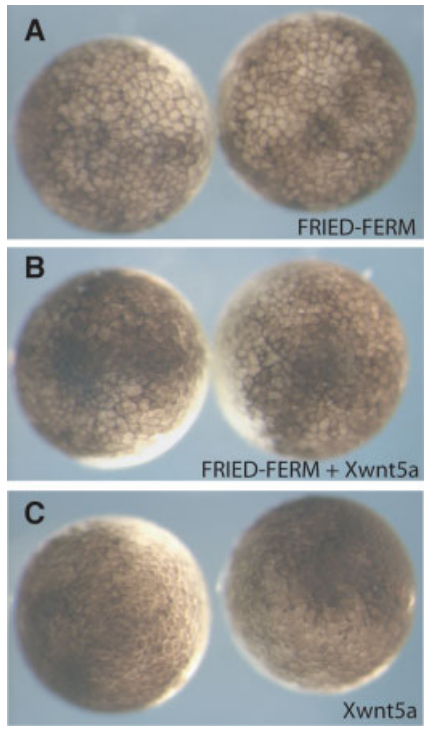


Fig. 5.



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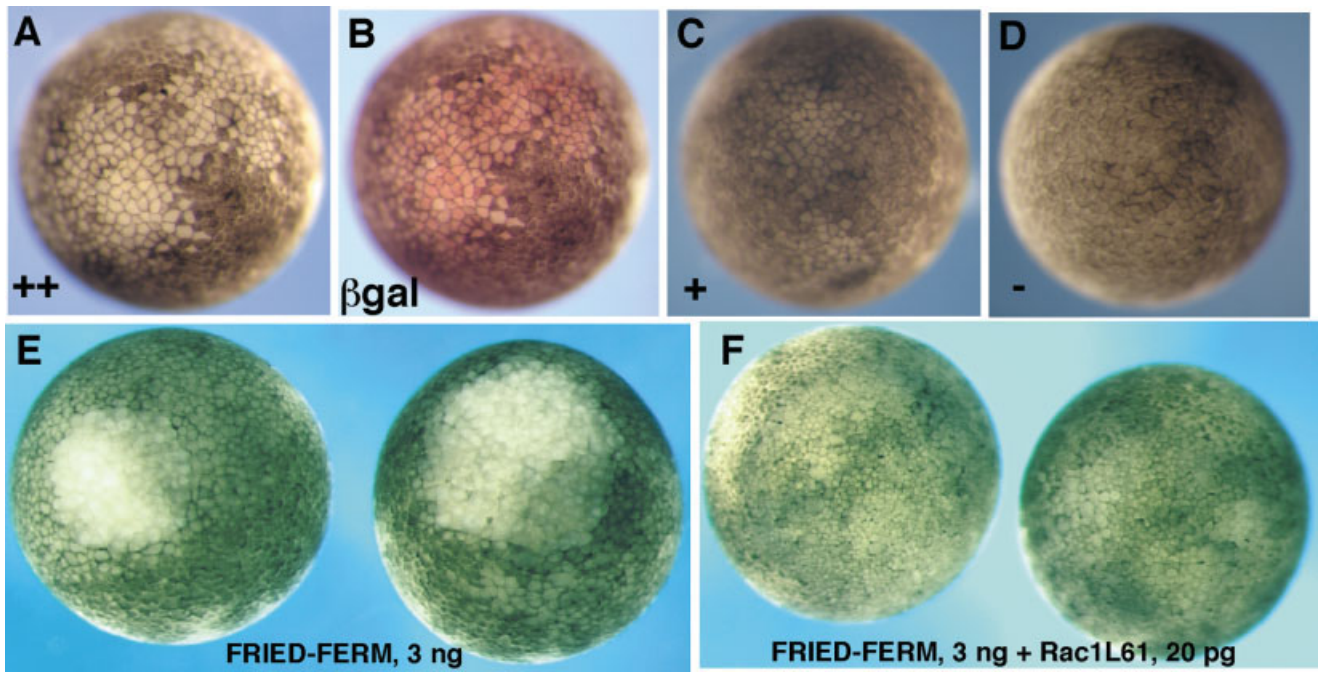

Fig. 8.

boundaries more distinctive (Fig. 5A). This phenotype was highly penetrant and was reproduced in more than 90% of embryos.

