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Identification of a novel Cdc42 GEF that is localized to the PAT-3-mediated adhesive structure

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Abstract

In the model organism *Caenorhabditis elegans*, UNC-112 is colocalized with PAT-3/β-integrin and is a critical protein in the formation of PAT-3-mediated adhesive structure in body-wall muscle cells. However, the signaling pathway downstream of PAT-3/UNC-112 is largely unknown. To clarify the signaling pathway from PAT-3/UNC-112 to the actin cytoskeleton, we searched for and identified a novel Dbl homology/pleckstrin homology (DH/PH) domain containing protein, UIG-1 (UNC-112-interacting guanine nucleotide exchange factor-1). UIG-1 was colocalized with UNC-112 at dense bodies in body-wall muscle cells. UIG-1 showed CDC-42-specific GEF activity in vitro and induced filopodia formation in NIH 3T3 cells. Depletion of CDC-42 or PAT-3 in the developmental stage, by RNAi, prevented the formation of continuous actin filament in body-wall muscle cells. Taken together, these results suggest that UIG-1 links a PAT-3/UNC-112 complex to the CDC-42 signaling pathway during muscle formation.
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Keywords: Integrin; Cdc42; GEF; PAT-3; UNC-112; Signal transduction; *Caenorhabditis elegans*

The interaction between cell and extracellular matrix (ECM) plays crucial roles in the regulation of cell morphology, migration, growth, and differentiation. Focal adhesion (FA) is regarded as the cell-ECM adhesive structure that is developed in cultured cells. FA consists of clustered integrin ECM receptors that link external ECM components to internal actin cytoskeletons [1].

In the nematode *Caenorhabditis elegans*, many homologues of the components localized to FAs are found in the attachment structure between the myofilament lattice of a body-wall muscle cell membrane and adjacent basement membranes, called dense bodies and M-lines [2–5]. Dense bodies are comparable to vertebrate striated muscle Z-lines [2] and include α-integrin/PAT-2, β-integrin/PAT-3, vinculin/DEB-1 [6,7], α-actinin [6,8], talin [9], UNC-97/PINCH [10], and UNC-112/Mig-2 [11,12] proteins. UNC-112 is a *C. elegans* homologue of human Mig-2, which interacts with migfilin, and fillamin and is required for the recruitment of migfilin to FAs [12]. UNC-112 is known to be localized to dense bodies, which consist of PAT-3/β-integrin and DEB-1/vinculin, and to anchor actin filaments to the cell membrane [11]. The UNC-112 protein contains a region that is highly homologous with talin and members of the FERM superfamily proteins [11]. UNC-112 is thought to play an important role as an adaptor protein that recruits FA-associated proteins in an integrin cluster.

Integrin has been reported to regulate the activity of Rho-family small GTPases [13,14], via integrin-binding proteins, FA kinase, and paxillin [15]. Rho-family small GTPases play a pivotal role in cytoskeletal rearrangements and in cell adhesion in response to extracellular signals. Rho-family small GTPases have GDP-bound

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inactive and GTP-bound active forms, which are interchangeable by means of GDP/GTP exchange and GTPase reactions [16]. GDP/GTP exchange reactions are regulated by various guanine nucleotide exchange factors (GEFs). GTP-bound forms of Rho-family small GTPases exert their biological functions through interaction with specific effectors [17–19].

In this study, to investigate the signaling pathway from integrin, we identified the cDNA encoding the novel protein that interacts with UNC-112, by using yeast two-hybrid screening. This protein contains a Dbl homology (DH) domain opposed to the pleckstrin homology (PH) domain (DH/PH domain). The DH/PH domain is a characteristic sequence for the GEF of Rho-family small GTPases [20–22]. Thus, we named this novel protein "UNC-112-interacting GEF-1" (UIG-1).

Materials and methods

Caenorhabditis elegans culture. Caenorhabditis elegans was cultivated on NGM agar plates with OP50 bacteria, according to standard techniques [23]. Wild-type worms were the N2 strain of the Bristol variety (provided by Caenorhabditis Genetic Center, MN, USA). Nematode culture and observation were performed at 20 °C.

Cell culture. COS7 cells were grown in DMEM containing 10% FBS. NIH 3T3 cells were grown in DMEM containing 10% CS. For the transfection experiments of COS7 cells and NIH 3T3 cells, lipofectamine and lipofectamine plus (Invitrogen, Carlsbad, CA, USA) were used, respectively.

