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A Molecular Mechanism for the Requirement of PAT-4 (Integrin-linked Kinase (ILK)) for the Localization of UNC-112 (Kindlin) to Integrin Adhesion Sites*§

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Background: PAT-4 (ILK) is required for localization of UNC-112 (kindlin) to integrin adhesion sites.

Results: N- and C-terminal halves of UNC-112 interact, and mutations abolishing this interaction restore the ability of a mutant UNC-112 that cannot bind PAT-4 to localize.

Conclusion: UNC-112 exists in two conformations, and binding to PAT-4 converts UNC-112 to an open state.

Significance: This molecular mechanism may be conserved among kindlins.

Caenorhabditis elegans muscle cells attach to basement membrane through adhesion plaques. PAT-3 (β-integrin), UNC-112 (kindlin), and PAT-4 (integrin-linked kinase) are associated with these structures. Genetic analysis indicated that PAT-4 is required for UNC-112 to be properly localized. We investigated the molecular basis of this requirement. We show that the cytoplasmic tail of PAT-3 binds to full-length UNC-112 and that the N- and C-terminal halves of UNC-112 bind to each other. We demonstrate competition between the UNC-112 C-terminal half and PAT-4 for binding to the UNC-112 N-terminal half. The D382V mutation results in lack of binding to PAT-4 and lack of localization to adhesion structures. T346A or E349K mutations, which abolish interaction of the N- and C-terminal halves, permit D382V UNC-112 to localize to adhesion structures. The following model is proposed. UNC-112 exists in closed inactive and open active conformations, and upon binding of PAT-4 to the UNC-112 N-terminal half, UNC-112 is converted into the open state, able to bind to PAT-3.

Caenorhabditis elegans is an excellent model system in which to study muscle due to its optical transparency and the available genetic resources (1–3). The muscle used for locomotion is restricted to a narrow length of the animal in four quadrants. The myofibrils are organized around M-lines. All of the dense bodies and M-lines are anchored to the muscle cell membrane and extracellular matrix, which is attached to the hypodermis and cuticle. This allows the force of muscle contraction to be transmitted directly to the cuticle and allows movement of the whole animal. Thus, nematode muscle M-lines and dense bodies serve the function of analogous structures in vertebrate muscle, but, in addition, because all of these structures are attached to the cell membrane and consist of integrin and integrin-associated proteins (see below), they are also similar to costameres of vertebrate muscle and focal adhesions of non-muscle cells. Many components of C. elegans thick and thin filaments and their membrane-ECM attachment structures have been identified.

Most were first identified through mutations that result in one of two main phenotypic classes, Unc or Pat. In the uncoordinated or “Unc” class, animals develop into adults but are slow moving or paralyzed (4–6). About 40 genes contribute to this class. In the “Pat” class (paralyzed arrested at 2-fold), embryos do not move in the eggshell, and development arrests at the 2-fold embryonic stage (7). When first defined, there were about 16 genes in this class. Most recently, RNAi screens of more than 3300 muscle-expressed genes have identified 108 new genes crucial for myofilament lattice organization, including four genes with Pat phenotypes, whose roles in muscle were previously unknown (8). Significantly, about 60% of these new muscle genes have human homologs. In recent years, multiple protein complexes have been found that link the muscle cell membrane to thick filaments at the M-line in C. elegans. The cytoplasmic tail of integrin is associated with a complex of four conserved proteins (UNC-112 (kindlin), PAT-4 (integrin-linked kinase (ILK))†, PAT-6 (actopaxin), and UNC-97 (PINCH)) (9–12). UNC-97 links to myosin in thick filaments through four different complexes: via UNC-98, via LIM-9 (FHL) and UNC-96, via LIM-8, and via UNC-95 and LIM-8 (13–16).

UNC-112 (kindlin) is one of a number of conserved proteins that have been shown to be essential for muscle development in

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3 The abbreviations used are: ILK, integrin-linked kinase; MBP, maltose-binding protein; PH, pleckstrin homology.
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C. elegans (3). The first kindlin, which was originally called Mig2 and later called kindlin-2, was identified previously (17) as a novel protein induced in human fibroblasts upon exposure to serum and transition from $G_0$ to S phase in the cell cycle (hence, the name “mitogen-induced gene”). The first clue that kindlins have a role in integrin adhesion complexes came from work in C. elegans (9), with the molecular cloning of unc-112, a muscle gene that has a hypomorphemic Unc and null Pat embryonic lethal phenotype, and the report of its sequence homology to human Mig2. In C. elegans muscle, UNC-112 co-localizes with PAT-3 (β-integrin) at muscle focal adhesions (M-lines and dense bodies), and its localization is dependent upon the presence of PAT-3. Although C. elegans has only one kindlin, humans have three kindlins (18). Kindlin-1 is expressed primarily in epithelial cells, such as keratinocytes and intestinal epithelial cells. Kindlin-2 is expressed everywhere except for hematopoietic cells. Kindlin-3 is expressed in hematopoietic cells. Mutations in the genes for human kindlin-1 and kindlin-3 result in inherited diseases. Mutations in kindlin-1 result in “Kindler syndrome” (19), a type of hereditary “epidermolysis bullosa,” which displays very fragile skin and recurrent blister formation. In addition, some mutations in kindlin-1 result in neonates with blistering and severe colitis (20). Mutations in kindlin-3 result in “leukocyte adhesion deficiency type III,” which is characterized by severe bleeding and impaired adhesion of leukocytes to inflamed endothelia (21, 22). Although null mutations in kindlin-2 are probably embryonic lethal, partial loss of function mutations are speculated to result in certain types of inherited cardiomyopathy (18). This is suggested by the finding that morpholino-mediated knockdown of kindlin-2 in zebrafish results in ventricular hypoplasia, reduced ventricular contractility, and disorganized intercalated disks, where kindlin-2 is normally localized (23). Finally, kindlins may also be involved in human cancer; kindlin-1 is up-regulated in colorectal and lung tumors (24), and kindlin-2 is involved in breast cancer (25, 26). Kindlin-3 is expressed in hematopoietic cells. Kindlin-3 is expressed in hematopoietic cells, such as keratinocytes and intestinal epithelial cells. Kindlin-2 is expressed everywhere except for hematopoietic cells. Kindlin-3 is expressed in hematopoietic cells. Mutations in the genes for human kindlin-1 and kindlin-3 result in inherited diseases. Mutations in kindlin-1 result in “Kindler syndrome” (19), a type of hereditary “epidermolysis bullosa,” which displays very fragile skin and recurrent blister formation. In addition, some mutations in kindlin-1 result in neonates with blistering and severe colitis (20). Mutations in kindlin-3 result in “leukocyte adhesion deficiency type III,” which is characterized by severe bleeding and impaired adhesion of leukocytes to inflamed endothelia (21, 22). Although null mutations in kindlin-2 are probably embryonic lethal, partial loss of function mutations are speculated to result in certain types of inherited cardiomyopathy (18). This is suggested by the finding that morpholino-mediated knockdown of kindlin-2 in zebrafish results in ventricular hypoplasia, reduced ventricular contractility, and disorganized intercalated disks, where kindlin-2 is normally localized (23). Finally, kindlins may also be involved in human cancer; kindlin-1 is up-regulated in colorectal and lung tumors (24), and kindlin-3 is up-regulated in several B cell lymphomas (25). Although human kindlins are involved in many cellular processes via integrin activation, the mechanisms by which kindlins are regulated are unknown.

In this study, we show that UNC-112 binds directly to the cytoplasmic tail of PAT-3 and that the N- and C-terminal halves of UNC-112 bind to each other. In addition, we demonstrate that this intramolecular interaction within UNC-112 can be competed by interaction of PAT-4 with the UNC-112 N-terminal half. We show that a mutant UNC-112 (D382V) that cannot bind to PAT-4 in vitro and in vivo but can still engage in the intramolecular interaction fails to localize to integrin adhesion complexes in vivo. Finally, mutations (T346A or E349K), which abolish interaction of the N- and C-terminal halves of UNC-112, restore the ability of D382V UNC-112 to localize to muscle adhesion complexes. To explain our data, we propose a model in which a conformational change of UNC-112 between closed and open states is regulated by the binding of PAT-4 to the UNC-112 N-terminal half, thus locking it into an open state, which can then bind to PAT-3 (β-integrin).

