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LAD-1, the *Caenorhabditis elegans* L1CAM homologue, participates in embryonic and gonadal morphogenesis and is a substrate for fibroblast growth factor receptor pathway-dependent phosphotyrosine-based signaling

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This study shows that L1-like adhesion (LAD-1), the sole *Caenorhabditis elegans* homologue of the L1 family of neuronal adhesion molecules, is required for proper development of the germline and the early embryo and embryonic and gonadal morphogenesis. In addition, the ubiquitously expressed LAD-1, which binds to ankyrin-G, colocalizes with the *C. elegans* ankyrin, UNC-44, in multiple tissues at sites of cell–cell contact. Finally, we show that LAD-1 is phosphorylated in a fibroblast growth factor receptor (FGFR) pathway-dependent manner on a tyrosine residue in the highly conserved ankyrin-binding motif, FIGQY, which was shown previously to abolish the L1 family of cell adhesion molecule (L1CAM) binding to ankyrin in cultured cells. Immunofluorescence studies revealed that FIGQY-tyrosine–phosphorylated LAD-1 does not colocalize with nonphosphorylated LAD-1 or UNC-44 ankyrin but instead is localized to sites that undergo mechanical stress in polarized epithelia and axon–body wall muscle junctions. These findings suggest a novel ankyrin-independent role for LAD-1 related to FGFR signaling. Taken together, these results indicate that L1CAMs constitute a family of ubiquitous adhesion molecules, which participate in tissue morphogenesis and maintaining tissue integrity in metazoans.

Introduction

The L1 family of cell adhesion molecules (L1CAMs)* includes L1, CHL1, NrCAM, NgCAM, and neurofascin in vertebrates (for review see Brummendorf et al., 1998), neuroglian in *Drosophila melanogaster* (Bieber et al., 1989), and C18F3.2 in *Caenorhabditis elegans* (Hutter et al., 2000; Teichmann and Chothia, 2000). These transmembrane proteins participate in numerous functions during nervous system development, including neurite growth and fasciculation, and axon guidance (for review see Brummendorf et al., 1998; Cohen et al., 1998; for review see Fransen et al., 1998). Both L1 and NrCAM are also expressed in nonneuronal tissues (for review see Kadmon et al., 1998; Wang et al., 1998) with L1 implicated in kidney-branching morphogenesis (Debiec et al., 1998) and immune response–induced remodeling of lymph node fibroblastic reticular matrix (Di Sciullo et al., 1998).

The extracellular portion of L1CAMs mediates cell adhesion via homotypic and heterotypic interactions to other adhesion and extracellular matrix proteins (for review see Hortsch, 1996). In contrast, the cytoplasmic tail mediates linkage to the spectrin-actin cytoskeleton by its interaction with ankyrins (Davis and Bennett, 1994). Ankyrins are a family of cytoplasmic proteins that couple distinct integral membrane proteins (including L1CAMs, Na^+/K^+ ATPase, anion exchanger, and the voltage-gated Na^+ channel) to the spectrin-actin cytoskeleton (for review see Bennett and Chen, 2001). L1CAMs collaborate with ankyrin in the establishment of specialized membrane domains such as axon initial segments and the nodes of Ranvier in vertebrates (for review see Bennett and Chen, 2001).

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*Abbreviations used in this paper: DTC, distal tip cell; FGFR, fibroblast growth factor receptor; GFP, green fluorescent protein; LAD-1, L1-like adhesion; LAD-1P, FIGQY-tyrosine–phosphorylated LAD-1; LAD-1NP, FIGQY-tyrosine nonphosphorylated LAD-1; L1CAM, L1 family of cell adhesion molecule; Vabs, variable abnormal.

Key words: L1CAM; *C. elegans*; UNC-44 ankyrin; tyrosine phosphorylation; cell migration

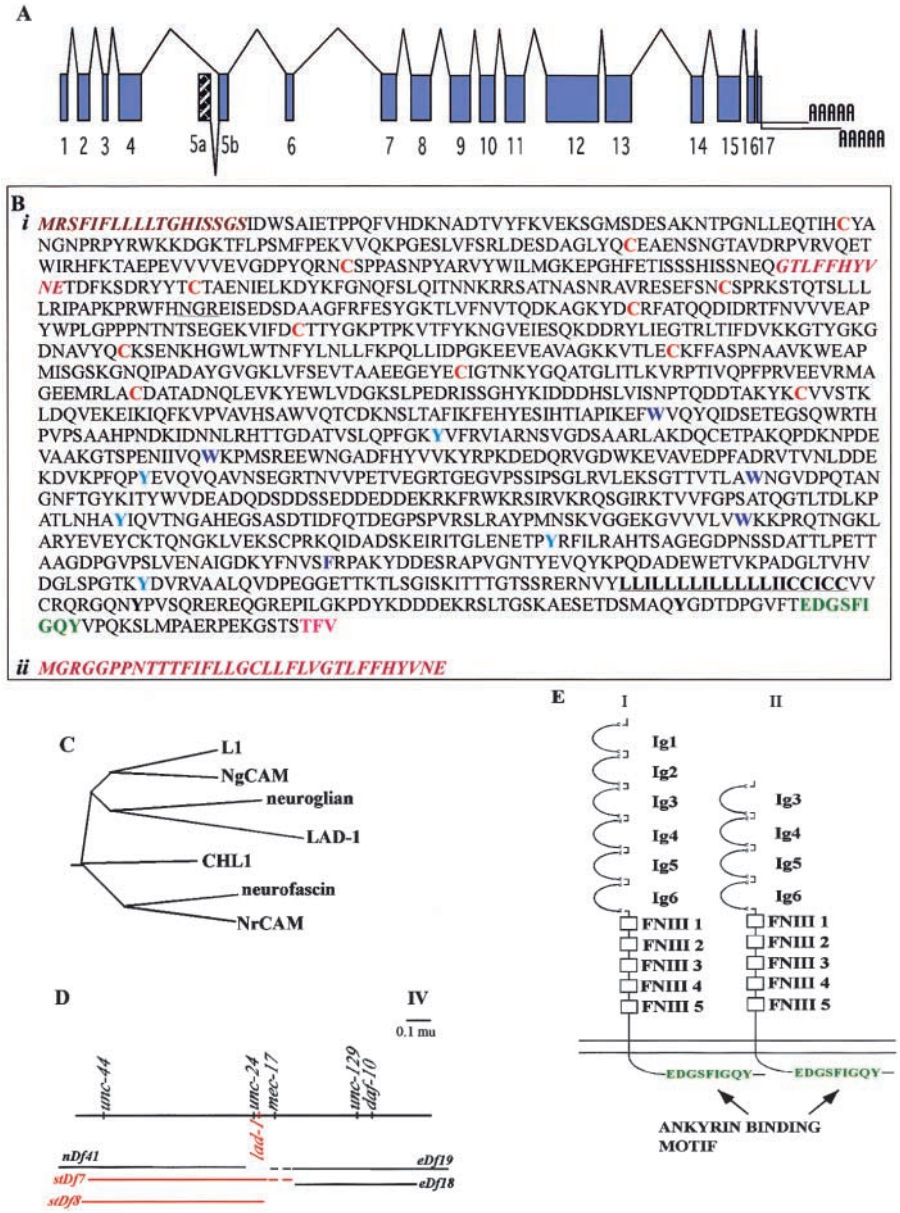

Figure 1. **LAD-1 is the sole *C. elegans* homologue of the L1CAM family.** (A) Intron-exon structure of LAD-1 (based on the cDNAs isolated). Blue boxes indicate exons. The hatched box indicates the unique 5′ coding region for the short cDNA isoform. Distinct 3′ UTRs and poly A tails of the cDNAs are also shown. Note that the shorter cDNA isoform lacks exons 1–4. (B, *i*) Predicted amino acid sequence for the longer *lad-1* cDNA. The italicized NH_2 terminus sequence in maroon indicates a predicted signal sequence peptide (SignalP; http://www.cbs.dtu.dk/services/SignalP). The ORF encodes the canonical L1CAM of six Ig domains (each Ig domain is flanked by red cysteines) followed by five fibronectin type III repeats. (Each repeat is flanked by a dark blue 5′ histidine or phenylalanine and a light blue 3′ tyrosine.) The transmembrane domain is bold and underlined, whereas the ankyrin-binding motif is highlighted in green. The last three amino acids marked in fuchsia are a consensus PDZ-binding motif. (B, *ii*) The pink italicized letters outline the start of the predicted signal sequence peptide for the second *lad-1* isoform. The pink italicized letters in B, *i*, completes this signal sequence peptide. The resulting cDNA encodes for a LAD-1 isoform, which lacks the first two Ig domains. The schematics for the two LAD-1 isoforms are shown in E. (C) A phylogenetic analysis of the L1CAM family members indicates LAD-1 is not an ortholog of any one vertebrate L1CAM family member. (D) Chromosomal deficiencies *stDf7* and *stDf8* remove the *lad-1* gene located on chromosome IV, whereas deficiencies *nDf41*, *eDf18*, and *eDf19* do not.

The L1CAM ankyrin association is abolished by phosphorylation of the conserved tyrosine residue in the ankyrin-binding motif, EDGSFIGQY (FIGQY), situated in the cytoplasmic tail (Garver et al., 1997; Tuvia et al., 1997). Neurofascin engineered to contain a FIGQY tyrosine-to-histidine (FIGQH) substitution mutation that was first identified in human L1, abolishes both FIGQY-tyrosine phosphorylation and ankyrin binding to neurofascin in cultured cells, suggesting either or both events are important for L1 function (Zhang et al., 1998). L1CAMs have also been linked to other signal transduction pathway molecules, which include src (Ignelzi et al., 1994), the Eph kinase Cek5 (Zisch et al., 1997), the fibroblast growth factor receptor (FGFR) (Saffell et al., 1997), and the mitogen-activated pathway kinase (Schaefer et al., 1999).

This paper reports the characterization of the sole *C. elegans* homologue of the L1CAM family, which we designated as LAD-1 (for L1-like adhesion). We show that LAD-1 is localized to the plasma membrane at sites of cell–cell contact in virtually all cells and that LAD-1 plays a role in embryonic and gonadal morphogenesis. We also demonstrate that the LAD-1 cytoplasmic tail, which contains the conserved ankyrin-binding motif, can bind mammalian ankyrin. Furthermore, we reveal that the *C. elegans* ankyrins, encoded by the *unc-44* gene (Otsuka et al., 1995), exhibit expression and localization patterns similar to that of LAD-1. Finally, this study presents in vivo evidence for FGFR-dependent tyrosine phosphorylation of LAD-1 in the conserved ankyrin-binding motif, FIGQY, and localization of FIGQY-tyrosine–phosphorylated LAD-1 (LAD-IP) in a complementary pattern to that of ankyrin at sites of mechanical stress. These results suggest alternate roles for L1CAMs as either general cell adhesion molecules coupled by ankyrins to the spectrin-actin skeleton or as mediators of FGFR signaling at specialized cell junctions.

