

# Essential kinase-independent role of a Fer-like non-receptor tyrosine kinase in *Caenorhabditis elegans* morphogenesis

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## Summary

Morphogenesis requires coordination of cell surface activity and cytoskeletal architecture. During the initial stage of morphogenesis in *Caenorhabditis elegans*, the concerted movement of surface epithelial cells results in enclosure of the embryo by the epidermis. We report that Fer-related kinase-1 (FRK-1), an ortholog of the mammalian non-receptor tyrosine kinase Fer, is necessary for embryonic enclosure and morphogenesis in *C. elegans*. Expression of FRK-1 in epidermal cells is sufficient to rescue a chromosomal deficiency that removes the *frk-1* locus, demonstrating its autonomous requirement in the epidermis. The essential function of FRK-1 is independent of its kinase domain, suggesting a non-enzymatic role in morphogenesis. Localization of FRK-1 to the plasma

membrane requires  $\beta$ -catenin, but not cadherin or  $\alpha$ -catenin, and muscle-expressed  $\beta$ -integrin is non-autonomously required for this localization; in the absence of these components FRK-1 becomes nuclear. Mouse FerT rescues the morphogenetic defects of *frk-1* mutants and expression of FRK-1 in mammalian cells results in loss of adhesion, implying a conserved function for FRK-1/FerT in cell adhesion and morphogenesis. Thus, FRK-1 performs a kinase-independent function in differentiation and morphogenesis of the *C. elegans* epidermis during embryogenesis.

Keywords: *C. elegans*, Fer kinase, Morphogenesis, Adhesion,  $\beta$ -catenin

## Introduction

The complex forms of animals are sculpted from amorphous collections of cells by alterations in cell shape and organized movements of epithelia. These dynamic events are directed by regulated changes in adhesive forces and remodeling of the cytoskeleton. The major morphogenetic events that create the form of the nematode *Caenorhabditis elegans* are driven by the outer epithelium, the epidermis. The epidermis is initially assembled into six bilaterally symmetric longitudinal rows assembled into a single dorsolateral epithelial sheet. The cells in the two dorsalmost rows intercalate to form a single row, followed by ventralward and anterior-directed epiboly of the epithelium, resulting in enclosure of the embryo (reviewed by Simske and Hardin, 2001). Following completion of enclosure, circumferential contraction of the actin cytoskeleton in the epidermis causes the embryo to elongate fourfold along its anteroposterior axis, transforming the spheroid embryo into the characteristic vermiform shape of the animal (Priess and Hirsh, 1986).

Modulation of cell adhesion mechanisms governs cell shape changes and migration during morphogenesis. Morphogenesis of the *C. elegans* epidermis requires cadherin adhesion complexes at the plasma membrane. Although *C. elegans* mutants defective in cadherin,  $\alpha$ -catenin or  $\beta$ -catenin function are able to undergo epidermal enclosure, they do not elongate properly, resulting in lumpy larvae that arrest at the first larval stage (Costa et al., 1998). Adhesion and migration during

ventral enclosure are also mediated by the ephrin signaling pathway. A fraction of embryos lacking either the VAB-1 Eph receptor or the VAB-2 ephrin ligand arrest without undergoing enclosure by the epidermis (Chin-Sang et al., 1999; George et al., 1998). The interaction of this ephrin/Eph receptor pair in underlying neurons creates a substrate for the migration of the epidermis during epiboly. The cell adhesion roles of the cadherin and Eph signaling systems are each associated with regulation of tyrosine kinase activity (Tepass et al., 2002; Zantek et al., 1999). Whereas cadherins control localization and phosphorylation of the Eph receptors by stabilizing cell adhesion, the activated Eph receptors are capable of autophosphorylation, which promotes their interaction with a variety of cytoskeletal and signaling proteins through SH2 domains.

Another type of kinase shown to participate in adhesion complexes in epithelial cells is Fer, a member of the Fes/Fps proto-oncogene family of non-receptor tyrosine kinases (NRTKs), none of which contains a membrane-anchoring domain. Fer interacts with cadherin complex components including N- and E-cadherins,  $\beta$ -catenin, and p120 catenin, and is thought to stabilize the interactions within the complex via its kinase activity (Piedra et al., 2003; Rosato et al., 1998; Xu et al., 2004). Fer has also been shown to move between cadherin and integrin complexes while mediating cell adhesion (Arregui et al., 2000). Two forms of mammalian Fer exist: a ubiquitously expressed, full-length version (Letwin et al.,

1988; Pawson et al., 1989), and a nuclear, truncated version, FerT, which has been observed only in primary spermatocytes (Fischman et al., 1990). While Fer has been implicated in cell adhesion complexes, the essential *in vivo* function of *fer* has not been reported, with the exception that a mouse knock-in of a kinase-inactive form of the protein showed no obvious developmental defects (Craig et al., 2001). Although it was previously reported that there were probably no Fer homologs in *C. elegans* (Greer, 2002; Plowman et al., 1999), *fer*-like genes have been found in sponges (Cetkovic et al., 1998), flies (Katzen et al., 1991; Paulson et al., 1997), birds and mammals (Fischman et al., 1990; Pawson et al., 1989; Feldman et al., 1986).

In this study, we demonstrate the essential *in vivo* role of a Fer-like protein, FRK-1, in morphogenesis of developing *C. elegans* embryos. We report that expression of FRK-1 in the epidermis is required for enclosure of the embryo at the onset of morphogenesis. We made the unexpected finding that the kinase activity of FRK-1 is not required for its role in morphogenesis, suggesting that FRK-1 may act to stabilize adhesion complexes independent of its enzymatic activity. This kinase-independent function of FRK-1 is also required cell-autonomously in the lateral row of epidermal cells, the seam cells, for late stages of their differentiation. FRK-1 is normally localized at the plasma membrane beginning at the onset of morphogenesis; however, it relocates to the nucleus in the absence of either the cadherin-associated  $\beta$ -catenin, HMP-2, or the muscle-expressed  $\beta$ -integrin homolog, PAT-3; the latter indicates a cell-non-autonomous action of  $\beta$ -integrin on FRK-1 function. Mouse FerT can substitute for FRK-1 in worms, and expression of FRK-1 in mammalian cells causes loss of adhesion, similar to the effects of overexpressing Fer in mammalian cells. These findings suggest that the crucial role for Fer-like proteins in epithelial morphogenesis is conserved throughout animal phylogeny.

## Materials and methods

### Strains and alleles

The wild-type strain used was the Bristol N2 strain, as described by Brenner (Brenner, 1974). The *elt-1(zu180)* mutation from J. Pries was used to make JR784 (*elt-1(zu180) unc-43(e408)unc-24(e138) dpy-20(e1282) IV. hmp-1(zu278)* [JJ862], *hmp-2(zu364)* [JJ1068] and *hmr-1(zu389)* [JJ1079] (Costa et al., 1998) were obtained from the Caenorhabditis Genetics Center (CGC). Strain RW3600, carrying *pat-3(st564)* (Williams and Waterston, 1994), was obtained from the CGC. The *mDf7* chromosomal deficiency strain JR2482 (*mDf7 dpy-13/unc-24 dpy-4*) was rebalanced from DR793 (Terns et al., 1997). Strains for rescue experiments are as follows: JR2557 was made from JR2482 and contains an extrachromosomal array (*wEx1529*) consisting of the *frk-1* coding region and the *sur-5::GFP* (green fluorescent protein) marker (pTG96) (Yochem et al., 1998), which allowed for observation of embryos containing the rescuing array. JR2559 was made from JR2482 and contains an extrachromosomal array (*wEx1531*) consisting of the *frk-1* promoter and the 3'-UTR region fused to the *ferT* mammalian cDNA (a generous gift of U. Nir) and pTG96. JR2729 was made from JR2482 and contains an extrachromosomal array (*wEx1633*) consisting of the *elt-1* promoter region fused to the *frk-1* coding region and pTG96. JR2732 was made from JR2482 and contains an extrachromosomal array (*wEx1634*) consisting of the *elt-3* promoter region fused to the *frk-1* coding region and pTG96. A minimum of three independent arrays were analyzed in each rescue experiment performed.