EXPERIMENTAL PROCEDURES

Nematode Strains and Culture—Wild type strain N2 was used in these studies. Nematodes were grown at 20 °C on NGM agar plates with Escherichia coli strain OP50 (4). Yeast Two-hybrid Screens and Assays—The general methods used for screening a C. elegans cDNA yeast two-hybrid library and for performing yeast two-hybrid assays were described previously (10, 14). The bait region for the PAT-3 cytoplasmic tail included residues 765–809 (pGBD-U112-36 (cyto)), and the bait region for the PAT-3 transmembrane and cytoplasmic tail included residues 735–809 (pGBD-U112-36 (TM/cyto)). To construct a bait plasmid that contained residues 765–809 of PAT-3, PCR was used to produce from a cDNA pool the corresponding cDNA using 5′ primer CGC GGA TCA GTT CGG GTT GCC GAC AGC GCA ATT with added BamHI site and 3′ primer GCG CTC GAG TTA GTT GGC TTT TCC AGC GTA with added XhoI site, insertion into pBluescript, finding an error-free clone, and finally moving the same fragment into pGDU-CI (26). For amplification of the region of PAT-3 residues 735–809, the following primers were used: 5′ primer, CGC GGA TCA CCT GTC CCT GTG CTC GCA ATT with added BamHI site; 3′ primer, GCG CTC GAG TTA GTT GCC GAC AGC GCA ATT with added XhoI site. Prey clones harboring UNC-112 residues 2–592, 2–547, and 2–390, respectively, are prey clones isolated from a screen of the yeast two-hybrid library RB2. Bait constructs of UNC-112 full-length and deletion series were described previously (10). Prey constructs of UNC-112 were made using the following procedure. BamHI-Xhol (residues 1–720), BamHI-BglII (residues 1–396), BglII-Xhol (residues 397–720), StyI-Xhol (residues 32–720), and SnaBI-Xhol (residues 556–720) fragments from pDM#225 (10) were cloned into a pGAD vector. The bait plasmid of full-length PAT-4 (pGBD-U112-36) was described previously (10).

Expression and Purification of UNC-112 Fusion Protein—An unc-112 cDNA (U112–36, C-terminal half without STOP codon) was amplified by PCR using primers U112–3 (GCC GGA TCA CCT GTC CCT GTG CTC GCA ATT) and U112–6 (GCC CTC GAG AGC CCA TCC TGT AAG TTT), cloned into pBluescript, and confirmed by DNA sequencing. To obtain full-length unc-112 cDNA without a STOP codon, the cDNA for the N-terminal half of unc-112 (U112-12) (10) was cloned into pBluescript-U112-36, resulting in in pBluescript-U112-1236. This full-length cDNA of unc-112 (U112-1236) was cloned into pET24a for the addition of His$_6$ tag to the C-terminal end of UNC-112. From the pET24a-UNC-112-1236, UNC-112 cDNA with His$_6$ tag was cut out and cloned into pMAL-KK-1, resulting in pMAL-UNC-112-his.

The pMAL-UNC-112-his plasmid was transformed into E. coli BL21 (DE3) RIL, and growth was performed in liquid LB for 2 h with shaking at 37 °C. This culture was cooled on ice, protein expression was induced upon the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.1 mM and shaking at 15 °C for 8 h, and the bacteria was pelleted and stored at −80 °C. The MBP-UNC-112-His protein was purified using our standard procedure for purifying MBP fusion proteins (27), except that throughout the procedure, we used a
buffer consisting of 20 mM Tris, pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM DTT. The resulting protein was stored on ice (never frozen). From 1 liter of culture, we obtained ~2.2 mg of MBP-UNC-112-His.

Direct Binding between Purified UNC-112 and Purified PAT-3 Cytoplasmic Tail—A plasmid expressing the GST-PAT-3 cytoplasmic tail (residues 765–809) in E. coli was made by cloning the PCR-amplified cDNA as described above into pGEX-KK-1 (gift from Dr. Kozo Kaibuchi, Nagoya University). GST and GST-PAT-3 were expressed in E. coli and purified using glutathione-agarose beads as described previously (27). Purified GST or GST-PAT-3 (30 μg) were mixed in a total volume of 500 μl containing 60 μl of a 1:1 slurry of glutathione-agarose beads (Sigma), incubated for 1 h, and washed, resulting in GST- or GST-PAT-3-coated beads. Binding reactions were conducted using these beads together with equimolar amounts of either MBP (52 μg) or MBP-UNC-112-His (144 μg), in a total volume of 1 ml of 20 mM Tris, pH 7.5, 200 mM NaCl, and 1 mM EDTA, with mixing for 3 h at 4 °C. Beads were pelleted and washed three times using the same buffer, and the beads were transferred to fresh tubes and pelleted again. Elution of proteins bound to the beads was done by incubation in 30 μl of 2× Laemmli buffer at 95 °C for 5 min and then pelleting out the beads. From each reaction, the entire 30 μl were separated on a 10% SDS-PAGE, and stained overnight in Coomassie Blue.

GST Pull-down Assay Using a Wild Type Worm Lysate—A worm lysate was prepared by vortexing for 1 min a 10% (v/v) mixture of worm powder (extensively ground in a mortar and pestle in liquid nitrogen) in lysis buffer (20 mM Tris, pH 8.0, 10% glycerol, 0.5% Nonidet P-40, 2 mM EDTA, 150 mM NaCl, complete Mini protease inhibitors (Roche Applied Science)), spinning at top speed in a microcentrifuge for 10 min at 4 °C, and saving the supernatant. GST- or GST-PAT-3-coated beads (described above) were mixed with the worm lysate, incubated at 4 °C for 1 h, and washed; the proteins were eluted; and portions of each sample were run on two gels and blotted. One blot was reacted with anti-GST (Sigma-Aldrich H3663; 1:200 dilution), anti-UNC-95 (1:100 dilution), and the other blot was reacted with anti-GST (Sigma-Aldrich A7340) to detect the presence of the yeast expression plasmid containing either wild type and D382V HA-UNC-112N and purified GST-PAT-4 (kinase domain, catalytic). Competition between MBP-UNC-112C and MBP-PAT-4 was expressed in E. coli and purified as described previously (27). Using yeast lysates containing either wild type and D382V HA-UNC-112N and purified MBP or MBP-UNC-112C, HA pull-down assays were carried out as described previously (30).

Preparation of Anti-PAT-4 Antibody—The 72 residues of PAT-4 (amino acids 143–215) between the third ankyrin repeat and the protein kinase domain were expressed and purified in E. coli as both GST and MBP fusion proteins. Using the primers GCG GGA TCC TCT GAT TTC AAT GCA ATG GAA GTA TTT TTT AGT GAT CAA ATT CAG CGA for the 5’ end (with added BamHI site) and CGC CTC GAG CTA TTT CTT CTT GAT GAT CAA ATT CAG CGA for the 3’ end (with added XhoI site), the corresponding cDNA fragment was amplified by PCR and cloned into pGEX-KK1 and pMAL-KK1. After finding error-free clones, they were used for protein expression as described previously (27). The resulting GST-PAT-4 (143–215) antigen was shipped to Spring Valley Laboratories (Woodbine, MD) for generation of rabbit polyclonal antibodies. Anti-PAT-4 antibodies were affinity-purified against MBP-PAT-4 as described previously (13). We verified the specificity of the antibody by performing a Western blot and finding a decreased level of a protein of the expected size (~50 kDa) for PAT-4 in worms that had undergone pat-4 RNAi beginning at the L1 stage, as compared with worms that had been fed bacteria containing the RNAi empty vector (data not shown).

In Vivo Binding Assay Using GST-PAT-3 and MBP-PAT-4—A plasmid for expressing the MBP-PAT-4 (full-length) in E. coli was made by cloning the Xhol fragment of pDM#280 (10) into pMAL-KK-2. GST, GST-PAT-3, MBP, and MBP-PAT-4 were purified from bacteria as described above. An in vitro binding assay using purified GST and MBP fusions was performed as described previously (15).

Immunostaining—Worms were fixed using the method described previously (29). Antibody staining with anti-HA (Sigma Aldrich H3663; 1:200 dilution), anti-GFP (Invitrogen A11122, 1:200 dilution), and anti-UNC-95 (1:100 dilution) was performed as described previously (15). Secondary antibodies were anti-rabbit Alexa 488 (Invitrogen) for anti-UNC-95 and anti-GFP and anti-mouse Alexa 594 (Invitrogen) for anti-HA, each used at 1:200 dilution. Stained samples were mounted on a glass slide with a coverslip containing mounting solution (20 mM Tris (pH 8.0), 0.2 mM DABCO, and 90% glycerol). Images were captured at room temperature with a Zeiss confocal system (LSM510) equipped with an Axiovert 100M microscope and an Apochromat ×63/1.4 numerical aperture oil objective, in ×2.5 zoom mode. The color balances of the images were adjusted by using Adobe Photoshop.