To determine which domain of FRIED is involved in causing ectodermal abnormalities, we compared the effects of several FRIED constructs. FRIED-FERM, which contains only the FERM domain of FRIED, caused depigmentation of animal hemisphere cells similar to FRIED-ΔC (Fig. 5B), while FRIED-6PDZ lacking the FERM domain had no effect (Fig. 5C). These constructs were expressed in embryos at comparable levels (Fig. 5G). Furthermore, FRIED-443 and FRIED-PDZ1, lacking the FERM domain, had no effect on pigmentation, while FRIED-26 and FRIED-2, containing the FERM domain, induced depigmentation (data not shown). These results indicate that the activity responsible for the pigment loss localizes to the FERM domain. At higher doses (3–5 ng), FRIED-FERM RNA induced ectodermal lesions surrounded by depigmented cells, indicating more profound cell defects, including those in cell adhesiveness (Fig. 5E). This effect was observed in 40–90% of embryos in different embryo batches. As embryos reached early gastrula stage, ectodermal lesions healed, and depigmentation of animal pole cells decreased (Fig. 5F). This fading of the phenotype at gastrulation suggests that the activity of FRIED-FERM is developmentally downregulated. Taken together, our results show that ectoderm pigmentation and cell adhesion are regulated by the FRIED-FERM domain.

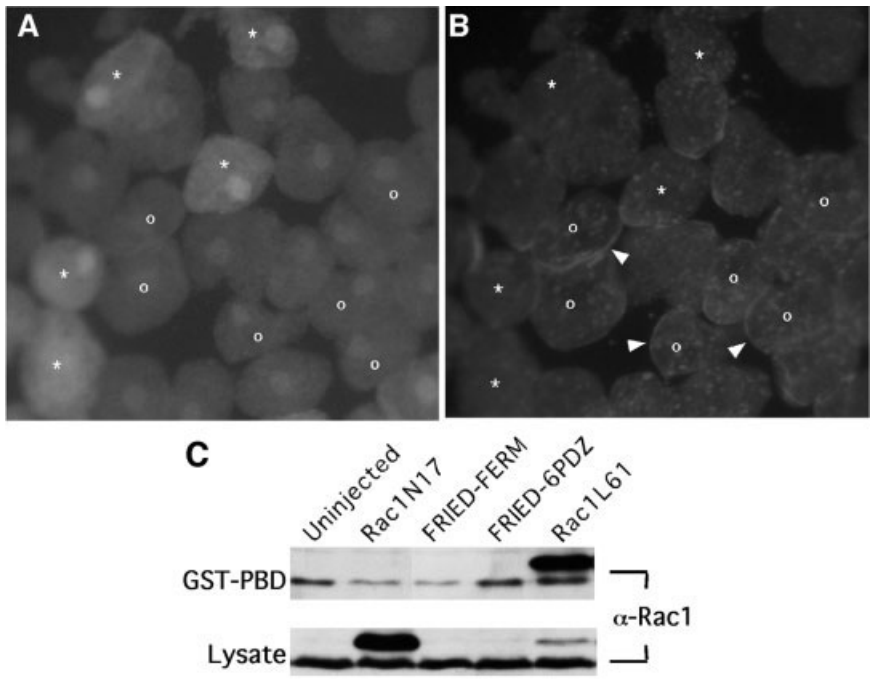

Fig. 7. FRIED reorganizes actin cytoskeleton and downregulates Rac1. A,B: FRIED disrupts cortical actin. Animal ventral blastomeres were injected at the 4-cell stage with FRIED-FERM and EGFP RNAs, 2 and 0.3 ng, respectively. Animal pole explants were isolated at stage 8 and analyzed by fluorescence microscopy. **A:** EGFP fluorescence of animal hemisphere cells. **B:** Rhodamine-phalloidine fluorescence of the same cells as in A. Only cells with low EGFP and FRIED-FERM expression (o) show cortical staining of F-actin (arrowheads). Cells with high EGFP and, thus, high FRIED-FERM expression (*) do not stain positively for F-actin. **C:** FRIED-FERM inhibits activity of endogenous Rac1. Four-cell embryos were injected with the following RNAs (eight injections per embryo): RacIN17, 2ng, FRIED-FERM, 2 ng, FRIED-6PDZ, 2 ng, Rac1L61, 0.6 ng. Embryo lysates collected at stage 8 were incubated with GST-PBD agarose to bind active Rac1. Bound protein complexes (top) and crude lysates (bottom) probed with anti-Rac1 antibody are shown. Myc-tagged Rac1L61 and Rac1N17 that migrate slower than endogenous Rac1 serve as positive and negative controls for GST-PBD binding.