To analyze the tissue distribution of *frk-1* expression, we created a transcriptional fusion construct containing 1.8 kb upstream of the *frk-1* gene fused to GFP and a nuclear localization sequence using pPD96.62 (Fire et al., 1990) (a gift from A. Fire). Arrays containing this construct and the *rol-6(su1006)* marker (pRF4) (Mello et al., 1991) were analyzed.

Expression of epidermis-specific genes in *frk-1(RNAi)* embryos was examined using the following strains: JR672 contains an integrated array (*wIs54*) of the seam-specific SCM::GFP marker (previously described by Terns et al. (Terns et al., 1997) plus *ajm-1::GFP* (a gift from J. Hardin). JG5 contains an *elt-3::GFP* fusion and was a gift from J. Gilleard. *nhr-72* (PY1215) and *nhr-81* (PY1282) GFP reporter lines were gifts from P. Sengupta (Miyabayashi et al., 1999). JR1736 contains *nhr-75::GFP* (P. Sengupta) and was made as described in Koh and Rothman (Koh and Rothman, 2001).

### RNA-mediated interference (RNAi)

RNA interference was performed by using dsRNA made from a PCR product containing the T7 promoter on each end fused to a genomic region of *frk-1* of approximately 1300 bp. dsRNA was synthesized and purified using the MEGAscript kit (Ambion) according to the manufacturer's protocol. dsRNA was either injected into young hermaphrodites (Fire et al., 1998) or used to soak young hermaphrodites overnight. Progeny were analyzed 8 to 20 hours after injection.

### Four-dimensional time-lapse analysis of development

Embryonic development in wild-type, *mDf7* and *frk-1(RNAi)* embryos was analyzed via time-lapse video microscopy as described previously (Moskowitz et al., 1994). Images were collected through ten focal planes, encompassing the entire embryo. Scans were made every 5 minutes for the first 8 hours of development (sufficient time for completion of ventral enclosure in wild-type embryos). For each mutant embryo recorded, a wild-type embryo was placed in the field of observation to allow for direct comparison of mutant versus wild type throughout development. At each time point the planes of focus that included developing epidermal cells were analyzed for cell division, shape and migration.

### In vitro kinase assays

*frk-1* and *frk-1(D308R)* PCR products were transcribed and translated *in vitro* using the TnT Quick Coupled kit (Promega) according to the manufacturer's protocol. The translated FRK-1 was purified by immunoprecipitation using FRK-1 antibodies and Sepharose A-agarose beads (Zymed) and resuspended in 1 × PBS buffer. Each tube of purified FRK-1 was then tested for autophosphorylation activity by adding 5 × tyrosine kinase buffer (100 mmol/l HEPES, 5 mmol/l MnCl<sub>2</sub>, 5 mmol/l DTT, 500 μmol/l Na<sub>3</sub>VO<sub>4</sub>), ATP (5 μmol/l final), 5 μCi [<sup>32</sup>P]ATP, and 2.75 μl dH<sub>2</sub>O. Each reaction was incubated at 30°C for 10 minutes and terminated by boiling. Unused radioactivity was eliminated by Micro bio-spin 6 chromatography columns (Biorad). Samples were boiled for 5 minutes and run on gradient (4–15%) tricine gels (Jule). Gels were dried on Whatman paper and exposed to radiographic film (Kodak).

### Antibody production and immunofluorescence

Anti-FRK-1 antibodies were raised against two FRK-1-specific peptides: FRK-1a (EKSSNNDASVTDDIRAE) and FRK-1b (QKNPEKRSTMDSIHKKLRE). The peptides were synthesized by United Biochemical Research. The sequences of the two peptides were derived from each end of the protein and chosen to minimize homology with other proteins. Two rabbits, UCSB64 and UCSB65, were immunized against each peptide by Cocalico Biologicals, Inc. Antiserum from each rabbit was affinity-purified with the original peptide using the SulfoLink kit (Pierce) and tested. Both antibodies gave identical staining patterns. Embryos were fixed and stained according to Sulston and Hodgkin (Sulston and Hodgkin, 1988) using a 1:5 dilution of each purified FRK-1 antibody.

MH27 is a mouse monoclonal antibody that recognizes epithelial apical junctions (Priess and Hirsh, 1986). NE2-1B4 is a mouse monoclonal antibody that recognizes an antigen expressed only in seam cells (Schnabel, 1991). LIN-26 antibodies (rabbit) were a generous gift from M. Labouesse.

### Immunoprecipitation

To test for physical interactions between FRK-1 and HMP-2/ $\beta$ -catenin, immunoprecipitation experiments were performed on cell lysates from worms expressing a transgenic version of FLAG-HMP-2 in which the N-terminus of HMP-2 was fused in-frame with the FLAG sequence. Complexes were precipitated with anti-FLAG antibody and electrophoresed. Western blots were probed with both anti-FLAG and anti-FRK-1 antibody B.

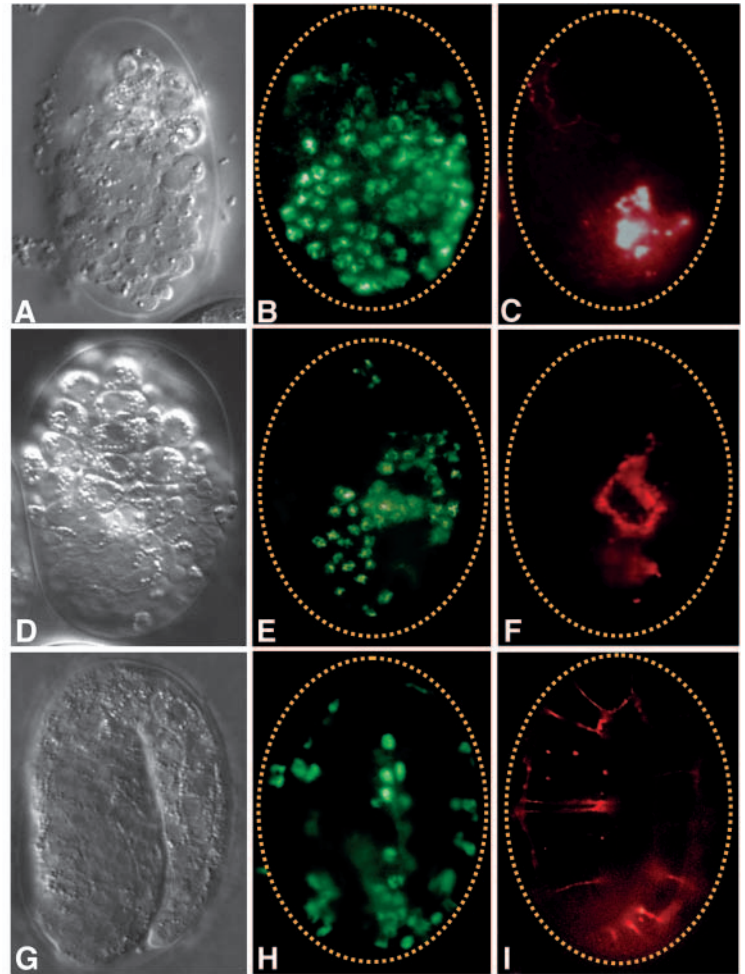
### Expression of FRK-1 in cultured human cells