HA Pull-down Assays—A yeast expression plasmid for HA-tagged wild type UNC-112 N terminus (residues 1–396) was constructed by two-step cloning. First, a PCR fragment using 5′ primer GCG GGA TCC TCT GAT TTC AAT GCA ATG GAA GTA TTT TTT AGT GAT CAA ATT CAG CGA for the 5′ end (with added BamHI site) and 3′ primer CGC CTC GAG CTA AAG ATC TGA TCG TCT GTT AAG with added stop codon and XhoI site was cloned into pKA-HA(Nhex2). Second, the NheI fragment containing HA-tagged UNC-112 N terminus was cloned into pGAP-C-Nhe (11, 12). Yeast expression plasmids for HA-tagged UNC-112 N terminus with a mutation (D382V) were prepared by two-step cloning similarly to the wild type fragment. A bacterial expression plasmid for the UNC-112 C terminus (residues 397–720) was made by cloning of the BglII-XhoI fragment of pDM#224 into pMAL-KK-3. MBP and MBP-UNC-112C were expressed in E. coli and purified as described previously (27). Using yeast lysates containing either wild type and D382V HA-UNC-112N and purified MBP or MBP-UNC-112C, HA pull-down assays were carried out as described previously (30).

Competition Assay—GST-PAT-4 (kinase domain, catalytically inactive) was prepared as described previously (10). Competition between MBP-UNC-112C and GST-PAT-4 for binding to HA-UNC-112N was carried out by using a modification of the HA pull-down procedure. Immunoprecipitated HA-UNC-112N from a yeast lysate (30) was mixed with MBP-UNC-112C (0.5 μg) and varying amounts of either GST or GST-PAT-4 (0, 10, 20 μg), incubated for 1 h in 20 mM Tris (pH 8.0), 0.2 mM DABCO, and 90% glycerol. Images were captured at room temperature with a Zeiss confocal system (LSM510) equipped with an Axiovert 100M microscope and an Apochromat ×63/1.4 numerical aperture oil objective, in ×2.5 zoom mode. The color balances of the images were adjusted by using Adobe Photoshop.
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reacted with anti-MBP (New England Biolabs E8038S) to detect possible binding with MBP-UNC-112C.

Screening for PAT-4 Non-binding Mutants—Mutations in UNC-112 were introduced randomly by error-prone PCR (31). To limit the mutagenized region to the N-terminal half, which is known to bind to PAT-4 (10), we amplified the N-terminal half of UNC-112. To clone these PCR-amplified and mutagenized fragments, we prepared an acceptor plasmid containing a wild type version of the C-terminal half of UNC-112 in the pACT vector. The acceptor plasmid was prepared by cloning the PCR-amplified C-terminal half fragment of UNC-112 (5′ primer, GGC GGA TCG TTT AAT GTC GAC AGA TCT TAC ACA AGT TCC CGA; 3′ primer, CGC GGA TCC CTC GAG CTA AGC CCA TCC TGG AAG) into a pACT vector from which we had eliminated the BglII enzyme site and identifying a clone that had a wild type sequence. The N-terminal UNC-112 fragment was amplified by the error-prone method using the following primers: 5′ primer, ACC AAA CCC AAA AAA AGA GAT CGG GCC CGG GGA TCC TCG AGA GTT CAC TCT TGT TGA AG; 3′ primer, GCT GCC AAT TTC TTT GGT TTC ATA TAT TTT. These primers contain 30 base pairs of sequence that overlap regions in the acceptor plasmid. Cloning of error-prone PCR-amplified fragments into the acceptor plasmid was performed by exploiting yeast recombination in vivo (32). The mixture of the amplified PCR fragments (~1 μg) and the acceptor plasmid (~1 μg) digested with BamHI and BglII was transformed into PJ69-4A harboring pGBDU-PAT-4 (10). Transformed yeast cells were spread onto −Leu−Ura−His and 2 mm 3-amino-1,2,4-triazole to screen for His+ colonies. His+ selection ensured that the mutagenized UNC-112N could still interact with the cytoplasmic tail of PAT-3. This step was essential for eliminating clones with premature stop mutations or with many other mutations. His+ colonies were streaked onto an −Ade plate and screened for His+Ade+ colonies. After streaking on a 5-fluoro-orotic acid plate to eliminate the URA3 marker bait plasmid (pGBDU-PAT-4), prey clones were isolated from yeast and amplified in E. coli. From a total of 96 His+Ade+ yeast colonies, 93 mutagenized clones were isolated. These prey clones were transformed separately into PJ69-4A carrying either pGBDU-UNC-112C or pGBDU-PAT-4 (full-length) to check for interaction with the C-terminal half of UNC-112 and interaction with full-length PAT-4. Among 93 mutagenized clones of UNC-112N, 87 clones showed binding to PAT-4, and 13 of these clones could not bind to UNC-112C. From DNA sequencing of these 13 clones, we identified two clones each with a single amino acid change, T346A and E349K. We prepared full-length UNC-112 with these two mutations and tested binding to PAT-4 and PAT-3 cytoplasmic tail. We also prepared full-length UNC-112 and the N-terminal half of UNC-112 containing T346A/D382V or E349K/D382V and tested binding to the UNC-112 C-terminal half, PAT-4, and PAT-3 cytoplasmic tail.

Expression of UNC-112 Mutants in C. elegans Using a Heat Shock Promoter—Full-length UNC-112 cDNA with no mutations, D382V, T346A, E349K, T346A/D382V, or E349K/D382V were cloned into the HA-tagged vector, pKS-HA(Nhex2) (33). From these pKS-HA (Nhex2) clones, Nhex fragments (containing HA-tagged UNC-112 cDNAs) were cloned into the Nhex site of C. elegans expression vectors, pPD49.78 and pPD49.83 (gifts from Dr. Andy Fire, Stanford University). These vectors contain two different heat shock promoters. pPD49.78/83-HA-UNC-112 (WT, D382V, T346A, E349K, T346A/D382V, and E349K/D382V) were mixed with pTG96 (SUR-5::NLS::GFP) as a transformation marker (34) and injected into wild type N2 worms. Transgenic lines with extrachromosomal arrays containing pPD49.78/83-HA-UNC-112 (WT (called sfEx40), D382V (called sfEx42), T346A (called sfEx43), E349K (called sfEx44), T346A/D382V (called sfEx45), and E349K/D382V (called sfEx46)) and pTG96 were established by picking GFP-positive worms using a GFP dissection microscope. Expression of the HA-tagged UNC-112 proteins (WT, D382V, T346A,
E349K, T346A/D382V, and E349K/D382V) was induced by incubation of the transgenic worms at 30 °C for 2 h (heat shock). Heat-shocked transgenic worms were fixed using the method described previously (29) and stained by anti-GFP (to verify the existence of extrachromosomal array), anti-UNC-95 (to identify dense bodies and M-lines in muscle cells), and anti-HA (to determine the localization of HA-tagged UNC-112 proteins). We prepared worm lysates from transgenic worms with or without heat shock and examined the expression of HA-tagged UNC-112 proteins by Western blot, reacting with anti-HA (Sigma-Aldrich H3663; 1:200 dilution).

Integration of Extrachromosomal Arrays——The extrachromosomal arrays containing pPD49.78/83-HA-UNC-112 (WT, D382V, T346A, and T346A/D382V) and pTG96 were integrated into the genome by UV irradiation (35) with some modifications. The resulting integrated nematode lines are called sfIs3 for WT, sfIs5 for D382V, sfIs6 for T346A, and sfIs7 for T346A/D382V.

Co-immunoprecipitation of HA-UNC-112 and Endogenous PAT——Transgenic lines carrying integrated arrays described above were induced to express either wild type, D382V, T346A, or T346A/D382V HA-tagged UNC-112 by heat shocking mostly adult animals by exposure to 30 °C for 3 h, and worm lysates were prepared as described above. To 500 μl of each worm lysate was added 50 μl of a 1:1 slurry of monoclonal anti-HA-agarose beads (Sigma-Aldrich A2095) followed by mixing at 4 °C for 2 h. The beads were pelleted and washed three times with lysis buffer, transferred to new tubes, and pelleted, and as much liquid was removed as possible. The proteins from these beads were eluted with 33 μl of 2X Laemmli sample buffer, and portions were separated on 10% SDS-PAGE gels and blotted. One set of lanes were reacted with rabbit anti-HA (Sigma-Aldrich H6908) at 1:200 dilution, and another set of lanes were reacted with affinity-purified anti-PAT-4 at 1:100 dilution, and the reactions were detected by ECL.

The Effect of Overexpressing UNC-112 Mutant Proteins in a Wild Type Background——The integrated lines, sfIs3, sfIs5, sfIs6, and sfIs7, were grown on four 10-cm NGM plates, either with or without heat shock or with heat shock at 30 °C for 2 h and then allowed to grow overnight at 20 °C. The worms were collected and prepared for immunostaining using the Nonet method (29) and stained with anti-myosin heavy chain A using the monoclonal antibody A2055.