Materials and chemicals. Genomic DNA of *uig-1* was amplified from the wild-type *C. elegans* genome, by polymerase chain reaction (PCR), and was cloned into pPD95.77 (provided by Dr. A. Fire). Full-length cDNA of *uig-1* was amplified from the *C. elegans* cDNA library RB2 (gift of Dr. Robert Barstead, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA). Full-length and deletion fragments of *uig-1* cDNA were cloned into mammalian expression vector pEGFP-C3. Full-length *unc-112* cDNA cloned into mammalian expression vector pEF-BOS-Myc. The following antibodies were used: anti-GFP polyclonal antibody (Medical and Biological Laboratories, Aichi, Japan); anti-GFP monoclonal antibody (Nacalai tesque, Kyoto, Japan); anti-Myc monoclonal antibody clone 9E10 (Amersham Bioscience, Piscataway, NJ, USA); anti-UNC-112 antibody was raised against the GST-fused 1–638 aa fragment of UNC-112 [39].

Microinjection of C. elegans. Microinjections of N2 hermaphrodites were performed as described in [24]. pKK100 {*uig-1p-uig-1*::*gfp*} plasmid DNAs (1 μg/ml) were injected into the gonad syncytium of wild-type hermaphrodites. The following strains were obtained: NR326 (kzEx100).

Immunohistochemical analysis. NIH 3T3 cells were serum starved for 12 h and then transfected with pEGFP constructs. The transfected cells were cultured in DMEM without serum for 12 h fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 10 min, and then treated with PBS containing 0.2% Triton X-100 for 10 min. Whole-mount worm immunostaining was performed on worms fixed in 1% formaldehyde, following a protocol described elsewhere [25]. For whole-mount worm phalloidin staining, worms were fixed with 100% MeOH and stained with Alexa fluor 488-conjugated phalloidin (Invitrogen, Carlsbad, CA, USA). Immunofluorescent images were obtained by using an LSM 510 laser-scanning microscope (Carl Zeiss, Oberkochen, Germany) built around a Zeiss Axio-vert 100M.

Yeast two-hybrid screening. The vectors pGAD-C1 and pGBDU-C1 and yeast strain PJ69-4A [26] were used for screening. Yeast was incubated at 30 °C in this study. Plasmid isolation from yeast was performed by use of the standard protocol [27].

Coimmunoprecipitation assay. COS7 cells were extracted by the addition of lysis buffer [20 mM Tris/HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1% (w/v) NP-40] and clarified by centrifugation at 100,000*g* for 20 min at 4 °C. The soluble supernatant was incubated with anti-GFP antibody. The immunocomplexes were then precipitated with protein G–Sepharose (Pharmacia LKB Biotechnology AB).

GTP-association assay. Effects of GEF fragments on the association of [^{35}S]GTPγS with Rho-family small GTPases were assayed as described previously [28]. The assay was carried out, at 25 °C, by addition of 10 μM [^{35}S]GTPγS and GST-UIG-1-NPH fragment to the reaction mixture [50 mM Tris/HCl (pH 8.0), 1 mM dithiothreitol, 10 mM $MgCl_2$, and 2.9 mM EDTA]. The reaction was stopped at the indicated time by addition of 2 ml of an ice-cold solution [20 mM Tris/HCl (pH 7.5), 20 mM $MgCl_2$, and 100 mM NaCl]. The diluted mixtures were filtered through nitrocellulose filters, and the radioactivity of the material trapped on the filters was counted.

L1-feeding RNAi. Feeding RNAi was performed according to [29]. Briefly, bacteria were cultured until an OD_{595} of 1.0 in LB supplemented with 100 μg/ml ampicillin and 100 μg/ml tetracycline, induced with IPTG (final volume of 100 μM) for 3 h, and seeded onto NGM agar supplemented with 100 μg/ml ampicillin and 100 μg/ml tetracycline. Eggs were laid onto the feeding plates for 12 h, and RNAi phenotypes were checked at 72 h after hatching. Full-length cDNA of *cdc-42* or *pat-3* was cloned into feeding RNAi vector pPD129.36 [30] and transformed into HT115 (DE3), an RNase III-deficient *Escherichia coli* strain with IPTG-inducible T7 polymerase activity [29].