The mammalian ecdysone-inducible expression system (Invitrogen) was used to express FRK-1 in cultured cells. A *frk-1* cDNA was amplified using oligonucleotides that included a 5' Kozak sequence necessary for efficient translation in mammalian cells and ligated into the *EcoRI* site of pIND-*lacZ* (Invitrogen). The pIND-*lacZ*; *frk-1* plasmid (320 ng DNA per 25  $\mu$ l medium) and pVgRXR (which expresses the ecdysone receptor) were then transiently transfected into the human embryonic kidney cell line HEK293, using Lipofectamine 2000 (Invitrogen). Cells were plated onto 96-well plates coated with poly-D-lysine (100  $\mu$ g/ml), human placental laminin-2 (500  $\mu$ g/ml; Sigma), or plasma fibronectin (10  $\mu$ g/ml; Invitrogen). FRK-1 expression was induced by exposing cells to the ecdysone analog Ponasterone A (5  $\mu$ mol/l) for 20 hours, according to the manufacturer's protocol. The cells were washed with PBS and fixed with 4% paraformaldehyde/4% sucrose. Cells transfected with pIND-*lacZ* were stained with BluOGal (5 mmol/l ferricyanide, 5 mmol/l ferrocyanide, 2 mmol/l MgCl<sub>2</sub>) to assess transformation efficiency (which was typically ~50%). Adhesion was quantified by staining triplicate wells with amido black (Davis and Camarillo, 1993).

## Results

### Essential role of the *C. elegans* Fer-like non-receptor tyrosine kinase in epidermal enclosure and differentiation

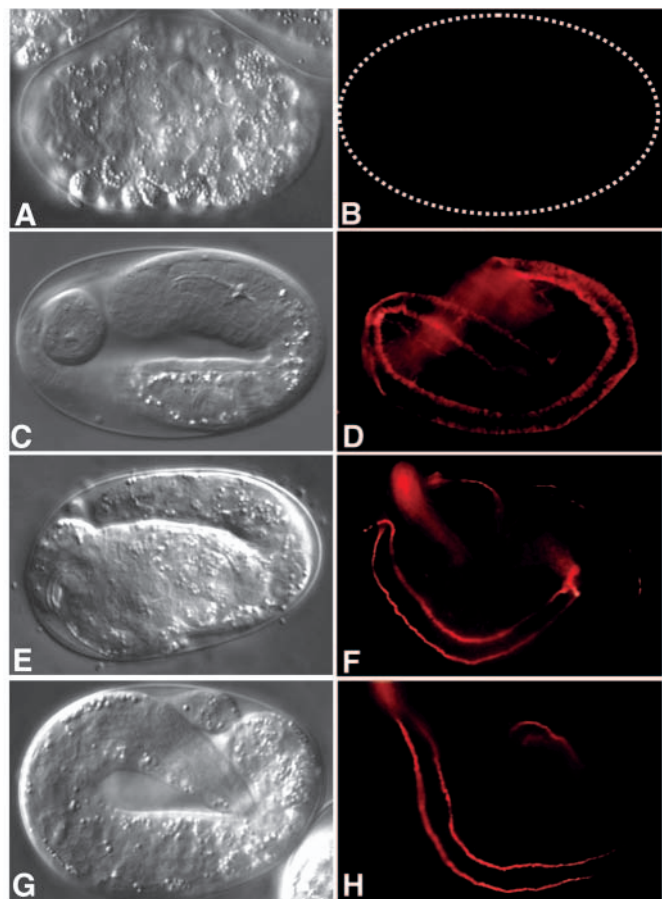
In classical genetic and functional genomics screens for genes required for embryonic morphogenesis, we identified mutants defective for the *C. elegans* ortholog of the mammalian non-receptor tyrosine kinase, Fer. Inhibition of this gene, fer-related kinase-1 (*frk-1*), by RNAi resulted in profound defects in ventral closure of the epidermis and therefore subsequent elongation and morphogenesis (Fig. 1). The phenotype observed for *frk-1(RNAi)* embryos resembled that seen in embryos homozygous for the chromosomal deficiency *mDf7*, which deletes *frk-1* (e.g. Chanal and Labouesse, 1997; Labouesse, 1997; Terns et al., 1997). While *mDf7* removes approximately 160 genes, we found that a 3.7-kb genomic fragment containing the *frk-1* gene was capable of restoring enclosure, elongation and embryonic viability to homozygous *mDf7* embryos (Fig. 2C,D), with the majority of the hatched larvae arresting at various post-embryonic stages. Given the large number of genes deleted by *mDf7*, we were surprised to find that some rescued *mDf7* homozygotes developed to adulthood. While most of these rescued adults were sterile, remarkably, some produced small



**Fig. 1.** Zygotic loss of FRK-1 blocks epidermal enclosure and elongation during embryogenesis. Differential interference contrast images of *frk-1(RNAi)* (A), *mDf7* homozygous (D), and wild-type (G) embryos are shown at approximately 8.5 hours post-fertilization. *frk-1(RNAi)* and *mDf7* embryos arrest without enclosing. The epidermal cells on the surface of the embryo round up and appear to be defective in cell adhesion. Epidermal cells do not migrate properly and become clustered together on the dorsal side of the embryo, as revealed with the nuclear marker LIN-26 (B,E,H). Apical junctions in the epidermis, revealed with antibody MH27, constrict and cluster dorsally (C,F,I). Embryos, as shown in this and subsequent figures, are ~50  $\mu$ m along the anteroposterior axis.

broods (10–20 embryos). It is possible that the loss of other genes in the deficiency could contribute to the overall embryonic phenotype of *mDf7*; however, the finding that the *frk-1* gene can rescue the homozygous deficiency embryos to adulthood strongly suggests that the primary defects we observed are attributable specifically to the removal of *frk-1* and not other genes eliminated by the deficiency. Thus, the gross embryonic phenotype of *mDf7* appears to reflect primarily the effect of removing zygotic *frk-1*. We therefore investigated the embryonic function of *frk-1* by characterizing both *frk-1(RNAi)* and *mDf7* embryos.