Results

lad-1 is the sole *C. elegans* homologue of the vertebrate L1CAM family

The *C. elegans* genome contains a single gene homologous to the vertebrate L1 family of neuronal adhesion molecules (Hutter et al., 2000; Teichmann and Chothia, 2000) (Fig. 1, C and D). We isolated cDNAs encoding two LAD-1 isoforms by a combination of 5′ rapid amplification of cDNA ends and screening of a *C. elegans* mixed stage cDNA library (Fig. 1, A, B, and E). One isoform encodes an LAD-1 protein, which shares the canonical L1CAM structure (for review see Hortsch, 1996) of six extracellular Ig domains linked to five fibronectin type III repeats followed by a transmembrane domain and a cytoplasmic tail, containing the ankyrin-binding motif, FIGQY (Fig. 1 E). The second isoform, a result of an alternative splicing event, encodes a shorter LAD-1 protein that has a unique signal sequence peptide and lacks the first two Ig domains (Fig. 1, B and E).

LAD-1 is localized to the plasma membrane at sites of cell–cell contact in all cells throughout development

Immunoblots of mixed stage worm lysates with an affinity purified antibody generated against the LAD-1 cytoplasmic tail detected a complex pattern of LAD-1 products with apparent molecular weights of 200, 185, 120, and 65 kD. This variety of LAD-1 products suggested a combination of alternative splicing, posttranslational cleavage, and differential glycosylation events, modifications known to occur in the L1CAM family (for review see Hortsch, 1996; Hassel et al., 1997). The polypeptides ranging between 20 and 30 kD that were also detected by immunoblots represent non–LAD-1–related products, since they also reacted with ^{125}I-labeled protein A alone (Fig. 2, A, *i*).

Specificity of this antibody for LAD-1 was established using embryos lacking the *lad-1* gene. Arrested embryos homozygous for the chromosomal deficiency *stDf7*, which removes the *lad-1* gene (Fig. 1 D), did not show immunoreactivity with the LAD-1 antibody (Fig. 2, *iii*). Immunostaining of the adherens junction protein, JAM-1, with the control monoclonal antibody, MH27 (Francis and Waterston, 1985; Raich et al., 1999) showed that these embryos arrest after intestinal development. On the contrary, embryos homozygous for *nDf41,* a similar chromosomal deficiency, which removes a majority of the genes deleted by *stDf7* but leaves the *lad-1* gene intact (Fig. 1 D), showed wild-type levels and localization of LAD-1 (Fig. 2, A, *ii*).

LAD-1 visualized by immunofluorescence was expressed in all cells throughout development (Fig. 2, B–I), starting at the onset of embryogenesis with its expression increasing dramatically during embryonic morphogenesis (Fig. 2 C, arrow and arrowhead). Similar to the vertebrate and *Drosophila* L1CAMs, LAD-1 was highly expressed in the nervous system. LAD-1 is localized to the plasma membrane of the cell bodies (Fig. 2 G, long arrow) and axonal and dendritic processes (Fig. 2, C, D, and G, arrowhead) and in nonneuronal tissues such as the pharynx (Fig. 2 G, short arrow), hypodermis (Fig. 2 D, short arrow), body wall muscles (Fig. 2 F, short arrow), and the germline (Fig. 2 I).

LAD-1 was strikingly localized to the plasma membrane at sites of cell–cell contact as early as just before the two-cell stage (Fig. 2 B, arrow). Localized LAD-1 was not detected in one-cell embryos, which lack sites of cellular contact (Fig. 2 B, arrowhead). LAD-1 localization at cellular contact sites was especially evident in the nematode germline where LAD-1 was present at sites of oocyte–oocyte contact (Fig. 2 I, arrowheads) and in the membranes between germline nuclei (Fig. 2 I, arrows).

LAD-1 recruits cytoplasmic ankyrin to the plasma membrane in cultured cells and colocalizes with UNC-44 ankyrins in vivo at sites of cellular contact

L1CAMs interact with ankyrins via the FIGQY ankyrin-binding motif located in their cytoplasmic tails (Garver et al., 1997; Zhang et al., 1998), which is also present in LAD-1. To determine if LAD-1 binds ankyrin, we performed a binding/recruitment assay using human embryonic kidney 293 cell cultures (Fig. 3, A and B) as described (Zhang et al., 1998). Green fluorescent protein (GFP)–tagged 270 kD rat ankyrin$_G$ transfected into 293 cells was primarily localized in the cytoplasm (Fig. 3 A) (Zhang et al., 1998). Upon cotransfection with a chimera construct comprised of the LAD-1 cytoplasmic tail fused to the entire rat neurofascin extracellular and transmembrane domains, the cytoplasmic ankyrin$_G$–GFP redistributed to the plasma membrane, colocalizing with the neurofascin–LAD-1 chimeric protein (Fig. 3 B). This result showed that the LAD-1 cytoplasmic tail could bind and recruit cytoplasmic mammalian ankyrin to the plasma membrane.

The above result suggests that LAD-1 could participate in vivo in localizing *C. elegans* UNC-44 ankyrin, which is 70% identical in the membrane-binding domains to vertebrate ankyrins (Otsuka et al., 1995). We therefore compared the expression and localization patterns of UNC-44 to that of LAD-1. UNC-44, visualized by immunofluorescence using an UNC-44 polyclonal antisera (a gift from A. Otsuka, Illinois State University, Normal, IL) was ubiquitously expressed in the gastrulating embryo and in multiple adult tissues (Fig. 3, C–F). Similarly, UNC-44 was detected at high levels in the nervous system (Fig. 3, D and E, arrows) and in nonneuronal tissues such as the pharynx (Fig. 3 F, short arrow), body wall muscles (Fig. 3 F, arrowheads), and the hypodermis (unpublished data). Similar to LAD-1, UNC-44 ankyrin was localized to the plasma membrane at sites of cell–cell contact (Fig. 3, C–F). Interestingly, cytoplasmic ankyrin could be detected in the intestinal primordium (Fig. 3 C, long arrows). Unlike *lad*-1, unc-*44* expression as determined with this antibody first occurred during gastrulation after *lad-1* expression was first detected (Fig. 3 C). In addition, *unc-44* is not expressed in the germline, indicating absence of a maternally derived UNC-44 protein.

These data suggest that LAD-1 could act as a general receptor for UNC-44 ankyrin at the plasma membrane in multiple tissues. Based on this hypothesis, mislocalization of LAD-1 was not expected in the absence of UNC-44. Indeed, in embryos homozygous for the chromosomal deficiency *nDf41*, which deletes *unc-44*, LAD-1 levels, and localization were unaltered (Fig. 2, A, *ii*). We were unable to