Results and discussion

UIG-1 is a UNC-112-interacting molecule

To clarify the signaling pathway from the integrin ECM receptor to the actin cytoskeleton, we searched for the molecules that interact with UNC-112 by using yeast two-hybrid screening [39] and identified a cDNA encoding 919 amino acids from the RB2 *C. elegans* cDNA library. The cDNA product was identical to the novel protein encoded by F32F2.1 cDNA. The F32F2.1 open-reading frame was identified by using the Genefinder program [31] and WormBase (www.wormbase.org; Fig. 1A). Adult animals homozygous for the *ok884* mutation (RB978) were healthy and fertile, however, disorganization of body-wall muscle filament was observed under a polarized microscope (Fig. 1B). F32F2.1 protein had a DH domain in tandem with a PH domain that is characteristic for the Dbl family of GEF proteins (Fig. 1C) [32]. The DH/PH domain of UIG-1 showed a sequence similarity (20.7%) to *Saccharomyces cerevisiae* Cdc24p [33]. In mammals, the common-site lymphoma/leukemia GEF (Clg) is reported to be a mouse homologue of F32F2.1 [34]. However, the localization or function of Clg is largely unknown.

To confirm the interaction between UIG-1 and UNC-112 in vivo, a coimmunoprecipitation assay was performed. COS7 cells were cotransfected with expression constructs for the full-length of enhanced GFP (EGFP)-fused UIG-1 (EGFP-UIG-1 full) and the