We next wanted to determine the subcellular distribution of the FRIED protein. To address this question, two animal blastomeres of 8-cell embryos were injected with FRIEDΔC, FRIED-6PDZ, or FRIED-FERM RNA encoding myc-tagged proteins. At stage 10, the injected embryos were fixed, sectioned, and stained with anti-myc antibodies. In superficial ectoderm cells, FRIED-ΔC and FRIED-FERM were localized preferentially in the apical

Fig. 5. The effect of FRIED constructs on embryonic ectoderm. Depigmentation of blastula animal pole cells induced by FRIED-ΔC (**A**), FRIED-FERM (**B**), but not FRIED-6PDZ (**C**) RNAs. **D:** Uninjected embryo. **E:** Stage 8.5, ectodermal lesion in an embryo injected with FRIED-FERM RNA. **F:** Stage 10, healing of the lesion shown in E. Bar in A (applies to B–D) = 80 μm. **G:** In vivo expression levels of FRIED mutant constructs in embryos shown in A–C. Loading is shown by a major yolk protein stained with Coomassie blue. RNAs were injected into each blastomere of four-cell embryos as indicated. **H:** The FERM domain of FRIED defines its cortical localization. Two animal blastomeres of 8-cell embryos were injected with the following RNAs: Myc-FRIEDΔC (1 ng), FRIED-6PDZ (1 ng), or FRIED-FERM (0.3 ng). When sibling embryos reached stage 10, ectoderm cells of the injected embryos were stained with anti-Myc antibodies. FRIEDΔC and FRIED-FERM are localized preferentially in the apical cortex (*), whereas FRIED-6PDZ is found mainly in the cytoplasm.

Fig. 6. Xwnt5a suppresses the effect of FRIED-FERM on ectoderm. Four animal blastomeres of 8-cell embryos were injected with 0.3 ng of FRIED-FERM, 0.8 ng of Xwnt5 RNA, separately (**A,C**) or in combination (**B**). Animal view of midblastula stage embryos is shown. The depigmentation of ectoderm cells overexpressing FRIED-FERM (A) is rescued by Xwnt5a RNA (B), restoring the normal pigmentation pattern.

Fig. 8. Rac1L61 rescues the effects of FRIED on embryonic ectoderm. **A–D:** Different degrees of depigmentation induced by FRIED RNA were scored as ++(A), + (C), and – (D, uninjected embryo). A,B: Cell autonomy of the FRIED effect. Embryo coinjected with 1 ng of FRIED-2 RNA and 0.1 ng of β-gal RNA reveals β-galactosidase activity (identified by RedGal staining) in the cells that undergo depigmentation (compare A and B). The same embryo before and after Red-Gal staining is shown (A, B). **E,F:** Rac1L61 RNA suppresses ectoderm lesions induced by higher doses of FRIED-FERM RNA.

TABLE 1. Effect of Rac1L61 and Dishevelled Mutants on FRIED-Dependent Ectoderm Depigmentation[a]

	RNA dose					
	Rac1L61		Dsh-ΔN		Dsh-DEP+	
	90 pg	6 pg	1 ng	0.2 ng	1 ng	0.2 ng
Exp. 1	++	+	−	−	−	+/−
Exp. 2	++	−	+/−	−	−	−
Exp. 3	+	−	−	−	−	−
Exp. 4	++	+	NA	NA	NA	NA

[a]Scoring criteria: (−), depigmentation caused by 1 ng of injected FRIED-FERM RNA (as in Fig. 8A), partial (+, as in Fig. 8C) or complete (++, as in Fig. 8D) rescue of normal pigmentation, was observed in embryos coinjected with Rac1L61 RNA. At least 80% of embryos per group had indicated phenotype. Each group contained between 18 to 32 embryos. Four different experiments are shown. NA, not tested.

cortical region, whereas FRIED-6PDZ was found mainly in the cytoplasm (Fig. 5H). These observations indicate that the FERM domain of FRIED defines its cortical localization, which may be important for understanding of the molecular function of FRIED.