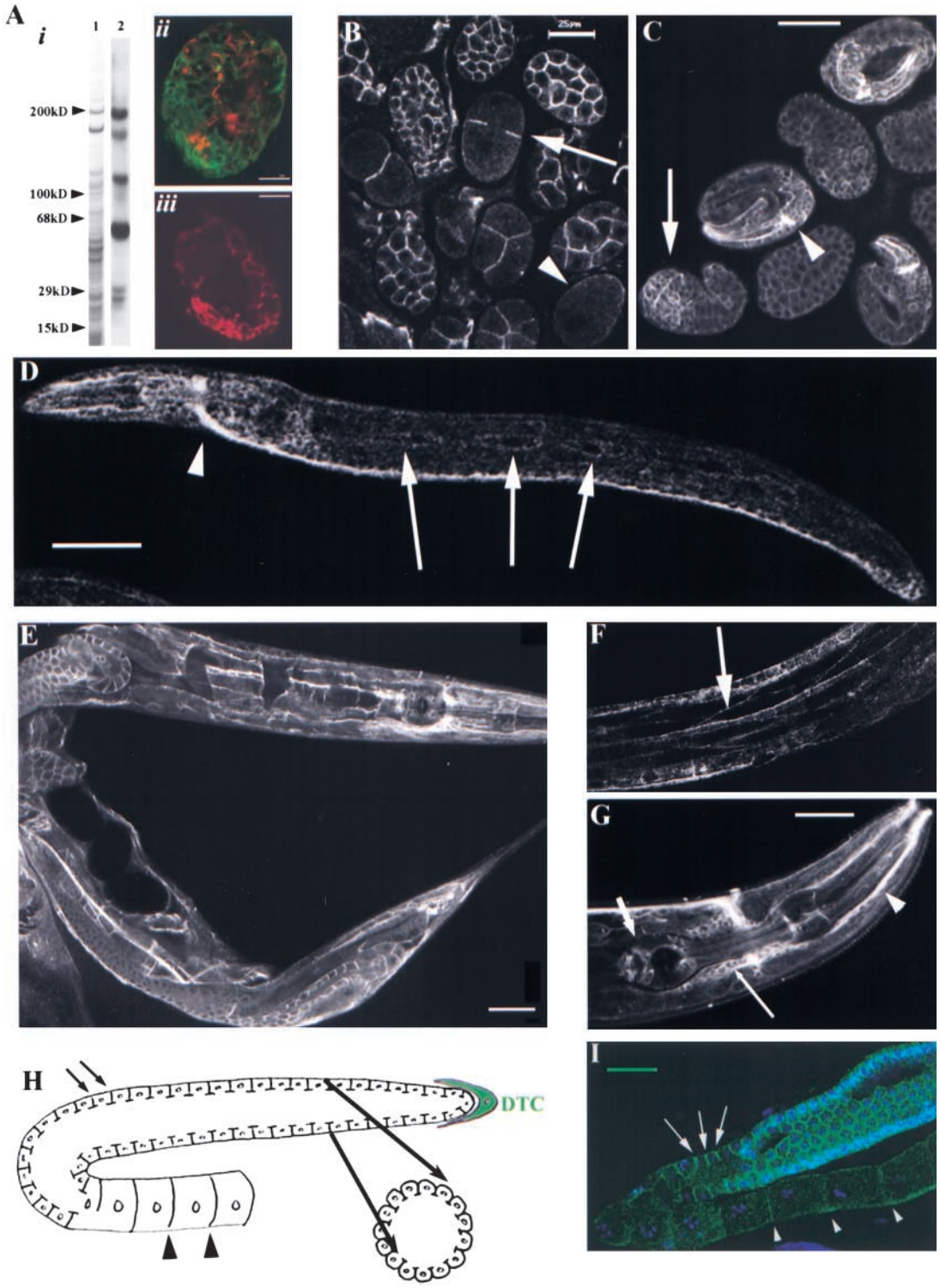

Figure 2. **LAD-1 is localized to the plasma membrane at sites of cell–cell contact in all cells throughout development.** (A, *i*) An immunoblot of total *C. elegans* lysates reveals four major LAD-1 polypeptides (200, 185, 120, and 65 kD) that were detected by a rabbit polyclonal antibody raised against the LAD-1 cytoplasmic domain (lane 2). The lower bands are not LAD-1 related, since they are also recognized by ^{125} I-labeled protein A (lane 3). (Lane 1) Coomassie blue staining of total *C. elegans* lysate. Equivalent lysate amounts. (*ii*) *nDf41* homozygous embryos, which contains the *lad-1* gene, show LAD-1 immunodetection (green) unlike *stDf7* homozygous embryos, which lack the *lad-1* gene; only control antibody staining of JAM-1 is seen in red (*iii*). B and C show immunodetection of LAD-1 in embryos. (B) LAD-1 is localized to sites of cell–cell contact, including the partial plasma membrane of a developing two-cell staged embryo (arrow). A one-cell embryo, which lacks contact sites, does not show LAD-1 localization (arrowhead). (C) LAD-1 is found in the developing nervous system (arrow) with robust LAD-1 staining in the nerve ring, the major *C. elegans* neuropil (arrowhead). D and E show LAD-1 expression in multiple tissues in a larva and adult, respectively. LAD-1 is present in the nerve ring and ventral nerve cord (D, arrowhead) and in the hypodermis (skin cells) (D, arrows), the plasma membrane of body wall muscles (F, arrow), neuronal cell bodies (G, long arrow), dendrites (G, arrowhead), and the pharynx (G, short arrow). LAD-1 is also present in the syncytial germline where its localization at contact sites is maintained. A schematic of the *C. elegans* germline is shown in H: arrowheads point to oocytes and arrows point to germ nuclei. The cross section of the cylindrical gonad reveals a single layer of germ nuclei around a cyto-

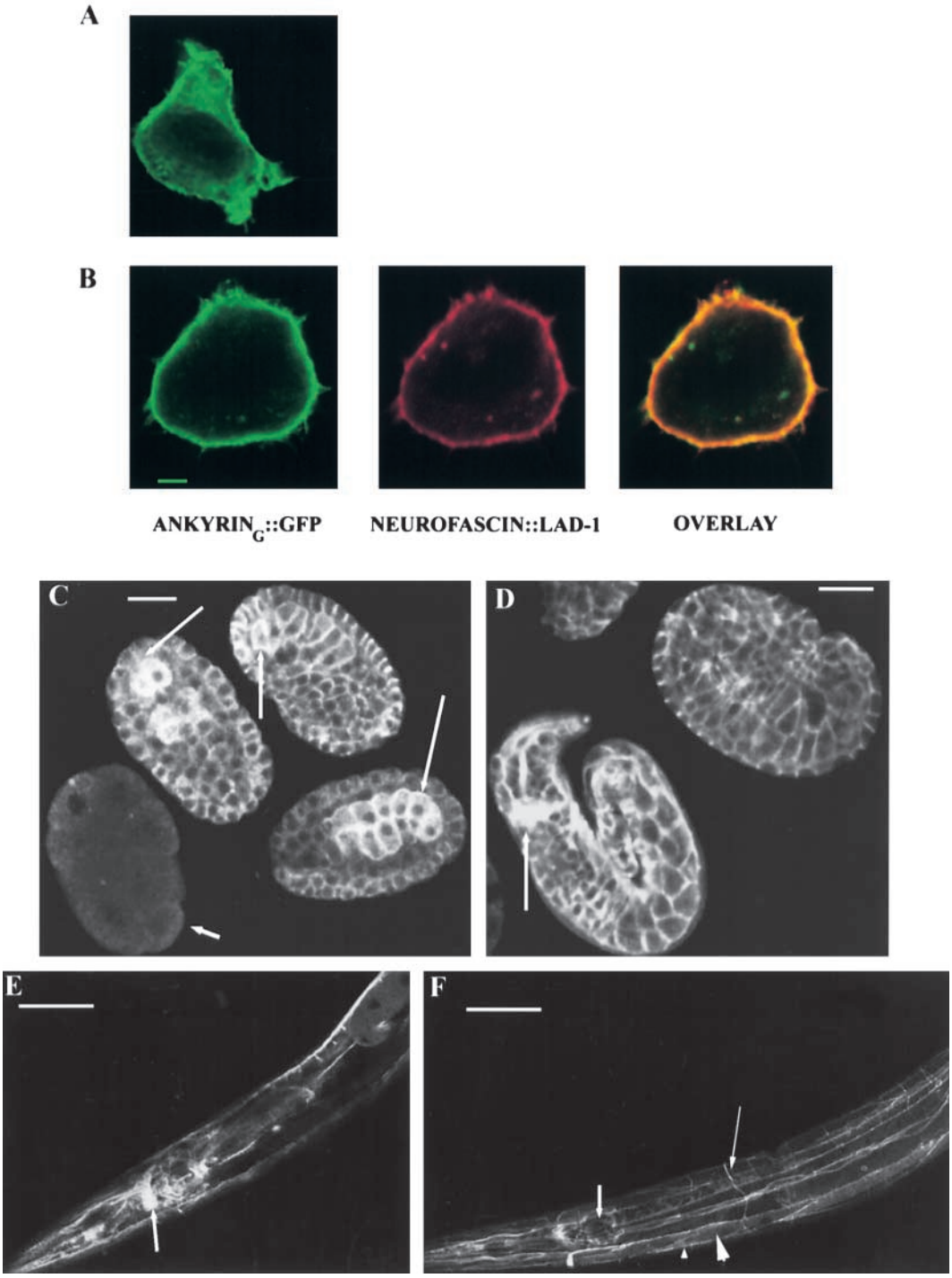

Figure 3. **The LAD-1 cytoplasmic tail binds and recruits mammalian ankyrin to the plasma membrane in cultured cells and similar to LAD-1 UNC-44 ankyrin is localized to the plasma membrane at sites of cell–cell contact in multiple tissues in *C. elegans*.** (A) Cytoplasmic localization of exogenous ankyrin in human embryonic kidney 293 cells transfected with murine GFP-tagged ankyrin$_G$ (Zhang et al., 1998). Upon cotransfection with a neurofascin–LAD-1 cytoplasmic tail chimera, cytoplasmic ankyrin$_G$ (green) is recruited to the plasma membrane, colocalizing with the L1CAM chimera (B, red). Immunofluorescence labeling of UNC-44 in multiple tissues in embryos as shown in C and D. The long arrows in C point to cytoplasmic ankyrin in the intestinal primordium. No *unc-44* ankyrin expression is evident in early embryos as indicated by the short arrow, which shows a 12-cell staged embryo. The arrows in D and E point to strong *unc-44* ankyrin expression in the nerve ring in the embryo and adult, respectively. In the adult (F), UNC-44 ankyrin is detected in the pharynx (short arrow), body wall muscles (small arrowhead), body wall muscle sarcomeres (large arrowhead), and commissural axons (long arrow). Bars: (A and B) 5 μm; (C and D) 10 μm; (E and F) 50 μm.

Figure 2 (continued)
plasmic core with T-shaped membranes between each nucleus. In a grazing section of the germline shown in I, LAD-1 is localized at oocyte–oocyte contact (arrowhead) and only the vertical part of the T-shaped membrane, which divides two neighboring germline nuclei (arrows). The top of the T does not show LAD-1 localization. Bars: (A) 20 μm; (B–I) 25 μm.