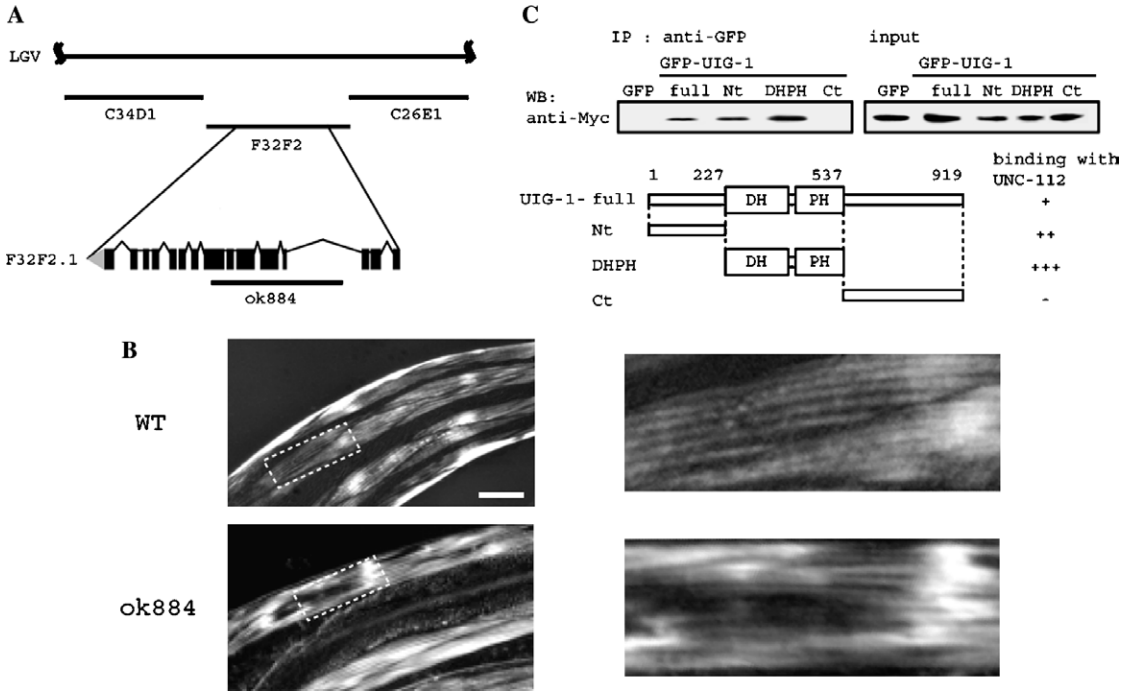

Fig. 1. UIG-1 is a novel UNC-112-interacting molecule. (A) Diagram of a 13-kb genomic DNA fragment containing ORF of F32F2.1/*uig-1*. The exons are represented as boxes. The sequence alteration corresponding to the *uig-1* deletion mutation allele (ok884) is also indicated. (B) Abnormal structure in body-wall muscle cells of RB978. Body-wall muscle cells of WT and RB978 (*ok887*) adult worms were observed under a polarized microscope. A bands are observed as white lines. Bar, 10 μm. (C) Coimmunoprecipitation assay of UIG-1 and UNC-112. Indicated *egfp-uig-1* fragments were cotransfected with full-length of *Myc-unc-112* into COS7 cells and immunoprecipitated with anti-GFP antibody. Coimmunoprecipitated Myc-UNC-112 (IP: anti-GFP) and input Myc-UNC-112 (input) are shown. The results are representative of three independent experiments. Summary of the interaction between UIG-1 and UNC-112 is shown below. The structures of UIG-1 and its deletion fragments are represented. DH, Dbl homology, PH, pleckstrin homology.

full-length of Myc-fused UNC-112 (Myc-UNC-112). When EGFP-UIG-1-full or EGFP was immunoprecipitated with anti-GFP antibody, Myc-UNC-112 was detected in the immunoprecipitate of EGFP-UIG-1-full but not in that of EGFP (Fig. 1C). This result indicates that UIG-1 interacts with UNC-112 in vivo. Next, to narrow down the binding region between UIG-1 and UNC-112, we performed a coimmunoprecipitation assay using deletion fragments of UIG-1, the UIG-1 N-terminal (Nt) region, DH/PH domain (DHPH), and C-terminal (Ct) region (Fig. 1C). When EGFP-UIG-1 fragments were immunoprecipitated with anti-GFP antibody, Myc-UNC-112 was detected in the immunoprecipitates of EGFP-UIG-1 Nt and DHPH but not in that of EGFP-UIG-1 Ct (Fig. 1C). Consistent results were obtained by yeast two-hybrid assay, using the same fragments (data not shown). These results indicate that the Nt region and/or the DH/PH domain of UIG-1 are necessary for the interaction with UNC-112.

UIG-1 is colocalized with UNC-112 at dense bodies in the body-wall muscle of C. elegans

To observe the localization of UIG-1, we generated a transgene containing the entire *uig-1* coding region and 2 kb of its upstream regulatory sequence, which was fused to GFP and injected into wild-type worms. GFP fluorescence in adult hermaphrodites carrying GFP-UIG-1 was observed in the body-wall, vulval, uterine, pharyngeal, and anal muscles (data not shown). In the body-wall muscle, GFP-UIG-1 was localized to dense bodies and was weakly localized to muscle-cell boundaries in regions of contact with adjacent muscle cells (Figs. 2A and B). In the same body-wall muscle cell, endogenous UNC-112 was localized to dense bodies, M-lines, and muscle-cell boundaries, and was colocalized, with GFP-UIG-1, to dense bodies (Figs. 2C–F). UNC-112 is reported to be colocalized with PAT-3/β-integrin at dense bodies and M-lines [11]. These results, together with previous observations, suggest that UIG-1 is colocalized with PAT-3/β-integrin at dense bodies.

UIG-1 shows Cdc42-specific GEF activity in vitro

Next, we examined the GEF activity of UIG-1 for Rho-family GTPases in vitro. In this assay, we used a fragment of UIG-1 containing the DH/PH domain (NPH fragment, 1–537 aa). The NPH fragment of UIG-1 enhanced the association of [^{35}S]GTPγS with CDC-42 but not with RHO-1, CED-10/Rac1, and MIG-2 (Fig. 3A). We next examined the GEF activity of UIG-1 for human small GTPases RhoA, Rac1, and