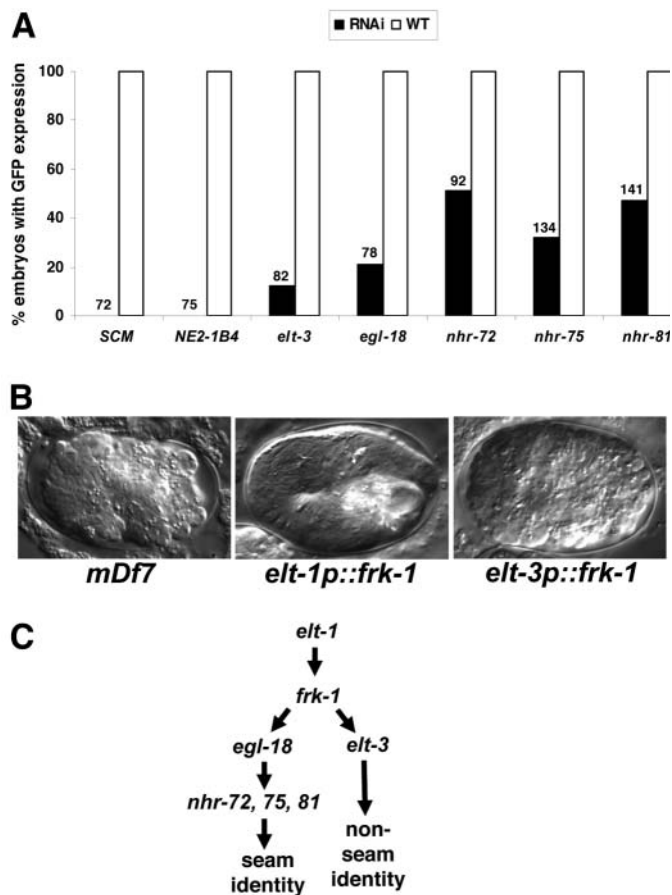
The predicted product of *frk-1*, FRK-1, is most similar to mammalian FerT, the truncated form of the protein that lacks the amino terminal coiled-coil domain of full-length Fer. FRK-1 is 67% similar and 39% identical to FerT. The similarity



**Fig. 2.** *frk-1* rescues the morphogenetic and differentiation defects of *mDf7* homozygous embryos. Embryos homozygous for *mDf7* (A) do not express the seam-cell-specific marker recognized by antibody NE2-1B4 (B). Expression of FRK-1 from an extrachromosomal array restores enclosure and elongation (C) and expression of the seam-specific NE2-1B4 antigen (D) to *mDf7* embryos. Mouse FerT similarly rescues *mDf7* embryos (E,F). A wild-type embryo is shown at the three-fold stage (G) with the NE2-1B4 marker (H).

between the *C. elegans* and mammalian proteins is more pronounced in the SH2 binding domain (86% similarity), which contains the signature FLVR element, and the tyrosine kinase domain (73% similarity), where the ATP binding site resides and conserved amino acid residues (numbers 252 and 308) required for kinase activity (Cole et al., 1999) are located. The predicted FRK-1 protein may be the only isoform of FRK-1 in *C. elegans*, as corroborated by RT-PCR and Western blotting, which revealed a single transcript and polypeptide of approximately 1180 bp and 45 kDa, respectively (data not shown).

To analyze the morphogenetic defects of mutants lacking *frk-1* function, we followed the development of *frk-1(RNAi)* and *mDf7* homozygous embryos by 4-D time-lapse video microscopy (see Materials and methods) and compared it to that of wild-type embryos in the same recordings. These studies showed that depletion of FRK-1 disrupted the normal dorsal intercalation and ventralward migration of epidermal cells, preventing enclosure and elongation of the embryo (Fig. 1A). The organization of epidermal cells appeared similar to



**Fig. 3.** FRK-1 is required for late epidermal differentiation. (A) Percent of embryos with a wild-type (empty bars) and *frk-1(RNAi)* (filled bars) genotype expressing the indicated marker. Numbers of *frk-1(RNAi)* embryos scored are shown above each bar. More than 100 embryos were scored for all markers in wild-type embryos. Seam cell marker (SCM) is a transgenic reporter that expresses a GFP- $\beta$ -galactosidase fusion protein specifically in seam cells driven by an uncharacterized promoter (Terns et al., 1997). (B) Nomarski images of mutant and rescued embryos. Expression of the *frk-1* coding region from a pan-epidermal promoter, *elt-1p*, restores enclosure and elongation to homozygous *mDf7* embryos. Expression in non-seam epidermis only, using the *elt-3* promoter, is not sufficient to allow enclosure or elongation. (C) Genetic model for the pathway of epidermal differentiation, showing position of FRK-1 action based on gene expression data.

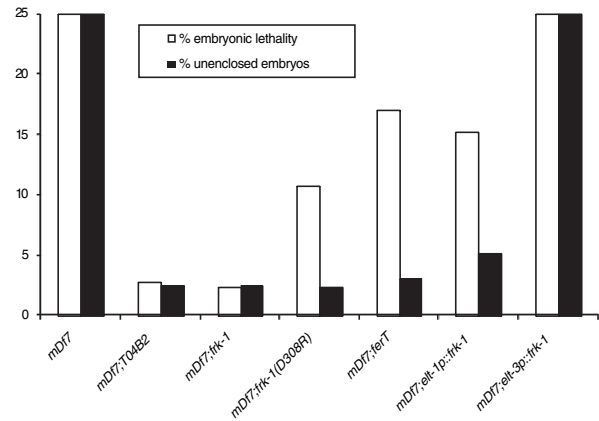
that in wild-type embryos until the onset of ventral migration, at which point these cells either initiated ventral migration and then retracted dorsally, or never initiated ventral migration and instead only contracted toward the dorsal side until development arrested in the mutants. This dorsal contraction of the epidermis (Fig. 1B) exposed normally internal tissues (e.g. intestine, pharynx and neurons) on the ventral surface. The epidermal cells appeared to be defective in adhesion, with most cells rounding up rather than flattening in a smooth sheet. The rounded-up appearance of these cells is unlikely to be a secondary consequence of the enclosure defect, but probably reflects a more direct role for FRK-1 in cell adhesion, as a large number of enclosure-defective mutants identified in several genetic screens (Chanal and Labouesse, 1997; Terns et al.,

1997) (J.H.R., unpublished) revealed that most embryos defective in ventral enclosure do not show the rounded-up epidermal cells. Other mutants defective in enclosure often look lumpy in appearance but retain some organization in the epidermis, as observed by the pattern of adherens junction staining (Chanal and Labouesse, 1997; Terns et al., 1997; Labouesse, 1997) (J.H.R., unpublished).

To determine whether the defects in epidermal morphogenesis in embryos lacking FRK-1 might be attributable to a failure to make the proper number of epidermal cells, we conceptually divided embryos into three sections and quantified the number of epidermal cells expressing nuclear LIN-26 protein, as described by Chanal and Labouesse (Chanal and Labouesse, 1997). We found that the epidermis in mutant embryos contained an approximately normal number of LIN-26-positive nuclei (an average of 56 nuclei for *frk-1(RNAi)*, 52 for *mDf7* homozygotes, and 53 in wild-type embryos;  $n > 50$  embryos for each genotype). However, as expected based on the time-lapse analysis of development, the positions of these cells was abnormal in the mutants, with most epidermal apical junctions clustering on the dorsal side of the embryo (Fig. 1C,F). Although the epidermis did not enclose the embryo in these mutants, epidermal cells contracted, suggesting normal assembly of the acto-myosin network involved in contraction and elongation (Costa et al., 1997). While epidermal cells in *frk-1(RNAi)* embryos did not align into discrete rows, as occurs in wild-type embryos, we found that the epidermis did organize to some degree in some *mDf7* embryos (compare Fig. 1B,E).