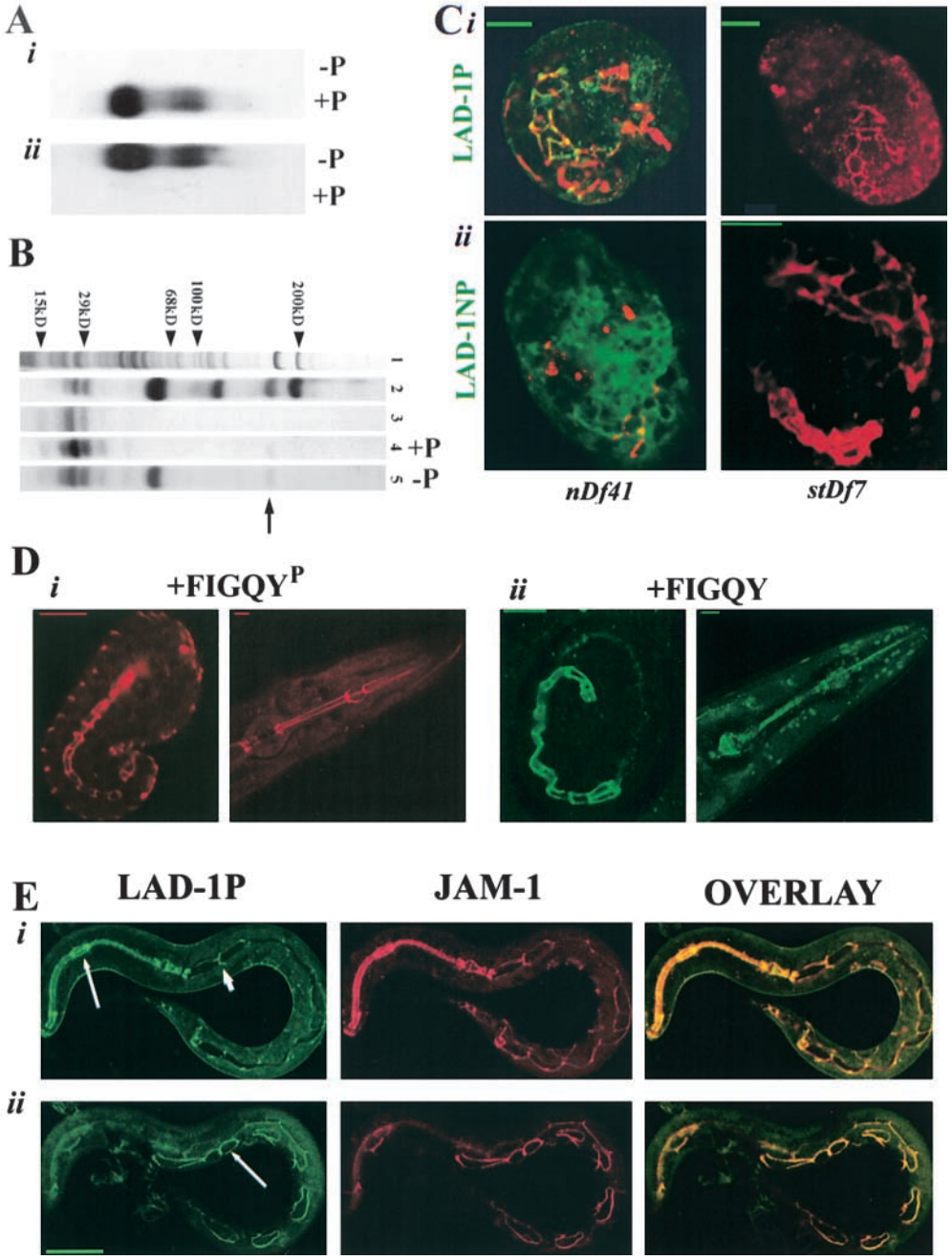

Figure 4. **Phosphorylation of LAD-1 at the conserved tyrosine residue of the ankyrin-binding motif, FIGQY, occurs in vivo, and LAD-1P localizes to polarized sites in epithelial tissues.** (A) Immunoblots of BSA-coupled LAD-1 peptides containing phosphorylated or nonphosphorylated FIGQY-tyrosine (+P and −P, respectively): *i*, LAD-1P antibody; *ii*, LAD-1NP antibody. (B) Immunoblots of total *C. elegans* lysates blotted with antibodies against the LAD-1 cytoplasmic tail (lane 2), LAD-1P (lane 4), LAD-1NP (lane 5), and ^{125}I-labeled protein A (lane 3). Lane 5 shows that the 185 and 65 kD LAD-1 polypeptides contain the FIGQY epitope; only the 185-kD product is phosphorylated (lane 4, arrow). Lane 1 shows a Coomassie blue–stained *C. elegans* lysate and relative molecular weight markers. (C) Control *nDf41* homozygous embryos, which contains the *lad-1* gene, show LAD-1P (*i*) and LAD-1NP (*ii*) staining (green) unlike embryos homozygous for *stDf7*, which lacks the *lad-1* gene; positive staining of JAM-1 is in red. (D) Preincubation of the LAD-1P antibody with the phosphorylated FIGQY peptide eliminated LAD-1P signal in stained animals; only JAM-1 immunostaining in red was detected (*i*). LAD-1P signal (green) is not displaced by preincubation of the LAD-1P antibody with the nonphosphorylated FIGQY peptide (*ii*). (E) Immunodetection of LAD-1P is localized in the pharynx and intestine (*i*, long and short arrow, respectively) and in the hypodermis (*ii*, arrow). LAD-1P (green) colocalized with JAM-1 (red) at the adherens junctions of hypodermal and intestinal cells. This colocalization is seen in the overlay panels as a yellow signal. Bar: (C and D) 10 μm; (E) 20 μm.

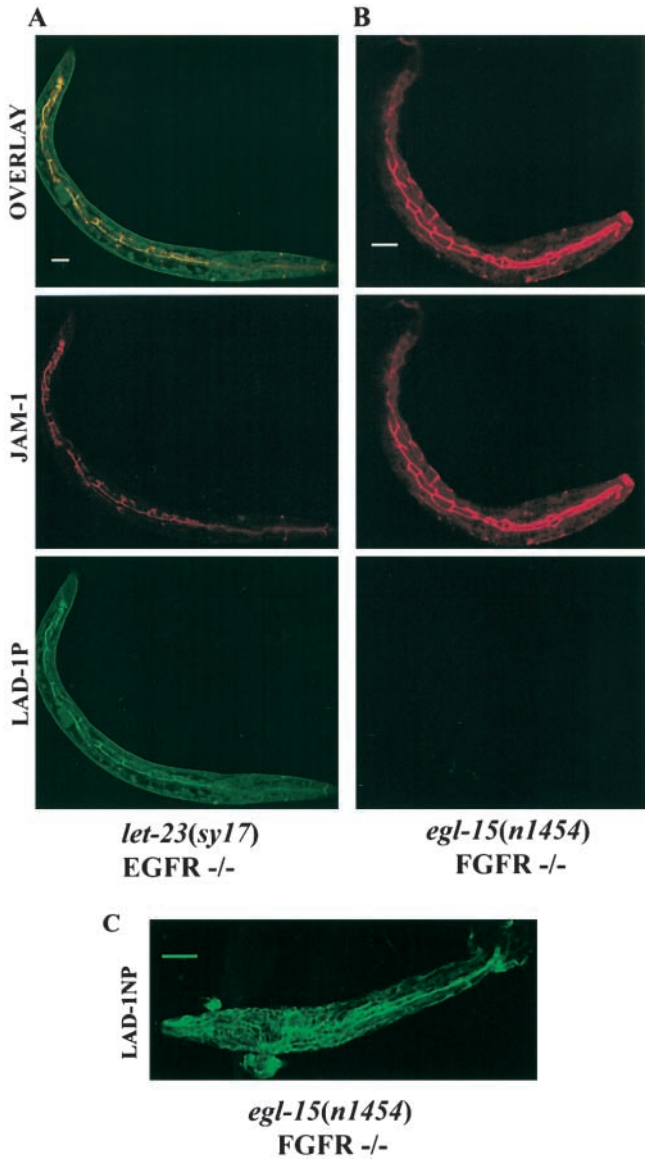

Figure 5. **LAD-1 FIGQY-tyrosine phosphorylation requires the FGFR pathway.** In *egl-15(n1454)* homozygous-null larvae (B), LAD-1P levels (green) are dramatically reduced compared with similarly arrested *let-23(sy17)* homozygous-null larvae (A). JAM-1 immunostaining is red. Levels and localization of LAD-1NP are not affected in the *egl-15* (*n1454*) homozygous animals (C). Bar, 10 μm.

determine if UNC-44 localization was affected by the loss of LAD-1, since *stDf7* removes both *unc-44* and *lad-1*.

LAD-1 is phosphorylated in vivo at the FIGQY-tyrosine residue of the ankyrin-binding motif

Neurofascin–ankyrin interactions are abolished in cultured cells upon phosphorylation of the FIGQY-tyrosine residue (Garver et al., 1997; Tuvia et al., 1997). We generated two affinity purified peptide antibodies able to recognize the LAD-1 FIGQY motif that was phosphorylated and nonphosphorylated on the tyrosine residue, respectively, to determine whether this tyrosine phosphorylation occurred in vivo.

Both antibodies were specific against LAD-1, since no LAD-1 immunofluorescence signal could be detected with either antibody in arrested *stDf7* homozygous embryos, which lack the *lad-1* gene (Fig. 4 C); only immunodetection of JAM-1 by the control antibody, MH27, was seen. On the other hand, embryos homozygous for the *nDf41* deficiency, which does not remove the *lad-1* gene, showed wild- type levels and localization of LAD-1. Moreover, the FIGQY sequence against which the antibodies were generated is not duplicated in the *C. elegans* genome. Taken together, these results indicated that these two peptide antibodies are specific for LAD-1. We next confirmed that the phosphorylated FIGQY-tyrosine LAD-1 antibody was indeed phosphotyrosine specific. Preincubation of the antibody with the tyrosine-phosphorylated FIGQY peptide before addition to methanol-fixed animals eliminated immunostaining but left the JAM-1 signal by the control antibody, MH27, intact (Fig. 4, D, *i*, red signal). On the contrary, preincubation of the antibody with the nonphosphorylated peptide did not eliminate the phosphorylated LAD-1 signal (Fig. 4, D, *ii*, green signal).

The nonphosphorylated LAD-1 (LAD-1NP) antibody recognized only the 185 and 65 kD LAD-1 polypeptides, (Fig. 4 B, lane 5), whereas the phosphorylated FIGQY-tyrosine antibody recognized only the 185 kD LAD-1 product (Fig. 4 B, lane 4). These results demonstrate that only the 185 and 65 kD LAD-1 products contain the ankyrin-binding motif, FIGQY, of which only the 185 kD LAD-1 product was phosphorylated. The faintness of the 185-kD band detected by the phosphorylated FIGQY-tyrosine antibody is consistent with relatively low levels of phosphorylated LAD-1 levels in *C. elegans* (Fig. 4 E). In brief, LAD-1 is indeed phosphorylated at the FIGQY-tyrosine residue in vivo, but this site-specific phosphorylation occurs differentially, suggesting distinct roles for the different LAD-1 products.

LAD-1 FIGQY-tyrosine phosphorylation is dependent on the FGFR pathway

We determined the tyrosine kinase pathway that was responsible for LAD-1 phosphorylation by screening the following tyrosine kinase-deficient or -null mutants for decreased levels of phosphorylated LAD-1 (LAD-1P): *vab-1* (Eph receptor kinase; George et al., 1998), *kin-8* (Ror kinase; Koga et al., 1999), *let-23* (EGFR; Aroian et al., 1990), and *egl-15* (FGFR; DeVore et al., 1995). By immunofluorescence, LAD-1P levels were dramatically reduced only in the *egl-15*–null background, whereas the other three kinase mutant backgrounds showed wild-type levels (Fig. 5, A and B). Expression levels and localization of nonphosphorylated LAD-1 appeared unaltered in the *egl-15*–null background (Fig. 5 C). This result indicates that LAD-1 FIGQY-tyrosine phosphorylation is dependent on the FGFR pathway and provides further evidence for the antibody specificity against phosphorylated FIGQY-tyrosine.

LAD-1P is localized in polarized epithelial tissues to sites distinct from LAD-1NP and ankyrin

LAD-1P (Fig. 4, green) by immunofluorescence is localized to polarized epithelial tissues such as the intestine and the pharynx (Fig. 4 E, *i*, short and long arrow, respectively), the hypodermis (Fig. 4 E, *ii*, arrow), and the vulva and the rectum (unpublished data). We compared the localization of