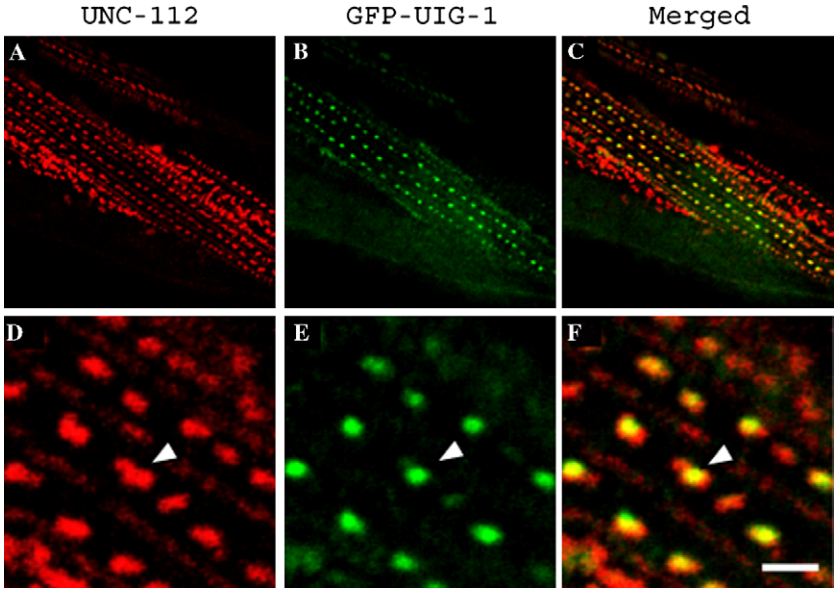

Fig. 2. UIG-1 colocalizes with UNC-112 at dense bodies. Subcellular localization of UIG-1 and UNC-112 in *C. elegans* body-wall muscle cells. NR326 (wild-type, *uig-1p-uig-1*::*gfp*) worms were grown for 72 h at 20 °C, and then fixed and doubly stained with anti-UNC-112 (A,D) and anti-GFP antibodies (B,E). The merged images are shown in (C,F). Higher-magnification images of A–C are shown in D–F, respectively. Arrowheads indicate dense bodies. Bar, 2 μm.

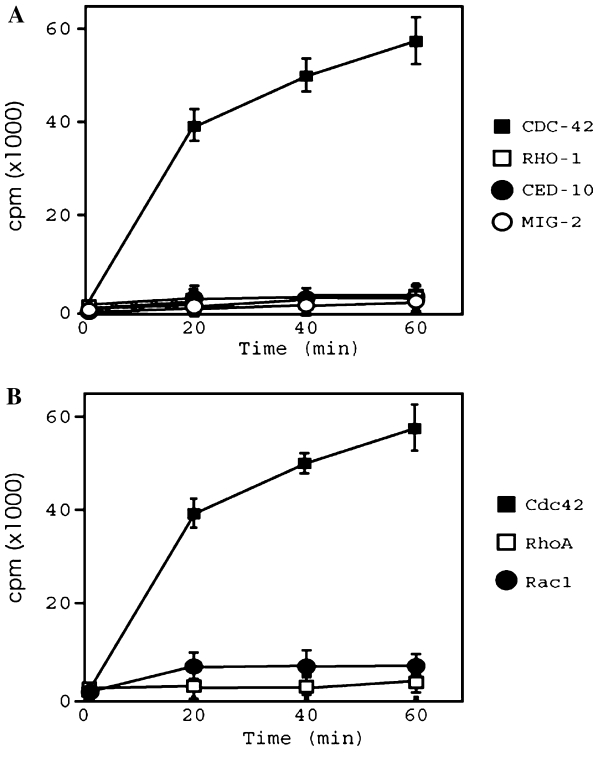

Fig. 3. UIG-1 shows Cdc-42-specific GEF activity in vitro. The effect of UIG-1 on the association of GTP for *C. elegans* (A) or mammalian (B) Rho-family small GTPases in vitro was observed. (A) CDC-42 (closed square), RHO-1 (open square), CED-10 (closed circle), and MIG-2 (open circle), (B) Cdc42 (closed square), RhoA (open square), and Rac1 (closed circle). The association of [^{35}S]GTPγS with the GDP-bound form of Rho-family small GTPases was carried out by addition of 10 μM [^{35}S]GTPγS and the reaction was stopped at 20, 40 or 60 min. Values are means of triplicate samples ± range. Similar results were obtained in two independent experiments.

Cdc42. The NPH fragment of UIG-1 also enhanced the association of [^{35}S]GTPγS specifically with Cdc42 but not with RhoA and Rac1 (Fig. 3B). These results indicate that UIG-1 is a *C. elegans* and mammalian Cdc42-specific GEF.

UIG-1 induces filopodia formation in NIH 3T3 cells

It has been reported that the constitutively active form of Cdc42 induces the formation of filopodia in Swiss 3T3 [35,36] and NIH 3T3 fibroblasts [37]. Since UIG-1 shows GEF activity toward mammalian Cdc42, we thought that NIH 3T3 cells were appropriate for evaluating the effects of UIG-1 as a Cdc42 GEF. Serum-deprived NIH 3T3 cells were transfected with an EGFP vector, EGFP-UIG-1-DHPH fragment (227–537 aa) or an EGFP-fused, constitutively active form of Cdc42 (Cdc42V12) (Fig. 4A). About 17% of control cells expressing control EGFP alone showed filopodia. Under this condition, Cdc42V12 induced the formation of filopodia in about 38% of cells, and the UIG-1 DHPH fragment induced the formation of filopodia in about 30% of cells (Fig. 4B). This induction of filopodia in NIH 3T3 cells may have been due to activation of Cdc42 by UIG-1. These results indicate that UIG-1 acts as a Cdc42 GEF in vivo.