The discrepancy between the two mutants may be attributable to partial rescue of *mDf7* by maternally contributed FRK-1; such maternal contribution is expected to be eliminated by RNAi. At least one other protein involved in embryonic morphogenesis, the cadherin HMR-1, shows a similar phenomenon: homozygous *hmr-1* embryos produced by heterozygous mothers are only partially defective in enclosure, whereas a substantial fraction of *hmr-1(RNAi)* embryos arrest without any signs of enclosure (Raich et al., 1999). We obtained evidence that maternally provided FRK-1 is required for embryogenesis by specifically eliminating it in *frk-1(RNAi)* homozygous embryos that were rescued zygotically with the mammalian *ferT* gene (see below), which is not susceptible to *frk-1(RNAi)* owing to the highly divergent DNA sequences of the two genes. All embryos specifically lacking maternal FRK-1/*FerT* function arrested with only 200–300 cells, far short of the number of cells required for enclosure. Elimination of FRK-1 by RNAi in wild-type embryos resulted in early arrest in a small fraction (~5%) of the embryos. Thus, the maternal FRK-1 contribution in *mDf7* probably functions both in cell proliferation in early embryos and in organizing the epidermis before enclosure in later embryos.

Analysis of *frk-1* mutant embryos demonstrated that FRK-1 function is required for late stages of epidermal differentiation. Early markers of epidermal differentiation, including the apical junction protein AJM-1 and LIN-26, a transcription factor expressed in all epidermal nuclei, were both observed on the dorsal side of the embryo. Two markers specific for late stages of differentiation of the lateral rows of epidermal cells, the seam cells, failed to be expressed in terminal embryos (Fig. 3A). However, an earlier marker of seam cells, the engrailed homolog CEH-16 was always expressed in arrested embryos (not shown). Expression of four other markers that normally



**Fig. 4.** Quantification of rescue experiments with homozygous *mDf7* embryos. Percent embryonic lethality (empty bars) and enclosure defective (filled bars) in progeny from *mDf7* heterozygotes is shown. All *mDf7* homozygous embryos (25% of the progeny for this fully recessive deficiency) fail to enclose and die. Both phenotypes are efficiently rescued with cosmid T04B2 or a fragment containing *frk-1*. The kinase-dead FRK-1(D308R) mutant, Mouse FerT, and *elt-1p*-driven *frk-1* all rescue enclosure effectively and embryonic lethality partially. Rescue of both phenotypes apparently requires expression in the lateral seam cells, as expression of *frk-1* from the *elt-3* promoter does not rescue *mDf7*. More than 300 embryos were scored for each independent array assayed in the rescue experiments.

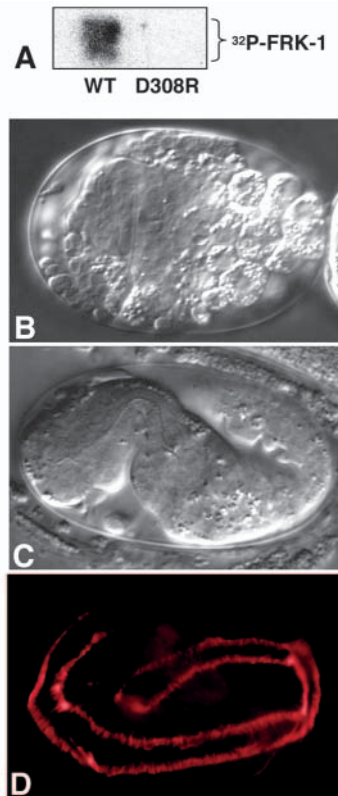
first appear as seam cells begin to differentiate was attenuated in arrested embryos (Fig. 3A). FRK-1 is also required for non-seam epidermal differentiation: *elt-3::GFP*, which is normally expressed in all epidermal cells except for seam cells, also showed attenuated expression in *frk-1* mutants (Fig. 3A). These results indicate that FRK-1 is required for completion, but not initiation, of epidermal differentiation.

#### Expression of FRK-1 in the epidermis is sufficient for morphogenesis

As FRK-1 is required to complete epidermal differentiation, we sought to determine whether expression of FRK-1 in some or all epidermal cells is sufficient to rescue the morphogenesis defect of *mDf7* homozygotes. We expressed FRK-1 throughout the epidermis by fusing its coding region to the promoter of the *elt-1* gene, which encodes the earliest acting transcription factor known to be required for specification of all epidermal cells (Page et al., 1997). This *elt-1p::frk-1* construct rescued the enclosure and elongation defects and embryonic lethality of *mDf7* embryos (Fig. 3B, Fig. 4). By contrast, expression of FRK-1 in all non-seam major epidermal cells, from the *elt-3* promoter (Gilleard et al., 1999), did not rescue the morphogenetic defect of *mDf7* homozygotes (Fig. 3B). However, the *elt-3p::frk-1* construct resulted in a failure in epidermal enclosure and embryonic lethality even in some wild-type embryos, raising the possibility that overexpression of FRK-1 in only some (non-seam) epidermal cells but not others (seam cells) results in defective signaling or adhesion in the epidermis leading to aberrant morphogenesis.

#### Mammalian FerT can substitute for *C. elegans* FRK-1

To investigate whether the function of Fer proteins in morphogenesis is evolutionarily conserved, we tested whether



**Fig. 5.** FRK-1 kinase activity is not required for enclosure and elongation. (A) In vitro assays demonstrate the autophosphorylation activity of FRK-1, which is eliminated in the FRK-1(D308R) mutant. The enclosure and elongation defects of *mDf7* homozygous embryos (B) are rescued by expression of the kinase-dead form of FRK-1 (C). The rescued embryos express the seam-cell-specific marker, NE2-1B4 (C), as is seen with rescue by wild-type FRK-1.

mammalian FerT could substitute for FRK-1. A mouse *ferT* cDNA was fused to the *frk-1* promoter and 3'-UTR and introduced into a strain carrying *mDf7*. We were able to isolate stable transgenic lines carrying this construct only when it was microinjected at very low concentrations, suggesting that high levels of FerT are toxic in *C. elegans*. However, several independent arrays containing the *frk-1p::ferT* transgene rescued the enclosure and elongation defects and embryonic lethality of *mDf7* (Fig. 2E,F), albeit somewhat less efficiently than FRK-1 (Fig. 4); thus, FerT family members perform a function that is conserved throughout metazoans.

### The kinase activity of FRK-1 is not essential for its morphogenetic function

We asked whether FRK-1, as suggested by its structure, is a bona fide kinase, and if so, if its essential role in embryonic morphogenesis is attributable to its kinase activity. As we do not know the normal substrates for FRK-1, we assayed its ability to autophosphorylate, an activity possessed by mammalian Fer. While FRK-1 lacks the longer N-terminal domain present in Fer, it was shown previously that removal of the N-terminal domain of Fer kinase does not block its autophosphorylation activity (Craig et al., 1999). Indeed, we found that in vitro translated FRK-1 was capable of efficiently phosphorylating itself in vitro (Fig. 5A). It was previously

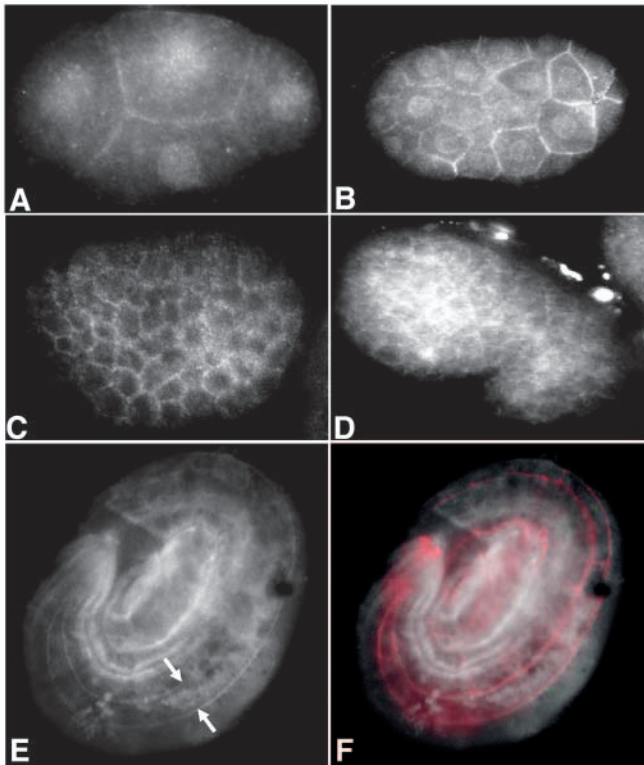
reported that mutation of a conserved aspartate residue to an arginine (D743R) eliminates the kinase activity of Fer (Cole et al., 1999). Consistent with these findings, introduction of the analogous point mutation in FRK-1 (D308R) completely abolished its autophosphorylation activity in the same assay (Fig. 5A).