RNAi Beginning at the L1 Stage——Feeding RNAi for pat-3, pat-4, and unc-112 was conducted as described previously (14). The resulting adult animals were fixed and immunostained for myosin, as described above. The feeding plasmids in the pPD129.36 vector were prepared by inserting 1.0–1.5-kb cDNAs of each gene.

Determination of Stoichiometries——To quantitate the ratio of proteins in the UNC-112N-UNC-112C complex presented in Fig. 3, the amount of each protein detected by immunoblotting was compared with blots containing a range of known amounts of purified proteins detected with the same antibodies. To determine the amount of HA-UNC-112N, we used MBP-UNC-112N; for MBP-UNC-112C, we compared with known amounts of the same protein. To quantitate the ratio of proteins in the UNC-112-PAT-4 complex presented in Fig. 6, the experiment was repeated, but this time, HA-UNC-112 was detected by using anti-UNC-112 rather than anti-HA. This allowed comparison with known amounts of MBP-UNC-112N using the same antibody. The quantity of PAT-4 in the complex was determined using anti-PAT-4 and known amounts of MBP-PAT-4 (full-length). The weight in ng was divided by the molecular weight of the pulled down proteins to obtain the mole numbers. These mole numbers were used to calculate the ratios for each complex (UNC-112N to UNC-112C and UNC-112 to PAT-4).

RESULTS

UNC-112 (Kindlin) Interacts with the Cytoplasmic Tail of PAT-3 (β-Integrin)——To search for molecules that interact with the cytoplasmic tail region of PAT-3, we screened a yeast two-hybrid cDNA library of C. elegans (RB2). From the screening of 2.4 × 10^7 colonies, we identified 80 His′ colonies, among which, 12 were also Ade+. After retransformation, only one clone was a true positive. This clone contained a cDNA encoding UNC-112 (residues 3–720). To clarify the importance of the cytoplasmic tail of PAT-3, we tested two bait constructs of PAT-3; one is the cytoplasmic region (used for screening), and the other is the transmembrane domain plus cytoplasmic region. Only the bait containing the cytoplasmic region of PAT-3 interacted with the UNC-112 (Fig. 1A). The original prey clone encoding UNC-112 was nearly a full-length cDNA. Next, we tested whether the N-terminal or C-terminal halves of UNC-112 are required for this interaction. The N-terminal half of UNC-112 was previously shown to bind PAT-4 (ILK) (10). However, neither the N-terminal nor the C-terminal half of UNC-112 bound PAT-3 (Fig. 1B), suggesting that interaction of PAT-3 with UNC-112 requires full-length UNC-112. To verify the interactions revealed by the yeast two-hybrid screen, we expressed and purified from bacteria GST and MBP fusion proteins for the cytoplasmic tail of PAT-3 and full-length UNC-112, respectively. As shown in Fig. 1C, GST-PAT-3 but not GST binds to MBP-UNC-112 and not MBP. The combination of yeast two-hybrid assays and in vitro binding with purified proteins indicates that UNC-112 directly binds to the cytoplasmic tail of PAT-3.

To obtain some evidence that native UNC-112 can interact with PAT-3, we next asked if the PAT-3 cytoplasmic tail can pull out UNC-112 from a worm lysate. Glutathione-agarose beads coated with either bacterially expressed and purified GST-PAT-3 cytoplasmic tail or GST (Fig. 2B) were incubated with a C. elegans lysate and washed. The proteins were eluted from the beads, separated on a gel, and subjected to Western blot analysis. As shown in Fig. 2A, GST-PAT-3 was able to pull down UNC-112 from the worm lysate, but GST was not. In addition, GST-PAT-3 was able to pull down PAT-4. However, as shown in Fig. 2C, GST-PAT-3 and MBP-PAT-4, as purified proteins, failed to interact (contrast with Fig. 1C). This result is consistent with the previous finding that the PAT-3 cytoplasmic tail fails to interact with PAT-4 by the two-hybrid method.

4 P. Barrett, personal communication.
Therefore, the pull-down of PAT-4 from a worm lysate with GST-PAT-3 is likely to occur through interaction of PAT-4 with UNC-112.

The N-terminal and C-terminal Halves of UNC-112 Interact with Each Other—Previously, we screened a two-hybrid library with full-length UNC-112 and identified two binding partners, PAT-4 (10) and UIG-1 (28). Although full-length UNC-112 is required for interaction with UIG-1, only the N-terminal half of UNC-112 is required for interaction with PAT-4. To obtain further insight into the function of the C-terminal half of UNC-112, it was used to screen the two-hybrid library. From 5.4 × 10⁸ colonies, we identified 185 His+ colonies, and after retransformation we identified four true positive clones. All four clones contained cDNAs encoding UNC-112 (one clone, residues 2–592; one clone, residues 2–547; two clones, residues 2–390; Fig. 3A). However, full-length UNC-112 did not show binding with the UNC-112 C-terminal half (Fig. 4), suggesting that the N-terminal half of UNC-112 can interact with the C-terminal half of UNC-112. This interaction between UNC-112N terminus and UNC-112 C terminus was confirmed by a biochemical method using purified proteins (Fig. 3, B and C). Briefly, yeast-expressed HA-tagged UNC-112 N-terminal half was shown to interact with bacterially expressed MBP-UNC-112 C-terminal half. The ratio of HA-UNC-112N to MBP-UNC-112C was 0.92.

This finding that the N- and C-terminal halves of UNC-112 interact could be explained as either an intramolecular interaction (i.e. one UNC-112 molecule is folded onto itself) or an intermolecular interaction (i.e. UNC-112 forms homodimers or multimers). As shown in Fig. 4, we have tested for binding of the N-terminal half, C-terminal half, and full-length UNC-112 to a series of deletion derivatives of UNC-112 by two-hybrid assays. Because full-length UNC-112 does not bind to any region of UNC-112 (including full-length), intramolecular interaction is most likely. The N-terminal half, containing part of the split FERM domain, can interact with as little as the C-terminal-most 164 residues (amino acids 556–720), containing the other part of the split FERM domain. The data also show that the PH domain is not involved in this intramolecular interaction.

PAT-4 Competes with UNC-112C for Binding to UNC-112N—In embryos, it has been shown that UNC-112 cannot localize to the muscle cell membrane in either pat-3 null mutants (9) or pat-4 null mutants (10), indicating that both PAT-3 and PAT-4 are required for the proper localization of UNC-112 in muscle cells. Using the yeast two-hybrid method, it was reported that PAT-4 does not interact with the cytoplasmic tail of PAT-3 (10). Although this came as a surprise, similar results were also reported from Drosophila (37). It was also reported that PAT-4 interacts with the N-terminal half of UNC-112 and that the two proteins are co-dependent for proper localization to adhesion

![FIGURE 1. UNC-112 interacts with the cytoplasmic tail of PAT-3 (β-integrin). A, to determine whether only the cytoplasmic region of PAT-3 is required for interaction with UNC-112, two bait constructs of PAT-3 were tested by the yeast two-hybrid method; one is the cytoplasmic region (used for screening), and the other is the transmembrane domain plus cytoplasmic region of PAT-3. As shown, only the bait containing the cytoplasmic region of PAT-3 showed interaction with UNC-112. B, the original library prey clone encoding UNC-112 was nearly a full-length cDNA. Neither the N-terminal nor the C-terminal half of UNC-112 interacts with PAT-3, suggesting that PAT-3 interaction with UNC-112 requires full-length UNC-112. In the schematic representation of UNC-112, the domains indicated were predicted by PFAM (see the Pfam 6.0 Web site). In A and B, the gray bars represent the minimal regions required for interaction. C, binding between GST-PAT-3 and MBP-UNC-112. Glutathione-agarose beads coated with either GST or GST-PAT-3 (cytoplasmic tail) were incubated with either MBP or MBP-UNC-112-His, washed, eluted, separated by SDS-PAGE, and stained with Coomassie Blue. The right-most lanes show 2 μg each of MBP and MBP-UNC-112-His. The purified MBP-UNC-112-His contains mostly the full-length fusion protein (~125 kDa). Note that GST-PAT-3, but not GST, binds to MBP-UNC-112-His (indicated by an arrowhead).]
Our new finding is that the N- and C-terminal halves of UNC-112 interact with each other (i.e. the binding sites for PAT-4 and the C-terminal half of UNC-112 overlap). We therefore hypothesize that UNC-112 exists in closed inactive and open active states and that binding of PAT-4 to the UNC-112 N terminus results in an opening up of UNC-112 and consequent binding of the UNC-112/PAT-4 complex to the cytoplasmic tail of PAT-3 (see Fig. 11).