CDC-42 is required for the organization of body-wall muscle structure in C. elegans

The *cdc-42* gene is a unique Cdc42 homologue in *C. elegans*. Depletion of CDC-42 by RNAi disrupts polar-

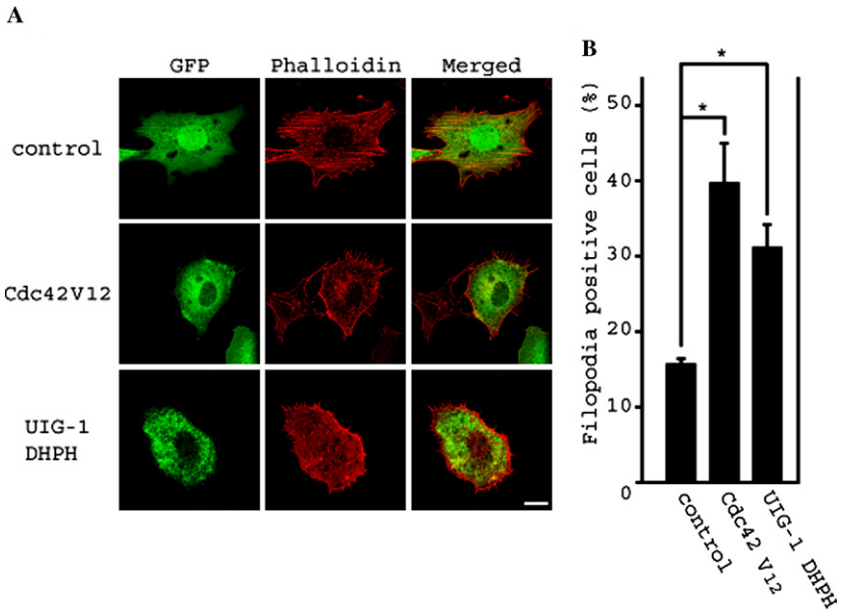

Fig. 4. UIG-1 induced filopodia formation in vivo. Effect of UIG-1 DH/PH fragment on the actin cytoskeleton in NIH 3T3 cells. (A) Serum-depleted NIH 3T3 cells were transfected with indicated constructs. Green color shows the EGFP (control), EGFP-Cdc42 V12 or EGFP-UIG-1 DHPH. (B) The ratio of the cells containing filopodia to the GFP-positive cells. Each value represents the mean ± SD of three independent experiments. Asterisks indicate statistical significance (Student's t test; $p < 0.01$). More than 50 cells were counted in each experiment. Bar, 10 μm.

ization in the early embryo, including the extent of pseudocleavage and spindle orientation at the two-cell stage, and causes early embryonic lethal [38]. To investigate whether *cdc-42* is required in the development of *C. elegans* body-wall muscle, we performed L1-feeding RNAi. L1 larvae of wild-type worms were grown on bacteria expressing dsRNA of *cdc-42*, for 72 h, and were then stained with Alexa fluor 488-labeled phalloidin. Depletion of CDC-42 resulted in a movement defect and inhibited the organization of consecutive actin filaments in body-wall muscles (Figs. 5B and E). On the other hand, *pat-3*/β-integrin-depleted adult worms showed both aggregated actin structure and contracted but continuous actin filaments (Figs. 5C and F). These results suggest that *cdc-42* is required for the formation of consecutive actin fiber, and that PAT-3/β-integrin is required for both organizing the actin fibers and anchoring the fibers to the adjacent basement membranes.