Xwnt5a Suppresses Ectoderm Depigmentation Induced by FRIED

To further demonstrate functional interaction of FRIED with Wnt signaling, we performed co-injections of FRIED-FERM and Xwnt5a, which is known to affect cytoskeletal organization and cell adhesion upon overexpression (Moon et al., 1993; Weeraratna et al., 2002). The depigmentation of ectoderm cells expressing FRIED-FERM was rescued by Xwnt5a RNA, resulting in a normal pigmentation pattern (Fig. 6). In addition, ectodermal lesions caused by higher doses of FRIED-FERM were suppressed by Xwnt5a overexpression (data not shown). We conclude that Xwnt5a rescues the effect of FRIED-FERM on embryonic ectoderm.

The Effect of FRIED-FERM on Cortical Actin and Rac1

Cortical actin anchors pigment granules in the submembranous layer of frog oocytes and embryonic cells (Gard et al., 1995). Disruption of filamentous actin leads to the loss of pigmentation of animal blastomeres (Moreau et al., 1999). Recently, the FERM domain of PTP-BL was shown to colocalize and cosediment with F-actin (Herrmann et al., 2003). To test whether FRIED might influence cortical actin cytoskeleton, we expressed FRIED-FERM together with GFP as a lineage tracer in embryonic ectoderm. In cells expressing high levels of GFP and, consequently, FRIED-FERM, cortical actin was stained with rhodamine-phalloidine weakly or not at all. However, cells with low expression of FRIED-FERM positively stained for cortical actin (Fig. 7A,B). This result suggests that overexpression of FRIED-FERM causes the rearrangement of actin cytoskeleton in blastula ectoderm cells.

Small GTPases of the Rho family, such as RhoA, Rac1, and Cdc42, are major regulators of actin cytoskeleton (Hall, 1998). Microinjection of the constitutively active RhoA-V14 mutant into *Xenopus* oocytes was reported to cause redistribution of surface pigment resulting in spotted oocytes (Mohr et al., 1990). Interestingly, the Rho GTPase activating protein PARG1 and Rho-dependent kinase PRK2 associate with different PDZ domains of PTP-BL (Saras et al., 1997; Vincent and Settleman, 1997). These data together with the observed effects of FRIED on cortical actin suggest that FRIED may function by regulating the activity of Rho GTPases. As Wnt/Fz signaling is known to activate RhoA and Rac1 (Habas et al., 2001, 2003), we analyzed activity of endogenous Rac1 and RhoA in embryo lysates. We found that Rac1 activity significantly decreased in embryos expressing FRIED-FERM (Fig. 7C), while active RhoA levels remained unchanged (not shown). To assess whether downregulation of Rac1 is critical for the effect of FRIED on ectoderm pigmentation, we attempted to rescue cell-autonomous ectoderm abnormalities induced by FRIED-FERM using a constitutively active Rac1L61 mutant (Fig. 8, Table 1). Rac1L61 RNA restored pigmentation of ectodermal cells in a dose-dependent manner (Table 1, Fig. 8A,C,D) and completely suppressed ectodermal lesions (Fig. 8E,F), while the dominant negative Rac1N17 did not have this effect (data not shown). These findings suggest that the effects of FRIED-FERM on pigmentation may involve inhibition of Rac1.

Since Dsh is known to regulate actin assembly in PCP pathway in *Drosophila*, we wanted to know whether Dsh mediates effects of FRIED on actin cytoskeleton. Therefore, we attempted to suppress FRIED-FERM-induced depigmentation with either Dsh-ΔN, a mutant that retains the activity in the planar cell polarity (PCP) pathway, or with Dsh-DEP+, a mutant that inhibits the PCP pathway (Tada and Smith, 2000). We were unsuccessful in rescuing the depigmentation phenotype with either mutant (Table 1). These data suggest that Dsh functions upstream of or parallel to FRIED or that the effect of FRIED-FERM on ectoderm pigmentation is unrelated to the PCP pathway.

DISCUSSION

We identified FRIED, a *Xenopus* protein tyrosine phosphatase, which interacts with Xfz8 and modulates ectoderm development. Sequence comparison revealed strong structural similarity of FRIED with the protein tyrosine phosphatase PTP-BAS/PTP-BL (Erdmann,

2003), with the exception of the sixth PDZ domain that is missing in PTP-BAS. We found that some FRIED constructs that contain the FERM domain can reorganize cortical actin and down-regulate activity of endogenous Rac1 GTPase. The observed functional interactions of FRIED constructs with over-expressed Xwnt8 and Xwnt5a indicate that FRIED may function as a link between Wnt/Frizzled signaling and actin cytoskeleton.