To obtain in vitro evidence for this model, we compared the amount of UNC-112C bound to UNC-112N in the presence or absence of PAT-4. To carry this out, we modified the method we had used to confirm the interaction between the N-terminal and C-terminal halves of UNC-112. HA-UNC-112N was

FIGURE 2. The cytoplasmic tail of PAT-3 can pull down a UNC-112-PAT-4 complex from a worm lysate. A, glutathione beads coated with GST-PAT-3 cytoplasmic tail were used to pull out UNC-112 from a C. elegans lysate. The worm lysate was incubated with glutathione-agarose coated with either GST or GST-PAT-3, extensively washed, eluted, separated by SDS-PAGE, and blotted to nitrocellulose membrane. Anti-UNC-112 antibodies (28) were used to detect UNC-112, UNC-112 could be detected (arrowhead) using GST-PAT-3 beads but not GST beads. The asterisk denotes the position of a derivative of GST-PAT-3, possibly a dimer (data not shown). Note that GST-PAT-3 beads could also pull out PAT-4 (indicated by an arrow). B, Coomassie Brilliant Blue (CBB) staining of an SDS-PAGE containing the GST and GST-PAT-3 used in A. C, glutathione beads coated with either GST or GST-PAT-3 cytoplasmic tail were incubated with either MBP or MBP-PAT-4 (full-length), pelleted, and washed, and the proteins were eluted precipitated with beads conjugated with anti-HA antibodies and mixed with bacterially expressed MBP or MBP-PAT-4 C-terminal half. After washing, bound proteins were detected by using anti-MBP antibodies. HA-UNC-112 N terminus can bind to MBP-PAT-4 (indicated by an arrow) but minimally to MBP itself. An SDS-polyacrylamide gel showing the proteins used for this experiment is shown in supplemental Fig. 1A.

FIGURE 3. The N-terminal and C-terminal halves of UNC-112 interact with each other. A, the C-terminal half of UNC-112 (residues 397–720) was used as a bait to screen a yeast two-hybrid library. From 5.4 × 10⁷ colonies, 185 His⁺ colonies were identified, and after retransformation, four true positive clones were identified. All four clones contained cDNAs encoding UNC-112 (one clone, residues 2–592; one clone, residues 2–547; two clones, residues 2–390). Because the smallest clones spanned residues 2–390, this suggests that the N-terminal half of UNC-112 (gray bar) can interact with the C-terminal half of UNC-112. B, the interaction between UNC-112 N-terminal and UNC-112 C-terminal halves was confirmed by using purified proteins. A lysate was prepared from yeast expressing the HA-tagged UNC-112 N-terminal half. HA-tagged proteins were precipitated with beads conjugated with anti-HA antibodies and mixed with bacterially expressed MBP or MBP-PAT-4 C-terminal half. After washing, bound proteins were detected by using anti-MBP antibodies. HA-UNC-112 N terminus can bind to MBP-PAT-4 C terminus but minimally to MBP itself. An SDS-polyacrylamide gel showing the proteins used for this experiment is shown in supplemental Fig. 1A.
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expressed in yeast, immunoprecipitated with anti-HA beads, and then mixed with MBP-UNC-112C with or without GST-PAT-4 or GST. After extensive washing, the amount of UNC-112C pulled down was determined by Western blot. As shown in Fig. 5, in the presence of GST-PAT-4, but not GST, the amount of MBP-UNC-112C bound to HA-UNC-112N was decreased, demonstrating a competition between the UNC-112 C-terminal half and PAT-4 for binding to the UNC-112 N-terminal half. This result supports our model (Fig. 11).

An UNC-112 Mutant That Cannot Bind to PAT-4 in Vitro Fails to Localize to Integrin Adhesion Complexes in Vivo—To obtain in vivo evidence for our model, we mutagenized and screened for UNC-112 mutants that continued to interact with PAT-3, but failed to interact with PAT-4, in the yeast two-hybrid system. We identified one such mutant with a single amino acid change in the N-terminal half of UNC-112, D382V (Fig. 6A). The aspartate at residue 382 lies at the boundary between FERM_M and PH domains but is not conserved among the human kindlin sequences. We had shown that for the wild type sequence, the N-terminal half interacts with the C-terminal half, we tested the N-terminal half of D382V, for its ability to interact with the C-terminal half of wild type UNC-112, by the two-hybrid method. As indicated in Fig. 6A, D382V retains this ability. To verify that the D382V mutant UNC-112 does not interact with PAT-4 in vivo, we expressed HA-tagged UNC-112 proteins (wild type or D382V) in worms, immunoprecipitated them using anti-HA antibodies, and tested for the presence of PAT-4. As shown in Fig. 6B, although wild type UNC-112 protein co-precipitated PAT-4, D382V UNC-112 failed to precipitate PAT-4. The ratio of wild type full-length HA-UNC-112 to endogenous PAT-4 was 0.62. The likely reason why this ratio is not closer to 1.0 is that the ectopic expression of HA-UNC-112 resulted in an excess of UNC-112 (endogenous UNC-112 plus HA-UNC-112) compared with endogenous PAT-4. To confirm that the D382V mutant UNC-112 protein retains the interaction between its N-terminal and C-terminal halves, we expressed HA-tagged wild type and D382V mutant N-terminal halves of UNC-112 in yeast, immunoprecipitated them with anti-HA, and reacted them with MBP-UNC-112 C-terminal half (wild type). As indicated in Fig. 6C, the N-terminal halves of wild type or D382V UNC-112 were able to interact with the C-terminal half of UNC-112 in vitro. In conclusion, the D382V mutant UNC-112 is unable to interact with PAT-4 but retains the ability of its N- and C-terminal halves to interact with each other. In addition, because D382V mutant UNC-112 retains the ability of its N- and C-terminal halves to interact and retains its ability to interact with PAT-3, this mutant protein is likely to be properly folded.

To determine whether the D382V mutant UNC-112 protein could localize to integrin adhesion complexes in vivo, we expressed wild type and D382V UNC-112 as HA-tagged proteins under the control of a heat shock promoter. As indicated in Fig. 7A, we confirmed the heat shock-induced expression of these HA-tagged UNC-112 proteins in transgenic worms. As shown in Fig. 7B, wild type HA-tagged UNC-112 is localized to both M-lines and dense bodies, as reported for GFP-tagged UNC-112 (9) and native UNC-112 detected by antibodies (28). However, D382V mutant HA-UNC-112 fails to localize to M-lines and dense bodies. The D382V result supports our model (Fig. 11) in which PAT-4 binding to the N-terminal half of UNC-112 is required for the association of UNC-112 with integrin adhesion complexes.

Mutations That Block the Interaction of UNC-112 N- and C-terminal Halves Overcome the Failure of D382V UNC-112 to Localize to Muscle Adhesion Sites—According to our model (Fig. 11), the failure of D382V UNC-112 to localize to muscle adhesion sites is due to the lack of binding to PAT-4, and consequently, the D382V UNC-112 is in a closed and inactive conformation. Thus, we wondered if mutations that block the interaction between N- and C-terminal halves, promoting a
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A mutation in the N-terminal half of UNC-112 (D382V) abolishes interaction with PAT-4 but retains the ability of the N- and C-terminal halves of UNC-112 to interact. A, error-prone PCR was used to introduce mutations in the N-terminal half of UNC-112. Full-length UNC-112 proteins containing these mutations were screened in two-hybrid assays for those that interacted with PAT-3 but failed to interact with PAT-4. One single amino acid change, D382V, was found that fulfilled these criteria. The N-terminal half of UNC-112 with D382V continued to bind to the C-terminal half of UNC-112. Two such clones, each with a single amino acid change, T346A and E349K, were identified (Fig. 8A). Thr-346 and Glu-349 reside in the FERM_M domain and are conserved residues in all three human kindlins (kindlins 1–3). Double mutants in the N-terminal half of UNC-112 were then created that contain each of these mutations plus D382V. In two-hybrid assays, the double mutants behaved as expected for each mutation acting independently; each double mutant fails to interact with both the C-terminal half of UNC-112 and PAT-4 (Fig. 8A). The single and double mutations were then introduced into full-length UNC-112 and tested for interaction with PAT-3 and PAT-4. All of these mutants bound to PAT-3, and both single mutants, T346A and E349K, bound to PAT-4. Only when D382V was introduced was the interaction with PAT-4 abolished (Fig. 8A, below the line). To confirm the two-hybrid results for PAT-4 binding in vivo, lysates were prepared from nematodes expressing from a heat shock promoter either wild type, T346A, or T346A/D382V HA-tagged UNC-112. Upon immunoprecipitation of the HA fusion proteins, UNC-112 wild type or UNC-112 with the T346A mutation co-purified endogenous PAT-4, but UNC-112 with T346A/D382V did not precipitate an appreciable amount of PAT-4 (Fig. 8B).