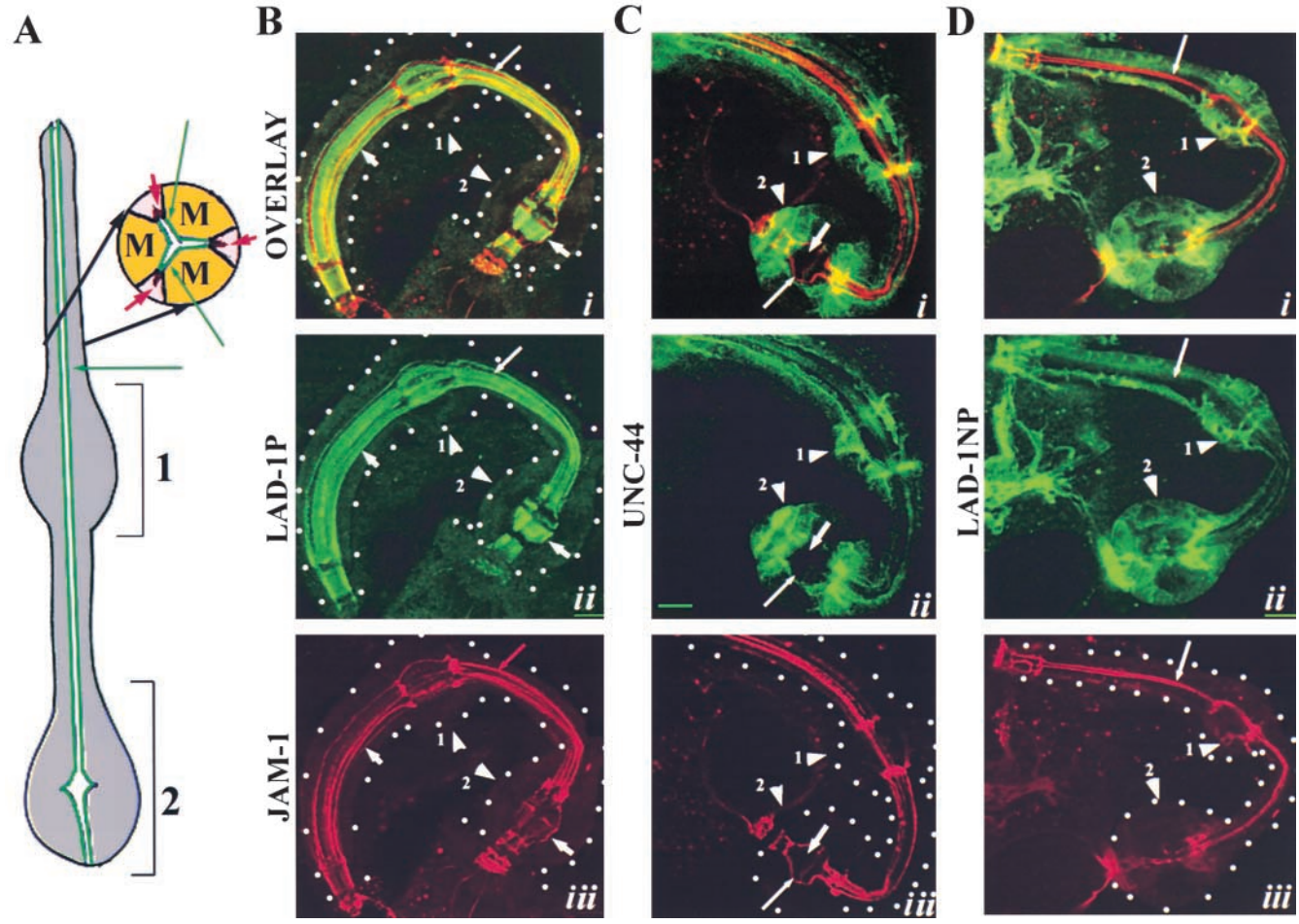

Figure 6. **Localization of LAD-1P is complementary to that of LAD-1NP and UNC-44 ankyrin in the pharynx.** (A) A schematic of the lateral view and cross-section of the pharynx. The pharynx has an anterior (1) and a posterior bulb (2). The cross-section shows the muscle cells (yellow and marked with "M") and the marginal cells (pink). The lumen-facing apical surface is outlined in green and indicated by the green arrow. The adherens junctions are in red in the cross-section and indicated by short red arrows. LAD-1P localization (B) is compared with those of UNC-44 ankyrin (C) and LAD-1NP (D) in the pharynx. The long arrow points to the adherens junction and the short arrow (C) points to the apical surface, whereas arrowhead 1 indicates the pharyngeal anterior bulb and arrowhead 2 the posterior bulb. The white dots in B, *i–iii,* C, *iii,* and D, *iii,* outline the entire pharyngeal organ. LAD-1P (green) appears to be localized to the pharyngeal apical surface (short arrows) outlined by the adherens junctions (red and long arrow) revealed by JAM-1 labeling. LAD-1NP and UNC-44 ankyrin does not colocalize with LAD-1P. Bar, 10 μm.

LAD-1P to that of LAD-1NP and UNC-44 ankyrin in the polarized epithelia, focusing on the pharynx (Fig. 6) and the intestine (Fig. 7). The pharynx is a single-cell–thick epithelial tube made of muscle and marginal cells, which are located with triradiate symmetry surrounding the pharyngeal lumen. The muscle and marginal cells are joined at the apices of the lumen by tight junctions thus dividing the membranes into apical and basal domains (Fig. 6 A, schematic). The apical surface (outlined in green) faces the lumen and secretes cuticle, whereas the basal surface is lined with basal lamina (White, 1988). In Fig. 6, B–D, the red signal indicates the staining pattern of JAM-1 at the adherens junctions, whereas the green signal indicates the respective staining patterns of LAD-1P, LAD-1NP, and UNC-44. In the pharynx, LAD-1P does not colocalize with JAM-1 (Fig. 6, B, *iii*, long arrow) but rather appears to be present in the pharyngeal apical surface (Fig. 6, B, *ii*, short arrows). However, because of the lack of apical pharyngeal markers further experiments have to be performed to confirm apical localization of LAD-1P. White dots were introduced to outline the pharynx in Fig. 6, B, C, *iii*, and D, *iii*, where it is difficult to discern the entire organ. On the other hand, LAD-1NP and UNC-44 ankyrin, although also expressed in the pharynx did not overlap with LAD-1P localization on the apical surface (Fig. 6, C and D).

Intestinal cells are connected to each other by adherens junctions, dividing the membranes into apical and basal domains (White, 1988). In Fig. 7, JAM-1 detection is shown in red, and LAD-1P, LAD-1NP, and UNC-44 detection is shown in green with the arrow pointing to the intestinal adherens junctions. In the intestine, LAD-1P was localized to the adherens junction (Fig. 7 A) unlike UNC-44 and LAD-1NP (Fig. 7, B and C), which were localized to the basolateral domains (Moorthy et al., 2000). The distinct localization of UNC-44 from LAD-1P in the pharynx and intestine is consistent with findings that ankyrin binding to neurofas-

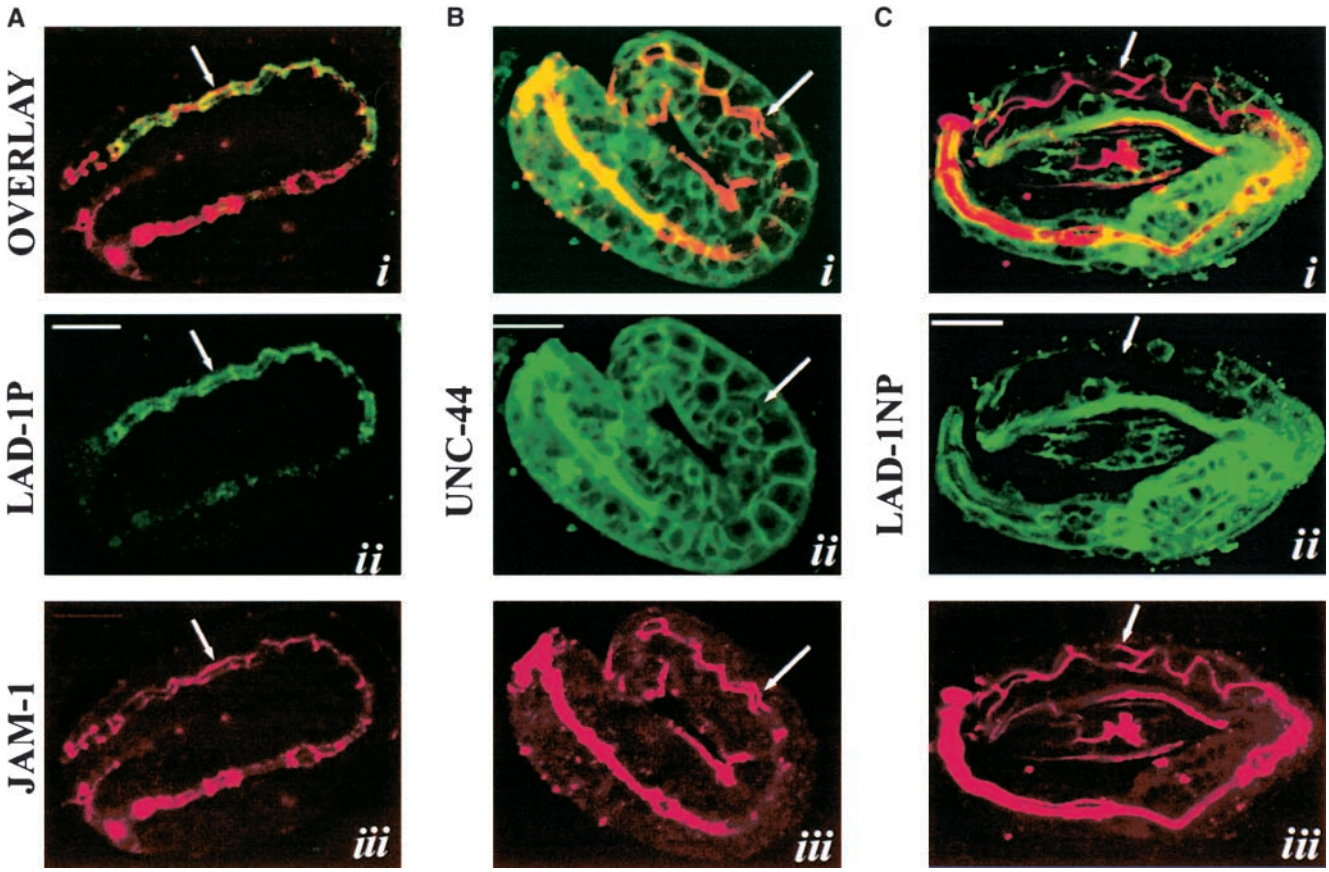

Figure 7. **LAD-1P (A, green) is colocalized with JAM-1 (red) at intestinal adherens junctions but is distinct from UNC-44 ankyrin (B) and LAD-1NP (C) localization.** Arrows in A–C point to the intestinal adherens junction. Bar, 20 μm.

cin in cultured cells is abolished upon FIGQY-tyrosine phosphorylation. Moreover, the complementary localizations of LAD-1P and LAD-1NP strongly suggest distinct roles for LAD-1, depending on its phosphorylation state.

Phosphorylated LAD-1 is localized in regularly spaced puncta at axon–body wall muscle junctions

The major longitudinal axon tracts in *C. elegans* include the ventral and dorsal nerve cords, which are flanked by the sublateral axon tracts (Fig. 8 A, schematic) (White et al., 1986). Sublateral axons grow along the body wall muscle basement membrane unlike the ventral and dorsal nerve cord. Commissural axons, originating from either the dorsal or ventral nerve cord, also grow along the body wall muscle basement membrane when they cross the sublateral axons (White et al., 1986).

LAD-1P was localized by immunofluorescence to regularly spaced puncta along the sublateral tracts (Fig. 8, B and C, arrow) and to the portion of commissural axons (Fig. 8, B and C, arrowheads) that are in contact with body wall muscles (Fig. 8 D, red outline). These puncta are not sites of synapse, since very few if any synapses are located along the sublateral nerve tracts and commissural axons overlaying the body wall muscles (White et al., 1986). Moreover, coimmunostaining for LAD-1P and the UNC-17 vesicular acetylcholine transporter (Duerr, J., and J.B. Rand, personal communication) did not reveal any colocalization. We hypothesize that these puncta are adhesion sites for maintenance of proper axonal positioning along the body wall muscle basement membrane.