Based on the rearrangement of cortical actin by FRIED constructs (Fig. 7A,B), we propose that FRIED-induced depigmentation of ectoderm is mediated by altered actin cytoskeleton. Pigment granules of *Xenopus* animal hemisphere cells are embedded in cortical networks of filamentous actin (Gard et al., 1995), and disruption of cortical actin may cause loss of pigment from the cell surface (Moreau et al., 1999). Ectodermal lesions induced by FRIED constructs are similar to the adhesion defects caused by a dominant negative form of E-cadherin (Levine et al., 1994) and the adaptor protein IQGAP that is known to disrupt the E-cadherin-β-catenin complex (Sokol et al., 2001; Kuroda et al., 1998). Since association of actin microfilaments with the E-cadherin-β-catenin complex is essential for cell adhesion (Yap et al., 1997), the disruption of actin may explain FRIED-induced ectodermal lesions.

Our structure-function analysis indicates that the FERM domain of FRIED is necessary and sufficient for the effects of FRIED constructs on pigmentation of *Xenopus* ectoderm cells. FERM domains are present in a diverse group of proteins, such as the actin-interacting linker ERM proteins (Bretscher et al., 2002), merlin (Brault et al., 2001), talin (Rees et al., 1990), several protein tyrosine phosphatases (Yang and Tonks, 1991; Gu et al., 1991), and focal adhesion kinase and Janus kinases (Girault et al., 1998). The common feature of FERM domains is their association with transmembrane proteins and lipids. For example, FERM domains of some ERM proteins bind the transmembrane receptors, such as CD44, ICAM-1, and ICAM-2, while the FERM domain of talin interacts with integrins (Vaheri et al., 1997; Pfaff et al., 1998). The FERM domain of PTP-BAS also associates with phosphatidylinositol-4,5-biphosphate (Bompard et al., 2003). Importantly, FERM domains of merlin, ezrin, and PTP-BL were shown to interact with filamentous actin (Herrmann et al., 2003; Xu et al., 1998; Brault et al., 2001; Veheri et al., 1997), supporting our conclusion on the role of FRIED in cytoskeletal reorganization. The effect of FRIED-FERM on pigmentation of *Xenopus* ectodermal cells establishes a unique in vivo assay for FERM domain activity. It should be helpful in further studies of FRIED/PTP-BL in the regulation of actin cytoskeleton.

Disorganization of cortical actin by FRIED-FERM can be mediated through the direct interaction with actin or through Rho GTPases (Hall, 1998). The FERM domains were proposed to activate Rho GTPases in a cell culture system through binding and suppression of a Rho-GDP dissociation inhibitor that keeps Rho GTPases inactive by complexing with their GDP-bound forms (Takahashi et al., 1997). In contrast, we show inhibition of Rac1 activity by FRIED-FERM. Consistent with the inhibitory effect of FRIED on Rac1, we demonstrate that the constitutively active Rac1L61 reversed the depigmentation and ectodermal lesions induced by FRIED-FERM. It is possible that individual Rho GTPases show considerable specificity in their responses to specialized FERM domains.

Currently, at least a dozen different proteins were shown to interact with various domains of PTP-BAS/PTP-BL (Erdmann, 2003); however, biological functions of this phosphatase remain largely unknown. Recently, a role for PTP-BL in cytokinesis has been proposed (Herrmann et al., 2003). The apical distribution of FRIED observed in our experiments, and the reported localization of PTP-BL in epithelial cells and the growth cones of axons, suggest a role in the maintenance of cell polarity (Thomas et al., 1998; Cuppen et al., 1999). The binding of FRIED to the cytoplasmic tail of Xfz8 and the observed functional interactions of FRIED with Xwnt5a and Xwnt8 suggest that FRIED participates in Wnt signaling to actin cytoskeleton. This view is also supported by the association of PTP-BL and adenomatous polyposis coli (APC), a component of Wnt signaling (Erdmann et al., 2000). A morpholino antisense oligonucleotide that was specifically designed to inhibit FRIED translation in vivo did not have an effect on embryonic development (data not shown), precluding the assessment of the function of FRIED in vertebrate embryos. Additional studies are needed to establish the connection between FRIED and Wnt/Frizzled signal transduction.