Surprisingly, we found that this kinase-dead FRK-1(D308R) mutant was capable of rescuing *mDf7* homozygotes (Fig. 5C,D) to hatching, implying that the kinase activity of zygotic FRK-1 is not required for its role in enclosure and elongation. Rescued, hatched larvae arrested at various stages of larval development (L1-L4), most often with a ruptured epidermis somewhere along the ventral midline. The only evidence we obtained that the kinase activity of FRK-1 could perform any function in the embryo came from observations of embryos overexpressing FRK-1. While overexpression of wild-type FRK-1 from an extrachromosomal transgenic array in wild-type worms often results in defective morphogenesis and lethality, this effect was eliminated by the D308R mutation, suggesting that it may be excessive kinase activity per se that causes the overexpression phenotype. The lack of a phenotype in the mouse line expressing kinase-inactive Fer has been attributed to possible redundancy with another kinase or its non-essential function in adhesion and/or morphogenetic pathways (Craig et al., 2001). Our findings instead suggest that FRK-1 performs an essential structural, rather than enzymatic, role in morphogenesis.

### FRK-1 shows dynamic localization to the cell surface and nucleus

Mammalian Fer was reported to localize to nuclei during the S-phase of mitosis (Ben-Dor et al., 1999) and co-purifies with the chromatin fraction in nuclear extracts (Hao et al., 1991). However, the in vivo localization of Fer remains a source of debate, as others reported that it never localizes to the nucleus (Zirngibl et al., 2001). We found that FRK-1 in *C. elegans* showed a dynamic localization pattern during embryogenesis that could account for both observations. Using two independent antibodies elicited to two different FRK-1 peptides (see Materials and methods), we found that immunoreactive FRK-1 was initially present both in nuclei and at cell-cell contact points of all cells in early embryos (Fig. 6A,B). Expression later became restricted to epithelial cells, body wall muscle and the germline, including mature sperm. Staining was eliminated in *frk-1(RNAi)* embryos, confirming specificity of the antibody (not shown). Epidermal expression of FRK-1, which is apparently sufficient for morphogenesis (see above), requires ELT-1, a transcription factor required to generate all epidermal cells (Page et al., 1997): FRK-1 was seen only in body wall muscle cells in late *elt-1(zu180)* mutant embryos.

Localization of FRK-1 to nuclei correlates with phases of active cell division: nuclear localization was prominent in early embryos and the adult germline (Fig. 6A,B and not shown). The protein remained in the nucleus and at the cell surface until shortly before embryonic enclosure, at which stage most cells became mitotically inactive and nuclear FRK-1 became undetectable (Fig. 6C,D). In elongated embryos, FRK-1 was present in the cytoplasm and at the membrane of epidermal cells; cell surface staining was especially prominent in the seam cells (Fig. 6E,F). While we cannot exclude a nuclear

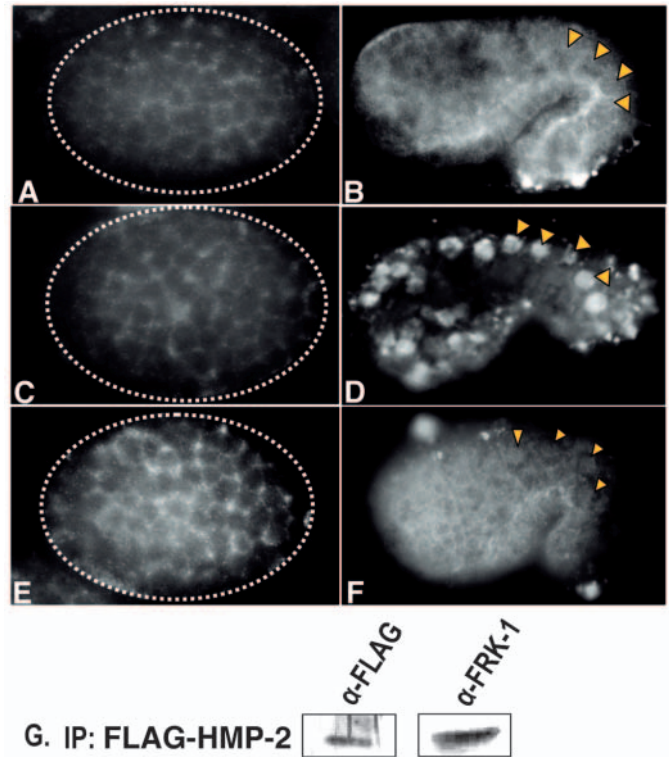


**Fig. 6.** FRK-1 is expressed ubiquitously in all cells during embryogenesis, and becomes nuclear excluded just before enclosure. FRK-1 is localized to the nucleus and at regions of cell-cell contacts in early embryos, as evident in 4-cell (A) and 40-cell (B) embryos. Immediately before enclosure, FRK-1 becomes nuclear-excluded, as seen in an embryo with approximately 550 cells (C). The protein remains nuclear-excluded throughout elongation (e.g. 1.25-fold stage; D), and cell surface localization is especially pronounced in the seam cells of elongated embryos (e.g. 3-fold embryo, E; overlay with MH27 apical junction antigen in F). Arrows point to apical junctions surrounding seam cells. Although both antibodies result in the same staining pattern, the images in this figure show localization using the antibody elicited to the FRK-1b peptide (see Materials and methods).

function for FRK-1 in morphogenesis, the protein was not detectable in nuclei during this phase of development, suggesting that it is the plasma membrane and cytoplasmic forms of the protein that underlie its essential morphogenetic function. Later, during larval development, FRK-1 reappeared in the nuclei of seam cells and in the nuclei of the developing germline, correlated with their active post-embryonic division. In the adult soma, FRK-1 stably localized to apical junctions of the intestine and somatic cells of the gonad (not shown).

#### Cell adhesion components are required for membrane localization and nuclear exclusion of FRK-1

Our findings that FRK-1 was nuclear-excluded and localized primarily to cell boundaries during embryonic enclosure and elongation suggest that it may perform its essential function in morphogenesis at the plasma membrane, perhaps by coordinating or regulating cell adhesion complexes. To assess possible interactions between FRK-1 and cell adhesion



**Fig. 7.** FRK-1 mislocalizes to the nucleus in mutants defective in cell adhesion components. In wild-type embryos, FRK-1 localizes to the plasma membrane of all cells immediately before enclosure (A), and remains nuclear-excluded throughout elongation (e.g. 1.5-fold embryo; B). In a mutant lacking the HMP-2  $\beta$ -catenin, FRK-1 becomes mislocalized to the nucleus, as is particularly evident in the seam cells (D). FRK-1 is similarly mislocalized to nuclei in a mutant lacking the PAT-3  $\beta$ -integrin (F), albeit to a less pronounced extent. In both cases, FRK-1 is localized to the plasma membrane before enclosure (C,E), demonstrating that the shift in localization takes place after enclosure is completed. Arrowheads indicate seam cells (B) or seam cell nuclei (D,F). Co-immunoprecipitation demonstrates that HMP-2 and FRK-1 physically interact in vitro (G). The Western analysis shows a single band at 45 kD when probed with the anti-FRK-1 antibody and a band at approximately 75 kD (HMP-2) when probed with anti-FLAG.

components, we examined its localization in mutants defective in cell adhesion mechanisms.