The LAD-1 pathway functions in the general adhesion of cells in the early embryo and is involved in the embryonic and gonadal morphogenesis

To assess the functions of LAD-1, we initially performed RNA-mediated interference on LAD-1 using standard methods (Fire et al., 1998). RNA-mediated interference did not reduce LAD-1 levels and resulted in no obvious phenotype (unpublished data). However, transgenic animals expressing a putative dominant negative LAD-1 construct exhibit multiple defects that suggested LAD-1 roles in embryonic and gonadal morphogenesis. Expressed under the native LAD-1 promoter, this construct comprises the first three Ig domains that are secreted extracellularly. We hypothesize that the second Ig domain in the transgene, which is required for vertebrate L1 homophilic binding (Zhao et al., 1998; De Angelis et al., 1999), binds the extracellular domains of the endogenous LAD-1, consequently impeding endogenous LAD-1 function. Transgenic animals displayed normal levels and lo-

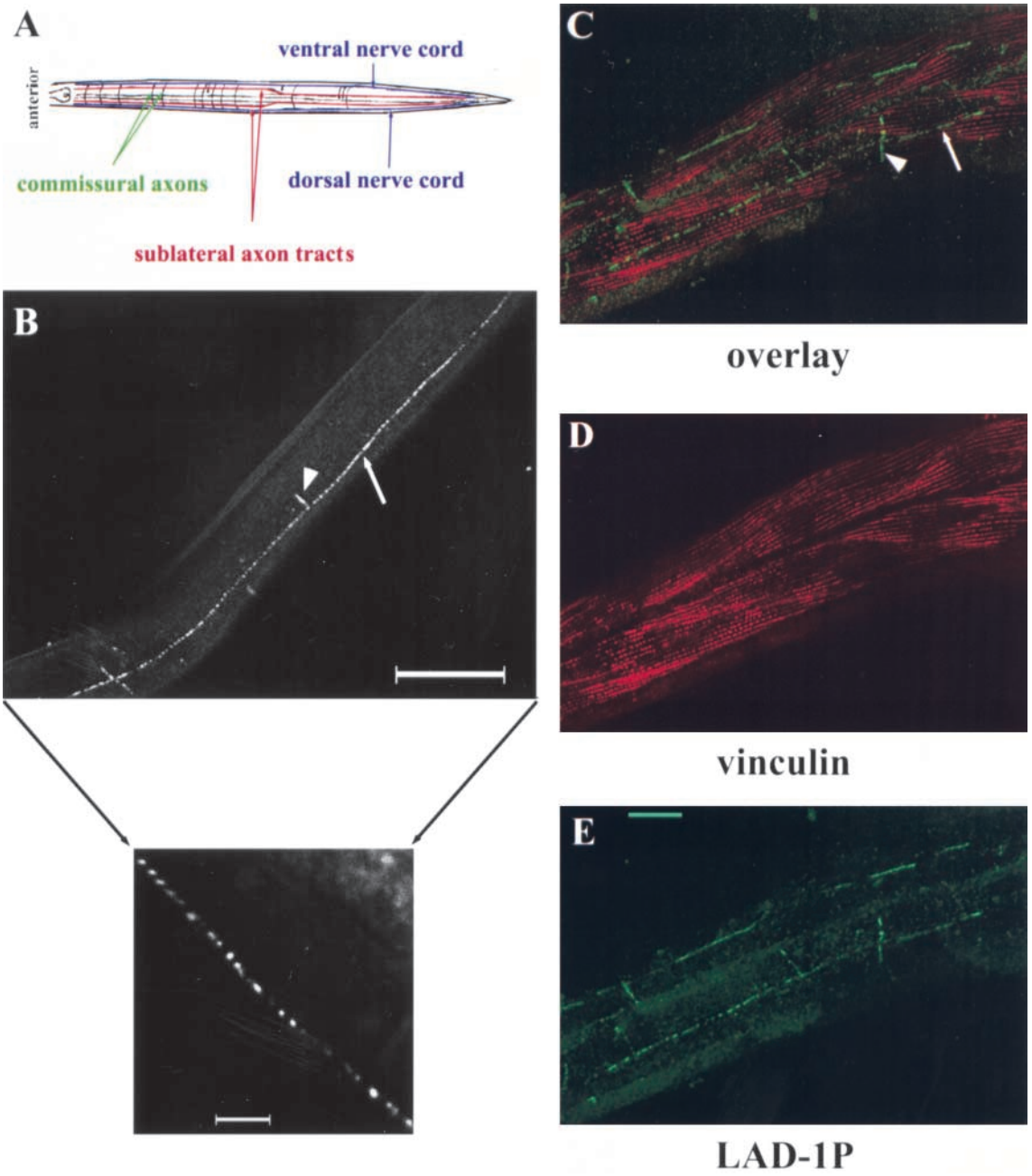

Figure 8. **LAD-1P is detected along the sublateral axons and commissural axons crossing the sublateral axons in a regularly spaced punctate pattern.** (A) A schematic of the *C. elegans* nervous system with two major nerve cords (ventral and dorsal) and smaller sublateral axon tracts on either side of the body. Sublateral axons grow along the body wall muscle basement membrane unlike the dorsal and ventral nerve cords. LAD-1P is localized specifically to sublateral axons (B, arrow) and the part of the commissural axons in contact with body wall muscles (B, arrowhead). A magnified portion of the axon tract shown in B highlights the regular punctate localization pattern of LAD-1P. (C and E) LAD-1P (green) is detected only in sublateral axons that overlay the body wall muscles (D, red, and E). The body wall muscle (red) is detected by MH24, a monoclonal antibody that recognizes vinculin. The arrow in C points to a sublateral axon tract, whereas the arrowhead points to the transversing commissural axon. Bars: (A) 50 μm; (B) 5 μm; (C) 20 μm.

calization of LAD-1 based on immunofluorescence (unpublished data), indicating that the expressed construct did not interfere with the regulation of endogenous *lad-1* expression.

Evidence that the Ig domain transgene interferes specifically with the endogenous LAD-1 pathway is based on the consideration that phenotypes conferred by dominant negative constructs are generally exacerbated by the reduction of the endogenous product targeted by the construct. Indeed, when the Ig domain transgene was crossed into hemizygous *lad-1* animals (see Materials and methods) essentially no cross progeny carrying both the deficiency and the transgene were identified. Instead, 83% of the transgenic cross progeny were wildtype for endogenous *lad-1*, whereas the remaining 17% were undetermined because the animals were either sterile or their development was prematurely arrested. The lack of a significant number of transgenic hemizygous

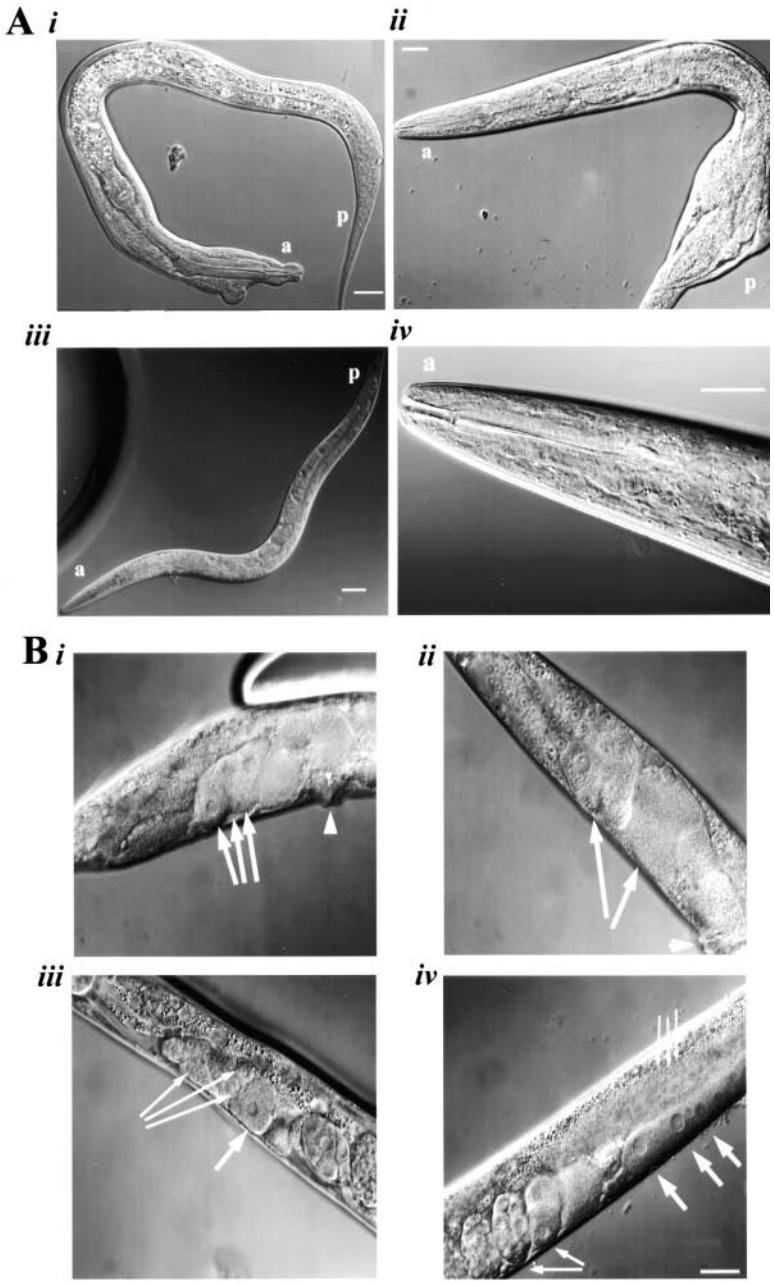

Figure 9. **LAD-1 plays a role in embryonic morphogenesis and in germline and early embryo development.** (A) Transgenic animals expressing a dominant negative form of LAD-1 display morphogenetic defects: anterior Vabs (*i*) and posterior Vabs (*ii*) (a, anterior; p, posterior). Wild-type animals are shown in *iii* and *iv*. (B) Apparent cell adhesion defects between oocytes result in mispositioned and misshapen oocytes, (*i*, arrows, and *iii*, short arrow). In addition, misplaced germ cells and immature-looking oocytes were detected (*iii*, long arrows). Apparent cell adhesion defects in early embryos result in misshapen cells and embryos, indicated by arrows in *ii*, that have yet to be laid through the vulva as designated by the arrowhead. The wild-type germline is displayed in *iv*, showing normal germline nuclei (long arrows), properly shaped and positioned oocytes (short arrows), and wild-type embryos (mid-sized arrows). Bar, 25 μm.