EXPERIMENTAL PROCEDURES

Yeast Two-Hybrid Screen

The cytoplasmic tail of Xfz8, consisting of 56 amino acids (aa), was used as bait in a two hybrid screen of a *Xenopus* gastrula cDNA library in pJG45 (Itoh et al., 2000) as described (Golemis et al., 1994). Approximately 1.2×10^6 colonies were screened and 38 colonies were isolated, which grew on leucine-deficient medium and turned blue on XGal-containing medium. Three isolated clones carried overlapping sequences with a novel open reading frame. One positive clone, termed FRIED443-BSSK, was used as a probe to obtain a full-length cDNA by screening a λgt10 *Xenopus* gastrula cDNA library. Two phage clones, 14 and 26, with large overlapping inserts were identified and together with an overlapping fragment of the *Xenopus* EST clone XL077h11 encompassed 8 kb cDNA encoding a protein of 2,500 a.a. The nucleotide sequence for FRIED gene and the deduced amino acid sequence of the corresponding protein have been deposited in the GenBank (accession number AY327257). Comparisons of amino acid sequences were performed using the BLAST program. Alignments of protein sequences were done using the ClustalW/DNAStar software.

Plasmid Constructs

To make a bait construct, a cDNA fragment encoding the cytoplasmic tail of Xfz8 (Xfz8-CT, aa 525–581) was PCR amplified with the sense primer 5′-CT-GGATCCGGTCGGGAAAGACT-3′ and the T3 anti-sense primer (Promega, Madison, WI) and subcloned into Bam HI and Xho I digested pEG202 (Golemis et al., 1994). In FRIEDΔC, cDNA frag-

ments from clones 14 and 26 were ligated into pXT7. To make myc-tagged FRIED-ΔC, an intermediate X/N-B-pXT7M construct was made initially by ligating Xho I/Bgl II fragment of FRIED-ΔC cDNA (bp 37 to 2,220) into Xho I and Bgl II-digested pXT7-6myc. The 5′-UTR of FRIED-ΔC was removed by re-ligation of the Xho I-digested and Nsi I-digested and blunted intermediate plasmid. Myc-tagged FRIED-ΔC was generated by subcloning an EcoR I-Age I fragment from X/N-B-pXT7M and Age I/Spe I fragment from FRIEDΔC-5′UTR-pXT7 into EcoR I and Spe I sites of pXT7-6myc. In FRIED-1-pXT7M (aa 3–1,339) EcoR I/Nsi I (blunted) fragment from FRIED-ΔCm was subcloned into EcoR I and Spe I (blunted) sites of pXT7-6myc. FRIED-2-pXT7M (aa 3-1446) was created by three-fragment ligation of EcoR I/Age I fragment from FRIED-ΔCmyc, Age I/Bcl I fragment from ΔXba-26-BSSK, and EcoR I/Bgl II-digested X/N-B-pXT7M. FRIED-3-pXT7M (aa 3–1,747) was generated by subcloning an EcoR I-Xho I-blunted fragment from FRIED-ΔCmyc into EcoR I-Spe I-blunted sites of pXT7-6myc. FRIED-6PDZ was created by four-piece ligation of EcoR I/Age I fragment from X/N-B-pXT7M, Dra I/Sal I fragment from ΔXba-26-BSSK and Sal I/Spe I fragment from ΔSal-26-BSSK into EcoR I and Spe I sites of pXT7-6myc. FRIED-FERM was generated by subcloning of the Age I/Pst I fragment from FRIED-ΔCm into Age I and Nsi I sites of FRIED-2-pXT7M. FRIED-26 was generated by subcloning EcoR I/Spe I fragment of 26-BSSK into EcoR I and Xba I sites of pCS2-myc. FRIED-PDZ1 was obtained by deleting FRIED-6PDZ at the Nsi I site. FRIED443-BSSK (aa 1,676–2,229) was subcloned into pXT7-myc to yield myc-FRIED-443. GST-CT was described previously (Lisovsky et al., 2002). To construct GST-CT-ARD, the cytoplasmic tail of Xfz8 was PCR amplified with the sense primer 5′-CTGGATCCGGTC-GGGAAAGACT-3′ and the anti-sense primer 5′-GTGTCGACTAGTCTCGAG-CTAAGGGCATTTGT-3′ and subcloned into pXT7-GST (Lisovsky et al., 2002). EGFP-C1 (Clontech, Palo Alto, CA) was subcloned into pXT7 for RNA microinjections. Myc-Rac1N17 and Myc-Rac1L61 plasmids were a gift of A. Hall. The GST-PBD plasmid was a gift of R. Habas. PTP-BL cDNA was a gift of W. Hendriks. The other plasmids used encoded Xwnt8 (Sokol et al., 1991), Xwnt5a (Moon et al., 1993), Flag-β-catenin (Liu et al., 1999), and nucβgal-pCS2 (Turner and Weintraub, 1994).