Next, we determined the localization of the single and double mutant UNC-112 proteins in nematode muscle. We created worm strains that carry extrachromosomal arrays containing constructs that would express the single and double mutant UNC-112 proteins with HA tags under the control of a heat shock promoter. As indicated in Fig. 9A, expression could be induced by heat shock. After induction of expression, nematodes were fixed and immunostained with anti-HA to determine localization of HA-UNC-112. As shown in Fig. 9B, each single mutant (T346A or E349K) UNC-112 localizes to M-lines and dense bodies, similar to wild type UNC-112 (Fig. 7B). Remarkably, in contrast to D382V UNC-112 that does not localize (Fig. 7B), the addition of either the T346A or E349K mutation to D382V largely restores the ability of UNC-112 to localize M-lines and dense bodies (Fig. 9B). Our interpretation is that although the double mutant proteins (T346A/D382V or E349K/D382V) still cannot bind to PAT-4, they are mostly in an open conformation and now better able to interact with PAT-3 to localize to the base of M-lines and dense bodies (Fig. 11C). In other words, mutations that result in a constitutively open UNC-112 bypass the requirement of UNC-112 to bind to PAT-4, in order to interact with PAT-3.

The Dominant Negative Effect of Overexpressing T346A/D382V UNC-112—To examine the effect of overexpressing wild type and various mutants of UNC-112, we utilized nematodes carrying integrated arrays containing HA-tagged UNC-112 under the control of a heat shock promoter. Without heat shock, these nematodes display normally organized thick filaments (Fig. 10A). Heat shock-induced overexpression of wild

**FIGURE 6.** A mutation in the N-terminal half of UNC-112 (D382V) abolishes interaction with PAT-4 but retains the ability of the N- and C-terminal halves of UNC-112 to interact. A, error-prone PCR was used to introduce mutations in the N-terminal half of UNC-112. Full-length UNC-112 proteins containing these mutations were screened in two-hybrid assays for those that interacted with PAT-3 but failed to interact with PAT-4. One single amino acid change, D382V, was found that fulfilled these criteria. The N-terminal half of UNC-112 with D382V continued to bind to the C-terminal half of UNC-112. B, lysates were prepared from worms expressing either wild type or D382V HA-tagged UNC-112. The HA-tagged UNC-112 proteins were immunoprecipitated (IP) with anti-HA beads and washed, and the bound proteins were eluted, separated by SDS-PAGE, and blotted. Separate lanes were reacted with anti-HA and anti-PAT-4. Although wild type co-precipitated PAT-4, D382V UNC-112 failed to do so. C, lysates were prepared from yeast expressing HA-tagged N-terminal halves of wild type and D382V UNC-112 proteins. These proteins were immunoprecipitated with anti-HA beads, washed and incubated with either MBP or MBP-UNC-112 C-terminal half, and washed, and the eluted proteins were separated by gel and blotted. Separate blots were reacted with anti-UNC-112 and anti-MBP. MBP-UNC-112C interacts with either wild type or D382V HA-UNC-112N proteins. (MBP-UNC-112C interacts minimally with the anti-HA beads (last lane)). The asterisk indicates the position of MBP on the blot.
type, D382V (PAT-4 non-binding), and T346A (N- to C-terminal non-binding) UNC-112 proteins also showed normal thick filament organization. However, overexpression of T346A/D382V UNC-112 resulted in large aggregates of myosin. This phenotype is similar to that obtained by RNAi-mediated knock-down of integrin and integrin-associated proteins, especially of PAT-4 (ILK) (Fig. 10B). Our interpretation is that HA UNC-112 with T346A/D382V can localize to muscle focal adhesions, as shown in Fig. 9, but because it cannot bind to PAT-4, it cannot recruit PAT-4 to these sites. Consequently, UNC-112 with T346A/D382V results in a similar phenotype as knocking down PAT-4. For wild type or T346A UNC-112 proteins, they can localize to adhesion sites (Figs. 7 and 9), and because they can bind to PAT-4, they can recruit PAT-4 to these sites. In the case of D382V UNC-112, because it cannot localize (Fig. 7), it will have no effect on sarcomere organization, despite the fact that it cannot bind to PAT-4. Therefore, these results provide further support for our model (Fig. 11).

DISCUSSION

Regulation of Kindlin Activity by Interaction with ILK—We demonstrate that UNC-112 interacts with the cytoplasmic tail of PAT-3. This result is compatible with the initial observation that UNC-112 cannot localize to the muscle cell membrane in pat-3 null mutants (9). This type of kindlin/integrin interaction has been demonstrated in other organisms and has been shown to be crucial for many cellular processes (18). Our results demonstrate that this interaction is conserved from worms to humans. Furthermore, we demonstrate that the N- and C-terminal halves of UNC-112 interact with each other, most likely as one UNC-112 molecule folded onto itself. It has been reported that the N-terminal half of UNC-112 interacts with PAT-4 (10). We also have shown that in vitro, PAT-4 can compete with the UNC-112 C-terminal half for binding to the UNC-112 N-terminal half. In addition, we have isolated one missense mutant of UNC-112 (D382V) that cannot bind to PAT-4 (Fig. 6, A and B). It is worth noting that D382V does not interfere with the interaction between the N- and C-terminal halves (Fig. 6, A and C). Significantly, this D382V mutant UNC-112 fails to localize to integrin adhesion complexes in vivo (Fig. 7B). Thus, our data are compatible with the observation that UNC-112 fails to localize to the muscle cell membrane in pat-4 null mutants (10). The inability of the D382V UNC-112 to localize to muscle adhesion sites can be overcome by second site mutations (T346A or E349K) that block the N- to C-terminal half interaction. Finally, the dominant negative effect of overexpressing T346A/D382V UNC-112 in a wild type background supports the idea that this mutant UNC-112 protein can localize without PAT-4 binding. Altogether, previous results and our new data can be interpreted in the following model: that UNC-112 exists in two interconvertible states, closed inactive and open active, and that upon binding of PAT-4 to the UNC-112 N-terminal half, this results in an opening up of UNC-112 and consequent binding of a UNC-112-PAT-4 complex to the cytoplasmic tail of PAT-3 (Fig. 11). One possible explanation for the requirement of an open conformation is that upon opening up, a portion of...
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We have shown that full-length UNC-112 is required for binding to the cytoplasmic tail of PAT-3 by the yeast two-hybrid method (Fig. 1B) and demonstrated that recombinant purified full-length UNC-112 can bind to PAT-3 cytoplasmic tail \textit{in vitro} (Fig. 1C). Furthermore, when we ectopically expressed HA-tagged full-length UNC-112, it could localize to integrin adhesion sites (Fig. 7). However, we have also found that, using a similar expression method, either the N-terminal half or C-terminal half of UNC-112 alone can also localize to the adhesion sites (data not shown), suggesting that the association with integrin may not require full-length UNC-112 \textit{in vivo}. Further experiments will be required to clarify the mechanisms by which the two halves of UNC-112 can localize.

There is an apparent discrepancy between our model and the results presented in Fig. 1; i.e., although we have evidence that PAT-4 binding is essential for the association of UNC-112 with PAT-3 \textit{in vivo}, we were able to show interaction between UNC-112 and PAT-3, using either a yeast two-hybrid assay or an \textit{in vitro} binding experiment with purified proteins. However, it should be pointed out that for both two-hybrid and biochemical assays, we used fusion proteins in which either MBP or GAL-4-AD reside N-terminal of UNC-112. We speculate that the presence of these substantial N-terminal tags leads to an artificial opening up of UNC-112. In the future, we would like to conduct the \textit{in vitro} binding experiments using purified wild type and constitutively opened up mutant forms of UNC-112 (T346A and E349K), with and without the MBP tag.

Interaction between kindlins and ILK has been implicated in mammalian endodermal cells (38) and in C2C12 myoblasts (39). However, no direct interaction has been demonstrated, although such an interaction has been shown with the orthologous proteins in \textit{C. elegans} (10). Significantly, our results and model suggest a possible function for a kindlin/ILK interaction in mammalian cells.

One alternative interpretation for our results is that the UNC-112 conformational change is regulated by phosphorylation. Although we cannot completely disqualify this possibility, several observations mitigate against this being likely. Because ILK has serine/threonine kinase activity, serine/threonine phosphorylation of UNC-112 (kindlin) might be important for this conformational change. It has been demonstrated that vertebrate ILK with the E359K mutation shows an 80% reduction in kinase activity as compared with the wild type enzyme (40).