LAD-1 cross progeny indicates that the Ig domain construct in combination with reduced levels of endogenous wild-type LAD-1 induced lethality, arrested development, or sterility.

Dominant negative LAD-1 transgenic animals displayed pleiotropic phenotypes. 57% of the transgenic animals were variable abnormal (Vabs) (Fig. 9, A, *i* and *ii*), which indicates morphogenic defects, 34% showed uncoordinated movements and variable lethality, and 40% showed defects in gonadal morphogenesis. Striking defects in germline and early embryonic development were also observed. In the wild-type germline (Fig. 9, B, *iv*), embryos are situated close to the vulva (mid-sized arrows), and the oocytes are lined up in the proximal gonad (short arrows), whereas meiotic germline nuclei are located more distally (long arrows). Loss of contact between oocytes was observed in these transgenic animals, resulting in oocytes with altered shapes and sizes (Fig. 9, B, *i* and *iii*). In addition, many germ cell nuclei were completely enclosed by a membrane (Fig. 9, B, *iii*, long arrows), suggesting the presence of premature oocytes. Many dominant negative LAD-1 transgenic embryos also had cells with altered shapes and positions (Fig. 9, B, *ii*). In the early *C. elegans* embryos, the invariant cell position is critical for proper embryogenesis, due to cell–cell signaling (Schnabel and Priess, 1997). Because of the apparent loss of cell contact in these transgenic embryos, many of these embryos did not complete embryogenesis.

The transgenic animals also displayed abnormal gonad morphology. The wild-type hermaphrodite gonad consists of two U-shaped tubular arms that are formed by distal tip cells (DTCs) located at the tip of each arm, which migrate during larval development along a characteristic path that originates at the ventral midbody (Fig. 10 A, schematic) (Kimble and Ward, 1988). Fig. 10 C shows a mild defect

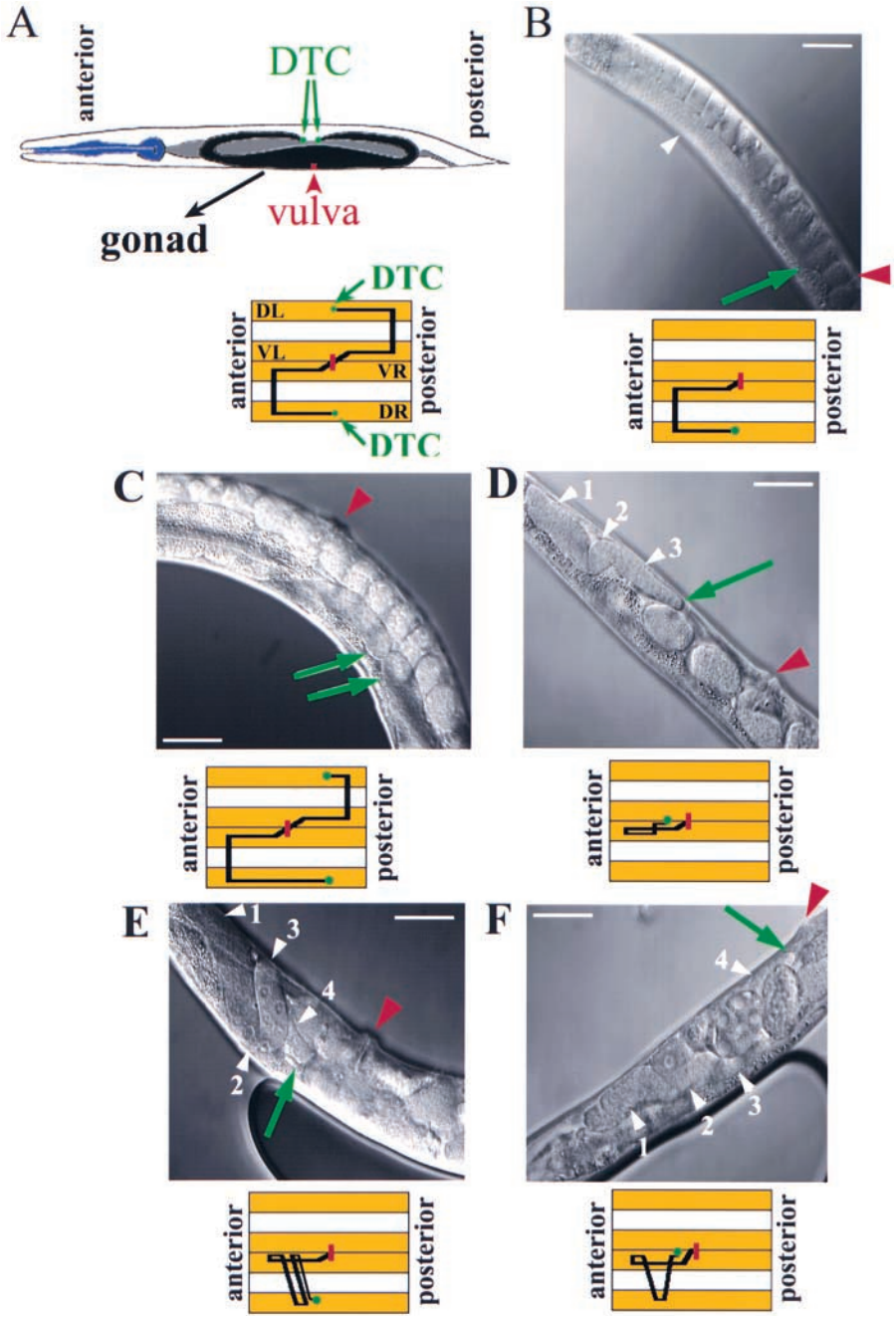

Figure 10. **LAD-1 is required for the gonad morphogenesis.** (A) A cartoon (not to scale) of a wild-type U-shaped gonad in *C. elegans*. Below each panel (A–F) is a schematic of an animal opened dorsally and spread out, displaying the migration path of the DTCs as seen in the corresponding DIC micrograph. In wild-type animals (A and B), the gonad arms, led by a DTC on each arm, grow in opposite directions along the ventral length of the body. Each DTC makes the appropriate turns to meet the other at the middle of the body. DL, dorsal left; VL, ventral left; VR, ventral right; DR, dorsal right; red bar, vulva. In C, one DTC migrated a little too far to meet the other DTC that did not migrate far enough. D–F show various gonadal morphogenesis defects due to abnormal cell adhesion and/or DTC migration. The green arrows in B–F indicate the DTC, the numbered arrowheads indicate the progression of the DTC migration, and the red arrowhead points to the vulva. Bar, 50 μm.

where the DTCs are positioned slightly posterior of the midbody, marked by the vulva (arrows indicate the ends of the gonad arms). More severe defects are shown in Fig. 10, D–F, where extra DTC turns were observed.

No changes in the levels and localization of either UNC-44 or LAD-1P were detected by immunofluorescence in dominant negative LAD-1 transgenic animals (unpublished data). These results indicated that the level of interference likely to occur extracellularly did not affect LAD-1 FIGQY-tyrosine phosphorylation or intracellular UNC-44 binding and localization. These results are consistent with recent data showing that ankyrin recruitment to the plasma membrane by vertebrate L1 is independent of L1 extracellular homo- and heterophilic interactions (Needham et al., 2001).

Discussion

This study reveals that LAD-1, the sole *C. elegans* L1CAM homologue, is ubiquitously expressed at sites of cell–cell contact and is required for germline development and embryonic and gonadal morphogenesis. In addition, both LAD-1 and ankyrin, which are shown to interact, are localized at sites of cellular contact in vivo. Moreover, tyrosine phosphorylation in the conserved ankyrin-binding motif FIGQY, which abolishes binding of neurofascin to ankyrin in cultured cells, does indeed occur in vivo in LAD-1. LAD-1P is localized to epithelial and axon–body wall muscle junctions and does not colocalize with either LAD-1NP or UNC-44 ankyrin, suggesting a novel ankyrin-independent pathway

for LAD-1. Finally, we show that this LAD-1 phosphorylation is dependent on the FGFR pathway. Our data, extrapolated to vertebrates, indicates that L1CAMs constitute a family of ubiquitous adhesion molecules, which participate in tissue morphogenesis and maintaining tissue integrity.

L1CAMs have been viewed primarily as neuronal adhesion molecules with few reports of nonneuronal expression and roles of L1CAMs in both vertebrates and *Drosophila* (Bieber et al., 1989; Debiec et al., 1998; for review see Kadmon et al., 1998; Wang et al., 1998). Our results suggest that L1CAMs function in multiple cell types early in embryonic development. The variable lethality and notched head phenotype displayed by the dominant negative LAD-1 transgenic animals are characteristic of mutants such as *vab-1*, which have defects in epithelial cell migration and cell adhesion (George et al., 1998). Likewise, the gonadal abnormalities may be due to defects in DTC migration or cell adhesion of gonadal arms to the body wall muscles or both. *Drosophila* neuroglian is expressed also in multiple tissues throughout development, and loss of neuroglian results in embryonic lethality (Bieber et al., 1989). Although the basis for lethality has not been determined, causes may include defects in cell adhesion and/or cell migration.

Based on the similar cellular localization of the ubiquitous LAD-1 and UNC-44 ankyrins in *C. elegans* and ability of LAD-1 to recruit ankyrin to cell membranes, we hypothesize that LAD-1, and by extrapolation vertebrate L1CAMs, can act as general ankyrin "receptors." Consistent with this idea is the finding that loss of neuroglian results in decreased levels of DANK2, the neuronal-specific ankyrin, in the *Drosophila* peripheral and central nervous systems (Bouley et al., 2000). In turn, ankyrins can cluster L1CAMs into microdomains due to multiple binding sites for the neurofascin (for review see Bennett and Chen, 2001); such clusters for L1CAMs would result in strong intercellular adhesion. Indeed, loss of ankyrin–neurofascin interaction results in reduced neurofascin-induced intercellular adhesion in cultured cells (Tuvia et al., 1997). Ankyrins are known to bind distinct tissue-specific integral membrane proteins, such as the Na^+/K^+ ATPase and the voltage-gated Na^+ channel and have nonoverlapping binding sites to these proteins and neurofascin (for review see Bennett and Chen, 2001). We predict that these proteins together with L1CAMs could act as tissue-specific ankyrin coreceptors to form microdomains required for tissue morphogenesis and function. Supporting this prediction is the loss of neurofascin/NrCAM and Na^+ channel coclustering at axon initial segment and nodes of Ranvier in ankyrin-G–deficient mice (for review see Bennett and Chen, 2001). By extrapolation, likely coreceptors for UNC-44 include the Na^+/K^+ ATPase encoded by *eat-6* (Davis et al., 1995) and the UNC-5 netrin receptor, which interacts with *unc-44* by genetic criteria (Colavita and Culotti, 1998).