While Fer was not reported to directly interact with cadherin, it was found to interact with the cadherin complex via  $\beta$ -catenin when overexpressed in cultured cells (Rosato et al., 1998). Previous studies demonstrated that components of the *C. elegans* cadherin complex, including the HMR-1 cadherin, the HMP-1  $\alpha$ -catenin and the HMP-2  $\beta$ -catenin, are required for proper enclosure and elongation during embryogenesis (Costa et al., 1998). We found that plasma membrane localization and nuclear exclusion of FRK-1 were normal in arrested *hmr-1(zu248)* or *hmp-1(zu278)* mutants. However, in *hmp-2(zu364)* mutant embryos, FRK-1 was dramatically mislocalized, becoming virtually entirely nuclear in most cells (Fig. 7D). Fer also interacts with the p120 catenin in cultured mammalian cells (Piedra et al., 2003). We observed that FRK-1 localized throughout cells, including in nuclei, in mutants defective for the *C. elegans* p120 catenin homolog, JAC-1 (not

shown). Thus, HMP-2 and JAC-1 are essential for membrane localization and nuclear exclusion of FRK-1, suggesting that these catenins may associate with FRK-1, mediate its association with the plasma membrane, and sequester it from the nucleus. Indeed, we found by co-immunoprecipitation experiments that HMP-2 and FRK-1 physically interacted in vitro (Fig. 7G). Moreover, FRK-1 is required for nuclear exclusion of HMP-2 (A.P.P. and J.H.R., unpublished). The failure of FRK-1 to be membrane localized, or its inappropriate activity in the nucleus, is likely to contribute to the morphogenetic defects in these mutants.

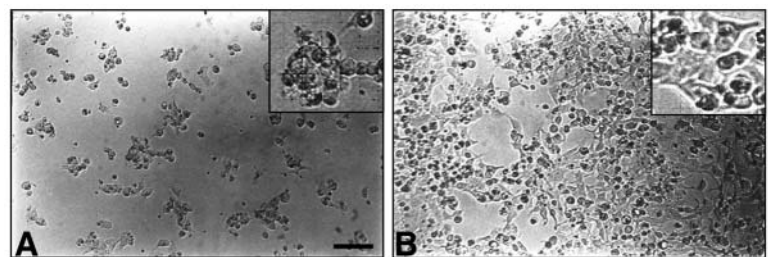
If plasma membrane-associated FRK-1 is essential for epidermal enclosure, these findings raise a paradox: while the absence of HMP-2 resulted in elimination of FRK-1 from the cell surface and its dramatic mislocalization to the nucleus, *hmp-2* mutants, unlike *frk-1* mutants, were nonetheless able to undergo epidermal enclosure. It is possible that an undetectable amount of FRK-1 remaining at the cell surface is sufficient to direct epidermal enclosure in *hmp-2(zu364)* mutants. However, we found that FRK-1 was localized at the plasma membrane before enclosure in *hmp-2(zu364)* mutant embryos and that its mislocalization to the nucleus occurred only once enclosure had initiated (Fig. 7C,D); thus, it is reasonable to suggest that by the time FRK-1 was absent from the plasma membrane in *hmp-2* mutants, it had already provided its essential function in epidermal enclosure. We considered the alternative possibility that FRK-1 may be required for epidermal enclosure only when  $\beta$ -catenin is present; however, this appears not to be the case, as we found that *frk-1; hmp-2* double mutants, like the *frk-1* single mutant, failed to enclose (not shown).

The integrin adhesion system is essential for normal morphogenesis in *C. elegans* (Gettner et al., 1995), and in vitro experiments suggest that Fer participates in cross-talk between the cadherin and integrin complexes (Arregui et al., 2000). We found that normal FRK-1 localization required the  $\beta$ -integrin homolog, PAT-3: in *pat-3(st564)* mutant embryos, FRK-1 partially mislocalized to the nucleus of epidermal cells (Fig. 7F). A startling aspect of this result is that PAT-3 was expressed only in the body wall muscle of *C. elegans* and was not detectable in the epidermis (Gettner et al., 1995). Thus, these results suggest cell non-autonomous signaling across the basement membrane that separates the body wall muscle from the epidermis. This effect might be

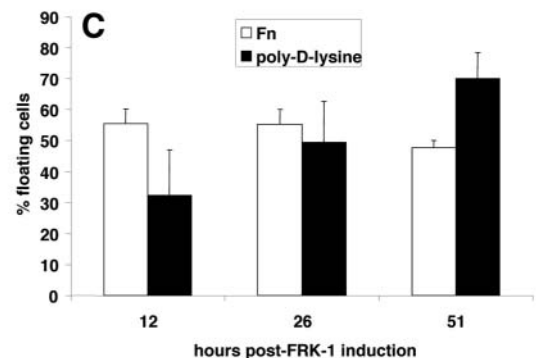
attributable to general disruption of the basement membrane adhesion system, or instead to a more specific, cell-non-autonomous role of muscle-expressed integrin on plasma membrane complexes containing FRK-1 in epidermal cells. We found that FRK-1 localization was not conspicuously altered in two mutants defective in different basement membrane collagens, *let-2* (Sibley et al., 1993) and *emb-9* (Guo et al., 1991), suggesting that the effect of the PAT-3  $\beta$ -integrin on FRK-1 localization in epidermal cells is not the result of a general perturbation of the basement membrane per se. The effect of the *pat-3* mutation on FRK-1 localization also raises the possibility that the elongation defect in this mutant may result in part from disruption of FRK-1 function.

### Expression of FRK-1 disrupts cell adhesion in cultured mammalian cells

The phenotypes of *frk-1* mutants and the localization of FRK-1 suggest that it may function at the plasma membrane to modulate cell adhesion complexes essential for morphogenesis. The finding that FRK-1 requires HMP-2  $\beta$ -catenin to localize to the plasma membrane raises the possibility that HMP-2 might be a substrate for FRK-1. Excessive phosphorylation of  $\beta$ -catenin has been shown to destabilize the cadherin complex, and thus loss of adhesion in cultured cells (Miravet et al., 2003). To investigate whether FRK-1 expression can perturb cell adhesion, we tested the effect of FRK-1 expression on the adhesive properties of mammalian cells. We found that expression of FRK-1 in HEK293 cells caused them to lose their adherent properties; FRK-1-expressing cells rounded up and detached from the culture plate (Fig. 8A). This effect was similar to that seen when full-length, mammalian Fer is overexpressed in adherent cell lines (Rosato et al., 1998). Adhesion assays showed that as much as 60% of the FRK-1 overexpressing cells detached from the substrate when compared with cells expressing the *LacZ* control; when normalized for the number of cells expressing the gene, based on parallel studies with *LacZ* transfections, this suggests that most or all of the FRK-1-