In addition, vertebrate ILK with the S343A mutation has no detectable activity (40). It is important to note that both \textit{pat-4} (E359K) and \textit{pat-4} (S343A) are equally effective in rescuing \textit{pat-4} null mutants (10). This suggests that \textit{PAT-4} kinase activity is not required for the localization of UNC-112 to focal adhesions, including interaction with PAT-3. At focal adhesions, many tyrosine kinases are located, such as focal adhesion kinase. However, we could show direct interaction between UNC-112 and PAT-3 \textit{in vitro} using bacterial expression of each protein (Fig. 1C). Because it has been reported that in bacteria there is almost no tyrosine, serine, or threonine phosphorylation (41), phosphorylation of PAT-3 or possibly of UNC-112 might not be required for this interaction. Thus, although it is still possible that in the nematode phosphorylation might regulate the strength of the interaction, phosphorylation probably

UNC-112 unavailable to interact with integrin in an UNC-112 closed conformation is consequently available to interact with integrin. In support of this idea, for mammalian kindlin 2, it has been demonstrated that the C-terminal portion of the FERM-M domain is the minimal integrin binding region \textit{in vitro} (38).
is not essential for the interaction of UNC-112 and integrin to occur.

Kindlin as a Member of the FERM Protein Superfamily; Conservation of Intramolecular Interaction—We have shown that N-terminal and C-terminal halves of UNC-112 can interact, and our additional data suggest that UNC-112, when in a closed conformation, is less able to bind to integrin. Such intramolecular masking is well known for the ERM proteins, ezrin, radixin, and moesin, which, like UNC-112 (kindlin), also contain a FERM domain. In the case of ERM proteins, the FERM domain is unable to interact with membrane proteins when it is bound to its C-terminal tail domain (42). The inactive ERM proteins become activated via phosphorylation of a threonine in the tail (43) and exposure to polyphosphatidylinositides (44), which weaken the FERM/tail interaction. Our data suggest a similar inactive state for UNC-112 (kindlin) when folded, but it should be pointed out that there are some differences. (i) As noted above, there is evidence that, instead of phosphorylation, binding of PAT-4 (ILK) causes the opening up and activation. (ii) The domain structures of UNC-112 (kindlin) and ERM proteins are rather different; according to PFAM (see the Pfam 6.0 Web site), ERM proteins consist of a FERM_N domain and then a FERM_M domain, a FERM C-terminal PH-like domain, and an ezrin/radixin/moesin (ERM) domain. In contrast, UNC-112 (kindlin) consists of a FERM_N domain, followed by a FERM_M domain curiously split by a PH domain. It will be interesting to obtain biophysical evidence, including a crystal structure, for a postulated closed conformation for UNC-112 (kindlin). So far, the structures of only two small portions of kindlins have been reported; there is an NMR structure of the ~100-residue N terminus (or F0 domain) of mouse kindlin-1 (45) and an NMR structure of the ~100-residue PH domain of human kindlin-2 (46).

Functional Implications of New unc-112 Mutants—Classical genetic analysis of unc-112 that led to its molecular cloning (9) identified one missense temperature-sensitive adult Unc mutant, unc-112(r367) (i.e. T85I), residing in sequence N-terminal of the FERM_N domain. This analysis also identified two premature stop mutations, st562 and st582, that result in a Pat...
embryonic lethal phenotype. By two-hybrid analysis, we have determined that full-length r367 (T85I) UNC-112 protein fails to interact with PAT-3 and PAT-4. Additionally, the N- and C-terminal halves of T85I UNC-112 failed to interact with each other. At this point, it is difficult to interpret these results. For example, it is possible that in yeast and in C. elegans, the r367 UNC-112 protein is unstable.

As described here, using random mutagenesis in vitro, we identified three more missense mutations (D382V, T346A, and E349K). Our new missense mutations in UNC-112 were analyzed for their inter- and intramolecular interactions, for their ability to localize to muscle adhesion sites, and for their ability to induce a dominant negative effect using transient ectopic expression. It will be interesting in the future to determine the function of these mutants in vivo. We would test for rescue of an unc-112 null mutant (e.g. st562) with D382V, T346A, or E349K mutant UNC-112 genes under the control of the authentic unc-112 promoter. UNC-112 is required to recruit PAT-4 to muscle adhesion sites; subsequently, PAT-4 recruits UNC-97 and PAT-6, each an essential protein, to these sites (10–12). Therefore, we would expect that D382V, which does not bind to PAT-4, would not be capable of rescuing unc-112 null. In contrast, either T346A or E349K mutant UNC-112 proteins might be expected to rescue.
given that they can interact with PAT-4 and localize to muscle adhesion sites.