A physical link between the FGFR and L1 via their Ig domains has been proposed (Doherty and Walsh, 1996; Saffell et al., 1997). However, subsequent studies showed that this cis interaction is unlikely due to structural hindrance of the putative binding motif, AAPYW (for review see Kamiguchi and Lemmon, 2000). More recently, genetic studies in *Drosophila* suggest that both the EGF and FGF receptors are downstream targets of neuroglian activity in growth cone decisions during sensory axon guidance (Garcia-Alonso et al., 2000). Our data indicates that LAD-1 is a substrate for FGFR pathway activity with preliminary data indicating that this LAD-1 phosphorylation occurs downstream of the FGFR-activated RAS pathway.

LAD-1P is localized in a polarized fashion in epithelial junctions and at axon–muscle junctions, which are sites lacking UNC-44 ankyrin in *C. elegans*. A similar localization of FIGQY-tyrosine–phosphorylated L1CAMs at epithelial adherens and neuromuscular junctions is conserved in both vertebrates and *Drosophila* (S. Jenkins, personal communication). *egl-15* FGFR is not known to be present at adherens junctions, and the ligand for the FGFR is not likely to be locally released in such a restricted manner. These considerations suggest the possibility that LAD-1 is phosphorylated elsewhere in the cell and transported to adherens junctions. We hypothesize that FIGQY-tyrosine phosphorylation creates a binding site for as yet unidentified proteins that could transport and localize LAD-1P to both epithelial and axon muscle junctions, sites where ankyrin is absent. Candidate proteins include PDZ proteins known for trafficking and localizing receptors to signaling pathways (Setou et al., 2000). The cytoplasmic tail of LAD-1 and several L1CAMs end in a consensus sequence for the PDZ-binding domain, and a recent finding indicates that neurofascin associates with the PDZ protein syntenin-1 (Koroll et al., 2001).

It is puzzling that LAD-1P, which does not bind ankyrin, is highly localized to the axon muscle and adherens junctions, sites that routinely experience mechanical stress, particularly since loss of ankyrin interaction leads to reduced L1CAM-induced intercellular adhesion in cultured cells (Tuvia et al., 1997). Proteins such as PDZ proteins, which participate in a variety of scaffolding and cytoskeletal linkage roles at epithelial and neuromuscular junctions (Dimitrakos et al., 1999) could bind, cluster, and associate LAD-1P to the actin cytoskeleton. Thus, LAD-1P could play a role in maintaining the integrity of both epithelial and axon muscle connections. Alternatively or additionally, LAD-1P may play a signaling role at cellular junctions.

FIGQY-tyrosine phosphorylation of LAD-1 may also create a binding site for as yet unidentified phosphotyrosine adaptor proteins, which in turn could mediate coupling to signaling pathways and cytoskeletal proteins such as microtubules or actin filaments. Preliminary data indicates that the microtubule-binding protein, doublecortin, binds FIGQY-tyrosine–phosphorylated L1CAMs (Kizhatil, K., personal communication). *zyg-8* is the *C. elegans* doublecortin homologue, which has roles in polarized division of the single-cell embryo. It will be of interest to investigate the possible interaction between *lad*-1 and zyg-*8*.

Taken together, the work on L1CAMs performed in *C. elegans*, *Drosophila*, and vertebrates suggest an expanded repertoire of roles for L1CAMs (Fig. 11). We propose that unphosphorylated LICAMs function as both cell adhesion molecules required for embryonic development and general ankyrin receptors at sites of cell–cell contact. Ankyrin reciprocally clusters L1CAMs and other ankyrin-associated integral membrane proteins to form specialized microdomains, which are required for proper tissue function. Additionally, microdomains of L1CAMs allow for strong

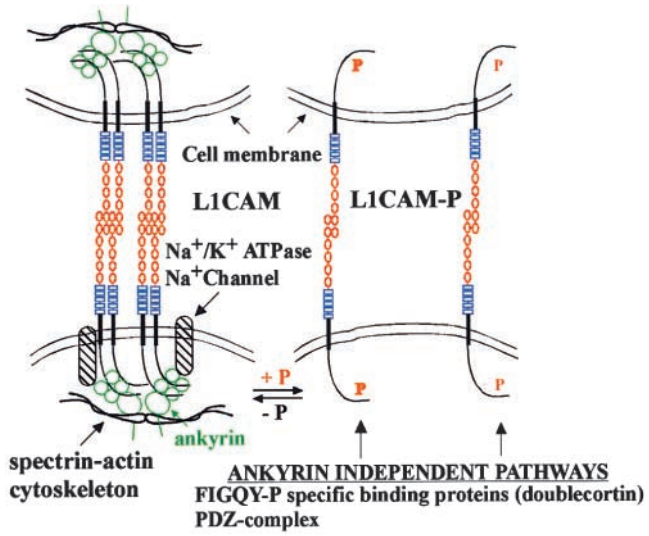

Figure 11. **A schematic model of L1CAM functions involving both unphosphorylated and phosphorylated states.**

intercellular adhesion. Thus L1CAMs may function to establish not only lateral membrane microdomains but also transcellular complexes. When phosphorylated, L1CAMs no longer associate with ankyrins and can interact with a different set of molecules, establishing a novel ankyrin-independent pathway related to FGFR signaling. These molecules are likely to include those that transport and localize phosphorylated L1CAMs to epithelial and axon muscle junctions where they may function to maintain integrity of those mechanically stressed sites. Phosphorylated L1CAMs may also act as signaling molecules by interacting with phosphotyrosine adaptor proteins, which in turn could mediate coupling to signaling pathways and cytoskeletal proteins. The roles of phosphorylated L1CAMs and the identity of signals promoting L1CAM phosphorylation, the kinase(s), and phosphatases involved, and the consequences of phosphorylation beyond loss of ankyrin-binding remain to be resolved. *C. elegans* and *Drosophila* with their potential for genetic analysis will provide powerful model systems to approach these questions with important implications for vertebrates.

Materials and methods

Reagents

DNA cDNA clone yk5b1 was from Y. Kohara (National Institute of Genetics, Mishuma, Japan); *C. elegans* mixed stage lambda ZAP cDNA library was from R. Barstead (Oklahoma Medical Research Foundation, Oklahoma City, OK); all cosmids were from the Sanger Center; plasmids pRF4 and pPD95.75 were from A. Fire (Carnegie Institute of Washington, Baltimore, MD); rat ankyrin$_G$–GFP and neurofascin–HA constructs were from Zhang et al., 1998; pEGFP-N1 was from CLONTECH.

Strains The following strains were sent from the *Caenorhabditis* Genetics Center: RW1333, RW1324, DA768, CB3823, CB3824, PS1423, and MT3460.

Antibodies Rabbit antiserum AO280 against the AO49 UNC-44 ankyrin regulatory domain (Otsuka et al., 1995) was provided by A. Otsuka, Illinois State University; monoclonal antibody MH27 recognizing JAM-1 and MH24 recognizing vinculin were from Francis and Waterston, 1985; monoclonal antibody 1403 recognizing UNC-17/VAChT was from J. Duerr and J.B. Rand, personal communications. Secondary antibodies conjugated with FITC and rhodamine were from Jackson ImmunoResearch Laboratories, Inc. DAPI was from Sigma-Aldrich.

Sequence analysis

Sequence alignments and the resulting dendrogram were made using MacVector V6.5.1 (Oxford Molecular Co.).

Constructs

***lad-1–GFP* dominant negative plasmid construct, pLC84** The plasmid pLC84 fuses GFP in parent vector pPD95.75 to 5 kb of upstream and 12 kb of genomic LAD-1 sequence downstream of the initiating ATG start site from cosmid C18F3.

Neurofascin–LAD-1 chimera LAD-1 cytoplasmic tail was fused to the previously constructed neurofascin cytoplasmic tail-deleted–HA construct (Zhang et al., 1998).

LAD-1 antibodies

6991 antibody Rabbits were immunized with LAD-1 cytoplasmic tail expressed in bacteria in-frame with glutathione *S*-transferase in a pGEX vector (Amersham Pharmacia Biotech). Sera were preadsorbed over a glutathione *S*-transferase column then affinity purified using an antigen fusion protein column.

LAD-1P antibody (1163P) and LAD-1NP antibody (1162NP) Rabbits were immunized with the peptide CTEDGSFIGQY^{P}VPQKS coupled to NEM-KLH (Pierce Chemical Co). Sera were preadsorbed over a column containing the nonphosphorylated peptide.

1162NP Antibodies from one rabbit eluted from this nonphosphorylated peptide column showed no cross-reaction with the phosphorylated peptide. The breakthrough from the column of nonphosphorylated peptide was then affinity purified using a phosphorylated peptide column. The final antibody preparation (1163P) showed no cross-reaction with the nonphosphorylated peptide.

Immunoblots

Adult animals were harvested, pelleted in 50 mM phosphate buffer, pH 7.5, containing protease inhibitors, 1 mM Na pervanadate, and 10 mM NaF, and frozen in liquid nitrogen before being directly dissolved in boiling SDS-PAGE buffer containing 40 mM DTT. Immunoblots were performed with affinity purified antibodies and visualized with ^{125}I-labeled protein A as described (Moorthy et al., 2000).

Immunofluorescence microscopy

Worms were fixed and stained for indirect immunofluorescence using standard protocols and the freeze-crack methanol fixation method (Epstein and Shakes, 1995). Primary antibodies were used at the following concentrations: 1:500 for 6991 LAD-1, 1:300 for 1163P and 1162NP LAD-1 and UNC-44, 1:1,500 for MH27, and 1:250 for MH24. Immunofluorescent images were obtained using a ZEISS LSM 410 laser scanning microscope.

Live animal visualization using Nomarski optics

Animals were placed on a 2% agarose pad using 1% 1-phenoxy-2-propanol in M9 buffer as anesthetic. The pad was gently sealed with a no. 1 coverslip.

Dominant negative LAD-1 transgenic animal characterization

Dominant negative LAD-1 transgenic animals were generated by standard transgenic technique (Epstein and Shakes, 1995). Transgenic strains carrying the extrachromosomal arrays (pLC84; pRF4) (see above) were selected using the pRF4 (*rol-6[su1006]* roller) phenotype as a dominant selectable marker. Transgenic hermaphrodites were cloned and their progeny analyzed and scored for various phenotypes.

To determine if the transgene acted in a dominant negative fashion, we crossed *stDf7* into the transgenic line in the following manner. Transgenic roller males were crossed into the RW1324 strain (*stDf7/fem-1[e1991]unc-24[e138]unc-22[s12]*). 53 F1 cross progeny rollers were cloned, and their genotypes and phenotypes were analyzed.

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