**Fig. 8.** Expression of FRK-1 causes loss of adhesion in cultured human cells. FRK-1 was transiently expressed from an ecdysone-inducible promoter in HEK293 cells. Cells expressing FRK-1 rounded up and detached from the laminin substrate (A), whereas expression of  $\beta$ -galactosidase from the same promoter had virtually no effect on adhesion of the cells to the substrate (B). Insets show higher magnification phase contrast micrographs. (C) Quantification of cell adhesion defects resulting from FRK-1 expression. The number of cells remaining attached to substrate was quantified as described in Materials and methods and the values normalized for transformation frequencies (~50%) and to the values determined from cells transformed with the empty pIND vector (defined as 100% attached, or 0% floating). Experiments were performed with either fibronectin (Fn) or poly-D-lysine as a substrate. Scale bars: 50  $\mu$ m.





expressing cells were defective in adhesion. Loss of adhesion was substrate-independent, as the cells acted in a similar manner whether cultured on plastic, fibronectin or poly-D-lysine. The detached cells did not lose their adhesive properties as a result of cell death, as Trypan Blue exclusion revealed that most (75-100%) of the detached cells remained alive, regained their ability to attach to the substrate and continued growing once FRK-1 expression, under control of an inducible promoter, was subsequently shut off (not shown).

## Discussion

Most studies of mammalian Fer have been performed on cultured cells, and the *in vivo* function of Fer-like proteins during development has not been determined. We report the first *in vivo* study of a Fer non-receptor tyrosine kinase family member. Our findings demonstrate that *C. elegans* FRK-1 is essential *in vivo* for morphogenesis and differentiation of the epidermis during embryonic development in *C. elegans*. These studies also suggest an earlier role for FRK-1, correlated with its localization to nuclei. Expression of FRK-1 in the epidermis was sufficient for its essential function during embryonic morphogenesis, suggesting that it may act autonomously within epidermal cells to regulate their proper adhesion and migration. While we have shown that FRK-1, like its mammalian counterpart, possesses kinase activity, surprisingly its essential role in enclosure of the embryonic epidermis is apparently independent of its kinase function. FRK-1 localized primarily to the cell surface during epidermal morphogenesis, where it probably interacts with cell adhesion components; indeed, the HMP-2  $\beta$ -catenin was required for cell surface localization (and nuclear exclusion) of FRK-1, and FRK-1 interacted with HMP-2 *in vitro*. Further evidence for the involvement of FRK-1 in cell adhesion systems is the unexpected finding that a  $\beta$ -integrin was required apparently cell-non-autonomously for proper FRK-1 localization and that expression of FRK-1 in cultured mammalian cells interfered with their adhesion.

The essential role of FRK-1 in morphogenesis appears to be functionally conserved across metazoans, as mammalian FerT could substitute for FRK-1 during enclosure and elongation in *C. elegans*. While FRK-1 is most similar in sequence to FerT, its pattern of expression broadly in the embryo, as well as in sperm, resembles that of both Fer, which is expressed ubiquitously, and FerT, which is specific for spermatocytes, in *Drosophila* and mammals. Fer contains an amino-terminal sequence not present on FerT, which determines its phosphorylation state (Craig et al., 1999) and which may control its cellular functions (Orlovsky et al., 2000). This sequence is apparently dispensable in *C. elegans*: unlike *Drosophila* and mammals, which both produce a full-length and truncated version of Fer, *C. elegans* appears to contain only a single Fer-like protein that apparently performs the function of both Fer forms in other animals.

### Kinase independence of FRK-1 function

Our finding that the kinase activity of FRK-1 was not required for embryonic morphogenesis suggests that the critical action of FRK-1 in morphogenesis depends not on its enzymatic activity, but on its interactions with other proteins; for example, it may be required for assembly of complexes at the plasma

membrane that mediate dynamic changes in adhesion and cell movement. Similar to our findings with FRK-1, elimination of the kinase activity of the integrin linked kinase (ILK) does not abolish its function in cell adhesion: a form of *Drosophila* ILK that carries the same sequence change as one in human ILK shown to drastically reduce kinase activity rescues as efficiently as the wild-type protein (Zervas et al., 2001). The same mutation in the *C. elegans* ILK homolog, PAT-4, does not prevent its binding to UNC-112, a cytoplasmic attachment protein (Mackinnon et al., 2002). Both studies support a kinase-independent role for ILK as an adaptor protein that stabilizes integrin complexes and facilitates muscle attachment.

Our findings do not rule out an essential kinase-dependent function for FRK-1. Maternally contributed FRK-1 was apparently required for completion of early embryogenesis, based on its early expression and the apparent rescue of the early embryonic arrest by maternally, but not zygotically, provided FRK-1 product. The kinase activity of FRK-1 may well be integral to this early role for the protein. It is during early embryogenesis that FRK-1 was nuclear-localized and it is interesting to note that Fer has been shown to phosphorylate nuclear localized factors such as Stat3 (Priel-Halachmi et al., 2000) and the TATA element modulatory factor (Schwartz et al., 1998). The role for nuclear FRK-1 may be of particular importance, as it has been shown that Fer kinase is upregulated in proliferating prostate cancer cells (Allard et al., 2000), and that inappropriately phosphorylated Fer prevents progression beyond the G0/G1 phase of the cell cycle (Orlovsky et al., 2000). At least some of the early function of FRK-1 as a kinase may also relate to its role in maternally directed Wnt-type signaling, as we have found that FRK-1 acts in concert with multiple components of the Wnt pathway (A.P.P. and J.H.R., unpublished).

It is conceivable that FRK-1 does function as a kinase later in embryogenesis but this activity is non-essential for epidermal morphogenesis owing to the redundant function of another kinase. Such redundancy has recently been suggested for the mammalian non-receptor kinases Fer and Fyn, and has been invoked as an explanation for the mild knockout phenotypes of each of the two genes in mice (Craig et al., 2001; Stein et al., 1992). While the region deleted by *mDf7* included two genes, in addition to *frk-1*, encoding SH2 domain-containing tyrosine kinases, the debilitation of either gene by RNAi did not result in a conspicuous embryonic phenotype; it remains to be determined whether another kinase can function as a redundant partner with FRK-1 during embryonic morphogenesis.

### FRK-1 and the pathway for epidermal development and differentiation

In addition to its role in promoting epidermal morphogenesis, FRK-1 was required for late stages of epidermal differentiation. Although early epidermal markers, such as LIN-26, which is required for specification and maintenance of epidermal cell fates (Page et al., 1997; Quintin et al., 2001), and CEH-16, are unaffected by the loss of *frk-1* function, and the appropriate number of epidermal cells appears to be specified, later markers of both seam and non-seam cells were either absent or severely reduced in *frk-1* mutants. The requirement for FRK-1 in epidermal differentiation is not

likely to be a consequence of the enclosure defect per se, as other mutants defective in enclosure, such as those lacking cadherin complex components, make differentiated seam cells (Costa et al., 1998; Hoier et al., 2000). Our results suggest that FRK-1 acts downstream of the regulators that initially specify epidermis, i.e. ELT-1, LIN-26 and CEH-16, but upstream of factors that confer final stages of differentiation on different subsets of the epidermis, e.g. EGL-18 (Koh and Rothman, 2001) in seam cells and possibly ELT-3 (Gilleard et al., 1999) in non-seam epidermis (Fig. 3C). Further studies will help to elucidate whether FRK-1 acts directly on the regulatory machinery for epidermal differentiation or instead results in cellular rearrangements that are required for the function of such regulators.

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