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REFERENCES
1. Waterston, R. H. (1988) Muscle in The Nematode Caenorhabditis elegans. (Wood, W. B., ed) pp. 281–335. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
2. Moerman, D. G., and Fire, A. (1997) Muscle. Structure, function, and development. in C. elegans II (Riddle, D. L., T. Blumenthal, T., Meyer, B. I., and Priess, J. R., eds) pp. 417–470. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
3. Moerman, D. G., and Williams, B. D. (2006) Sarcomere assembly in C. elegans muscle. in WormBook: The Online Review of C. elegans Biology, doi/10.1895/wormbook.1.81.1
4. Brenner, S. (1974) The genetics of Caenorhabditis elegans. Genetics 77, 71–94
5. Waterston, R. H., Thomson, J. N., and Brenner, S. (1980) Mutants with altered muscle structure of Caenorhabditis elegans. Dev. Biol. 77, 271–302
6. Zengel, J. M., and Epstein, H. F. (1980) Identification of genetic elements associated with muscle structure in the nematode Caenorhabditis elegans. Cell Motil. 1, 73–97
7. Williams, B. D., and Waterston, R. H. (1994) Genes critical for muscle development and function in Caenorhabditis elegans identified through lethal mutations. J. Cell Biol. 124, 475–490
8. Meissner, B., Warner, A., Wong, K., Norcini, S., I. Khattar, J., Rogalski, T., Somasiri, A., Chaudhry, I., Fox, R. M., Miller, D. M., 3rd, Baillie, D. L., Holt, R. A., Jones, S. I., Marra, M. A., and Moerman, D. G. (2009) An integrated strategy to study muscle development and myofilament structure in Caenorhabditis elegans. PLoS Genet. 5, e1000537
9. Rogalski, T. M., Muller, G. P., Gilbert, M. M., Williams, B. D., and Moerman, D. G. (2000) The UNC-112 gene in Caenorhabditis elegans encodes a novel component of cell-matrix adhesion structures required for integrin localization in the muscle cell membrane. J. Cell Biol. 150, 253–264
10. Mackinnon, A. C., Qadota, H., Norman, K. R., Moerman, D. G., and Williams, B. D. (2002) C. elegans PAT-4/ILK functions as an adaptor protein within integrin adhesion complexes. Curr. Biol. 12, 787–797
11. Lin, X., Qadota, H., Moerman, D. G., and Williams, B. D. (2003) C. elegans PAT-6/actopaxin plays a critical role in the assembly of integrin adhesion complexes in vivo. Curr. Biol. 13, 922–932
12. Norman, K. R., Cordes, S., Qadota, H., Rahmani, P., and Moerman, D. G. (2007) UNC-97/PINCH is involved in the assembly of integrin adhesion complexes in Caenorhabditis elegans body wall muscle. Dev. Biol. 309, 45–55
13. Mercer, K. B., Flaherty, D. B., Miller, R. K., Qadota, H., Tinley, T. L., Moerman, D. G., and Benian, G. M. (2003) Caenorhabditis elegans UNC-98, a C2H2 zinc finger protein, is a novel partner of UNC-97/PINCH in muscle adhesion complexes. Mol. Biol. Cell 14, 2492–2507
14. Miller, R. K., Qadota, H., Landsverk, M. L., Mercer, K. B., Epstein, H. F., and Benian, G. M. (2006) UNC-98 links an integrin-associated complex to thick filaments in Caenorhabditis elegans muscle. J. Cell Biol. 175, 853–859
15. Qadota, H., Mercer, K. B., Miller, R. K., Kibauchi, K., and Benian, G. M. (2007) Two LIM domain proteins and UNC-96 link UNC-97/pinch to myosin thick filaments in Caenorhabditis elegans muscle. Mol. Biol. Cell 18, 4317–4326
16. Qadota, H., and Benian, G. M. (2010) Molecular structure of sarcomere-to-membrane attachment at M-Lines in C. elegans muscle. J. Biomed. Biotechnol. 2010, 864749
17. Wick, M., Bürger, C., Brüsselbach, S., Lucibello, F. C., and Müller, R. (1994) A novel member of human tissue inhibitor of metalloproteinases (TIMP) gene family is regulated during G1 progression, mitogenic stimulation, differentiation, and senescence. J. Biol. Chem. 269, 18953–18960
18. Meves, A., Stremmel, C., Gottschalk, K., and Fässler, R. (2009) The Kindlin protein family. New members to the club of focal adhesion proteins. Trends Cell Biol. 19, 504–513
19. Siegel, D. H., Ashton, G. H., Penagos, H. G., Lee, J. V., Feiler, H. S., Wilm- helmens, K. C., South, A. P., Smith, F. J., Prescott, A. R., Wessagowitz, V., Oyama, N., Akiyama, M., Al Aboud, D., Al Aboud, K., Al Githami, A., Al Hawsawi, K., Al Ismailly, A., Al-Suwaidi, H., Sabin, T., Kaibuchi, K., and Benian, G. M. (2003) UNC-97/PINCH is involved in the assembly of integrin cell adhesion complexes. Cell 114, 431–443
20. Stoker, M., Bauer, M., Schmid, S., Ruppert, R., Schmidt, S., Sixt, M., Wang, H. Y., Sperandio, M., and Fässler, R. (2009) Kindlin-3 is required for beta1 integrin-mediated leukocyte adhesion to endothelial cells. Nat. Med. 15, 300–305
21. Svensson, L., Howarth, K., McDowall, A., Patzak, I., Evans, R., Ussar, S., Moser, M., Metin, A., Fried, M., Tomlinson, I., and Hogg, N. (2009) Leukocyte adhesion deficiency-III is caused by mutations in KIND1L3 affecting integrin activation. Nat. Med. 15, 306–312
22. Dowling, J. J., Gibbs, E., Russell, M., Goldman, D., Minarcik, J., Golden, J. A., and Feldman, E. L. (2008) Kindlin-2 is an essential component of intercalated discs and is required for vertebrate cardiac structure and function. Circ. Res. 102, 423–431
23. Weinstein, E. J., Bourner, M., Head, R., Zakeri, H., Bauer, C., and Mazza-rella, R. (2003) URP1. A member of a novel family of PH and FERM domain-containing membrane-associated proteins is significantly overexpressed in lung and colon carcinomas. Biochim. Biophys. Acta 1637, 207–216
24. Boyd, R. S., Adams, P. J., Patel, S., Loader, J. A., Berry, J., Redpath, N. T., Poyser, H. R., Fletcher, G. C., Burgess, N. A., Stamps, A. C., Hudson, L., Smith, P., Griffiths, M., Willis, T. G., Karran, E. L., Oscier, D. G., Catovery, D., Terrett, J. A., and Dyer, M. J. (2003) Proteomic analysis of the cell-surface membrane in chronic lymphocytic leukemia. Identification of two novel proteins, BCNP1 and MIG2B. Leukemia 17, 1605–1612
25. James, P., Halliday, I., and Craig, E. A. (1996) Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics 144, 1425–1436
26. Mercer, K. B., Miller, R. K., Tinley, T. L., Sheth, S., Qadota, H., and Benian, G. M. (2006) Caenorhabditis elegans UNC-96 is a new component of M-lines that interacts with UNC-98 and paramysosin and is required in adult muscle for assembly and/or maintenance of thin filaments. Mol. Biol. Cell 17, 3832–3847
27. Himms-Helmersson, K., K. Meyer, B. I., and Rand, J. B. (1993) Synaptic function is impaired but not eliminated in C. elegans mutants lacking synaptogamin. Cell 73, 1291–1305
28. Qadota, H., Blangy, A., Xiong, G., and Benian, G. M. (2008) The DH-PH region of the giant protein gene UNC-89 activates RHO-1 GTPase in Caenorhabditis elegans body wall muscle. J. Mol. Biol. 383, 747–752
29. Cadwell, R. C., and Joyce, G. F. (1992) Randomization of genes by PCR mutagenesis. PCR Methods Appl. 2, 28–33
30. Takita, Y., Takahara, M., Nogami, S., Anraku, I., and Ohyu, Y. (1997) Applications of the long and accurate polymerase chain reaction method in yeast molecular biology. Direct sequencing of the amplified DNA and

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its introduction into yeast. Yeast 13, 763–768
33. Qadota, H., McGaha, L. A., Mercer, K. B., Stark, T. J., Ferrara, T. M., and Benian, G. M. (2008) A novel protein phosphatase is a binding partner for the protein kinase domains of UNC-89 (Obscurin) in Caenorhabditis elegans. Mol. Biol. Cell 19, 2424–2432
34. Yochem, J., Gu, T., and Han, M. (1998) A new marker for mosaic analysis in Caenorhabditis elegans indicates a fusion between hyp6 and hyp7, two major components of the hypodermis. Genetics 149, 1323–1334
35. Mitani, S. (1995) Genetic regulation of mec-3 gene expression implicated in the specification of the mechanosensory neuron cell types in Caenorhabditis elegans. Dev. Growth Differ. 37, 551–557
36. Miller, D. M., 3rd, Ortiz, I., Berliner, G. C., and Epstein, H. F. (1983) Differential localization of two myosins within nematode thick filaments. Cell 34, 477–490
37. Zervas, C. G., Gregory, S. L., and Brown, N. H. (2001) Drosophila integrin-linked kinase is required at sites of integrin adhesion to link the cytoskeleton to the plasma membrane. J. Cell Biol. 152, 1007–1018
38. Montanez, E., Ussar, S., Schifferer, M., Böl, M., Zent, R., Moser, M., and Fässler, R. (2008) Kindlin-2 controls bidirectional signaling of integrins. Genes Dev. 22, 1325–1330
39. Dowling, J. J., Vreede, A. P., Kim, S., Golden, J., and Feldman, E. L. (2008) Kindlin-2 is required for myocyte elongation and is essential for myogenesis. BMC Cell Biol. 9, 36
40. Persad, S., Attwell, S., Gray, V., Mawji, N., Deng, J. T., Leung, D., Yan, J., Sanghera, J., Walsh, M. P., and Dedhar, S. (2001) Regulation of protein kinase B/Akt-serine 473 phosphorylation by integrin-linked kinase. Critical roles for kinase activity and amino acids arginine 211 and serine 343. J. Biol. Chem. 276, 27462–27469
41. Laub, M. T., and Goulian, M. (2007) Specificity in two-component signal transduction pathways. Annu. Rev. Genet. 41, 121–145
42. Reczek, D., and Bretscher, A. (1998) The carboxyl-terminal region of EBP50 binds to a site in the amino-terminal domain of ezrin that is masked in the dormant molecule. J. Biol. Chem. 273, 18452–18458
43. Pietromonaco, S. F., Simons, P. C., Altman, A., and Elias, L. (1998) Protein kinase C-θ phosphorylation of moesin in the actin-binding sequence. J. Biol. Chem. 273, 7594–7603
44. Matsui, T., Yonemura, S., and Tsukita, S. (1999) Activation of ERM proteins in vivo by Rho involves phosphatidylinositol 4-phosphate 5-kinase and not ROCK kinases. Curr. Biol. 9, 1259–1262
45. Goult, B. T., Bouaouina, M., Harburger, D. S., Bate, N., Patel, B., Anthis, N. J., Campbell, I. D., Calderwood, D. A., Barsukov, I. L., Roberts, G. C., and Critchley, D. R. (2009) The structure of the N terminus of kindlin-1. A domain important for αib3 integrin activation. J. Mol. Biol. 394, 944–956
46. Liu, J., Fukuda, K., Xu, Z., Ma, Y. Q., Hirbawi, J., Mao, X., Wu, C., Plow, E. F., and Qin, J. (2011) Structural basis of phosphoinositide binding to kindlin-2 protein pleckstrin homology domain in regulating integrin activation. J. Biol. Chem. 286, 43334–43342
47. Wilson, K. J., Qadota, H., Mains, P. E., and Benian, G. M. (2012) UNC-89 (obscurin) binds to MEL-26, a BTB domain protein, and affects the function of MEI-1 (katanin) in striated muscle of C. elegans. Mol. Biol. Cell 23, 2623–2634
Supplemental Figure 1

A

| kDa | MBP | MBP-UNC-112C |
|-----|-----|--------------|
| 150 |     |              |
| 100 |     |              |
| 75  |     |              |
| 50  |     |              |
| 37  |     |              |
| 25  |     |              |

CBB staining

B

| kDa | MBP-UNC-112C | GST | GST-PAT-4 |
|-----|--------------|-----|-----------|
| 150 |              |     |           |
| 100 |              |     |           |
| 75  |              |     |           |
| 50  |              |     |           |
| 37  |              |     |           |
| 25  |              |     |           |

CBB staining