Xenopus Embryos, RNA Synthesis, and Microinjections

Eggs were obtained from female *Xenopus laevis* after injection with 800 U of human chorionic gonadotropin. In vitro fertilization and embryo culture were performed as described (Newport and Kirschner, 1982). Staging was according to Nieuwkoop and Faber (1967). For microinjections, embryos were transferred to 3% Ficoll 400 in 0.5× MMR (1× MMR: 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM Hepes, pH 7.6, and 0.1 mM EDTA) (Newport and Kirschner, 1982). Capped RNAs for embryo microinjections were synthesized by in vitro transcription of linearized plasmid templates with SP6 or T7 RNA polymerases (Krieg and Melton, 1984) using mMessage mMachine kit (Ambion). RNA concentration was estimated in gel by ethidium bromide staining. Information on linearization and transcription of different DNA templates is available on request. Embryos were injected at the 4- to 8-cell stage with 10 nl of RNA solution at indicated doses. Before gastrulation, embryos were transferred to 0.1 × MMR with 50 μg/ml of gentamicin. Whole-mount in situ hybridization was carried out according to Harland (1991) with slight modifications as described previously (Hikasa and Sokol, 2004).

Immunocytochemistry, Actin Staining, and Lineage Tracing

Embryos injected with RNAs encoding Myc-tagged FRIED constructs were fixed at stage 9 with 100% methanol for 2 hr at −20°C and transferred to 100% ethanol at −20, 24, and 39°C, sequentially, over 3–4 hr. Ethanol was replaced with 50% polyethyleneglycol-400 distearate (PGD, 1 hr) and then 99% PGD plus 1% cetyl alcohol (4–6 hr) at 39°C before embedding. Embedded embryos were sectioned at 10 μm. The sections were washed in methanol, then in PBS and incubated with anti-Myc antibodies (9E10) and Cy3-conjugated anti-mouse antibodies. For cortical actin staining, FRIED-FERM and EGFP RNAs were injected into the animal pole of 4- to 8-cell embryos. At stage 8, vitelline membrane was removed and animal pole explants were cut and fixed in 4% paraformaldehyde in 1× phosphate-buffered saline (PBS). Embryos were stained with rhodamine-phalloidine (7.5 μg/ml, Sigma) in 1× PBS for 25 min at room temperature, then washed in 1× PBS and 0.1% Tween 20 for 20 min. EGFP and rhodamine fluorescence were visualized using a Zeiss Axiophot microscope with Omega XF22 and XF30 filters, respectively.

For lineage tracing, embryos were injected with FRIED-2 and nuclear β-galactosidase RNAs were fixed in 3.7% formaldehyde for 30 min. β-galactosidase activity was visualized with Red-Gal substrate (Research Organics). Embryos were photographed before and after Red-Gal staining.

Northern Blotting

For Northern blotting, total RNA from embryos at different stages of development was isolated as described and separated in formaldehyde-agarose gels (Itoh et al., 1995). Antisense RNA probe was generated by in vitro transcription of FRIED443-pXT7. Blotting, hybridization, and autoradiography were performed according to standard techniques (Sambrook et al., 1989).

GST Pull-Down Assays and Western Analysis

For analysis of Xfz8-FRIED interaction, 20 injected embryos per group were lysed in 500 μl of lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM Na_3VO_4) when embryos had reached early gastrula (stage 10). GST fusion proteins were recovered for 2 hr at 4°C using 20 μl of glutathione-agarose beads, resolved by 12% SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting as described previously using

anti-myc 9E10 and anti-GST antibodies (Itoh et al., 2000). Detection of activated Rac1 was performed exactly as described (Hens et al., 2002). Briefly, RNAs were injected four times dorsally and ventrally in all blastomeres at four- to eight-cell stage (eight injections per embryo). Twelve to eighteen embryos per group were lysed at stage 8 (20 μl of lysis buffer per embryo) on ice and incubated with GST-PBD agarose beads at 4°C. Agarose-bound protein complexes were resolved by SDS-PAGE and analyzed by Western blotting using anti-Rac1 antibodies (BD Transduction Laboratories).

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