Role of UNC-112 as an adaptor protein in mediation of the signaling complex

The molecular mechanisms by which the localization and/or activation of GEFs are modulated by extracellu-

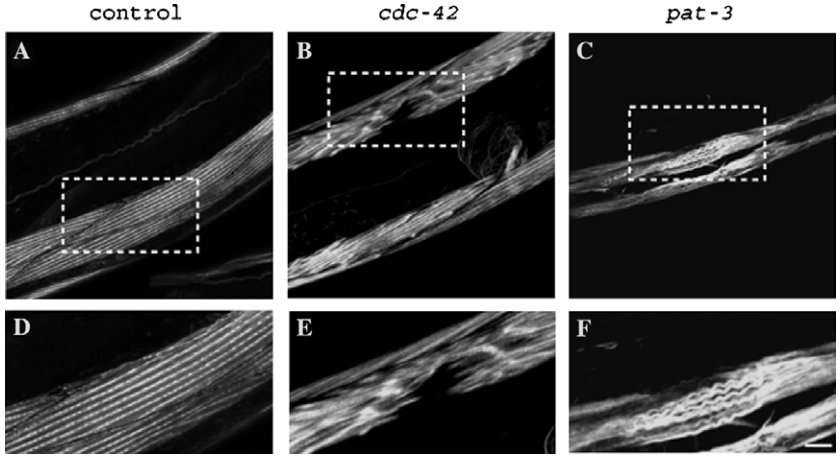

Fig. 5. CDC-42 and PAT-3 are required for normal development of body-wall muscle cells. Body-wall muscle actin fibers of L1-feeding RNAi-treated worms. Wild-type L1 larvae were fed with bacteria harboring pPD129.36 (control; A,D) pPD129.36-*cdc-42* (*cdc-42*; B,E) or pPD129.36- *pat-3* (*pat-3*; C,F) for 72 h at 20 °C and subjected to phalloidin staining. Higher-magnification images of A–C are shown in D–F, respectively. The results shown are representative of three independent experiments. Bar, 5 μm.

lar signals are largely unknown. In this study, we identified a novel Cdc42-specific GEF, UIG-1, as a UNC-112-interacting molecule. UIG-1 was constitutively colocalized with UNC-112 at dense bodies, suggesting that UNC-112 regulates the localization of UIG-1 but not the activation of UIG-1. It has been reported that UNC-112 is required for the localization of several dense body-localized proteins, including PAT-4/ILK, PAT-6/affixin/CH-ILKBP/actopaxin/parvin, and UNC-97/PINCH [10,39,40]. These reports and our results suggest that UNC-112 acts as a scaffold protein to link PAT-3/β-integrin to CDC-42. Since the activation of CDC-42 is necessary for the formation of muscle, PAT-3/β-integrin, UNC-112, and/or some other unidentified molecules may account for the activation of UIG-1. Further studies are necessary for understanding the mode of activation of UIG-1 downstream of PAT-3/β-integrin.

Role of Cdc42 in the integrin-mediated signaling pathway during muscle-cell development

Recent studies using cultured cells have shown that Cdc42 is activated during integrin-dependent cell spreading, via αPix, which is a Cdc42/Rac1-specific GEF [41,42]. In *C. elegans*, the role of CDC-42 in the integrin-mediated signaling pathway remains unclear. In this study, we examined whether CDC-42 is required during the developmental stage, by means of L1-feeding RNAi. The body-wall muscle cells of worms treated with *cdc-42* L1-feeding RNAi showed disorganized and discontinuous actin filament (Fig. 5). Mutant worms with a *uig-1* (*ok884*) deletion had discontinuous muscle fiber, like the worms treated with *cdc-42* RNAi (Figs. 1B, 5B and E). However, the penetrance was low, and the phenotype was weaker than that in the worms treated with *cdc-42* L1-feeding RNAi. The weakness of this phenotype suggests the redundant function of other CDC-42 GEFs, such as FGD/*exc-5* and/or βPix/K11E4.4, that have been reported to be expressed in muscle cells, in the integrin-mediated signaling pathway (*C. elegans* SAGE Project, http://elegans.bcgsc.ca/perl/sage_summary).

Role of UIG-1 in the adhesive structure

Genetic studies using *C. elegans* body-wall muscle cells have revealed that there are two parallel molecular complexes under PAT-3/β-integrin in dense bodies. One is UNC-112/Mig-2, PAT-4/ILK, PAT-6/actopaxin, and UNC-97/PINCH complex, and the other is DEB-1/vinculin complex [39]. To clarify the protein–protein interaction between all these molecules, we performed the binding assay by using a yeast two-hybrid system (data not shown). Interaction between DEB-1 and UNC-112, PAT-4, PAT-6 or UNC-97 was not observed, however,

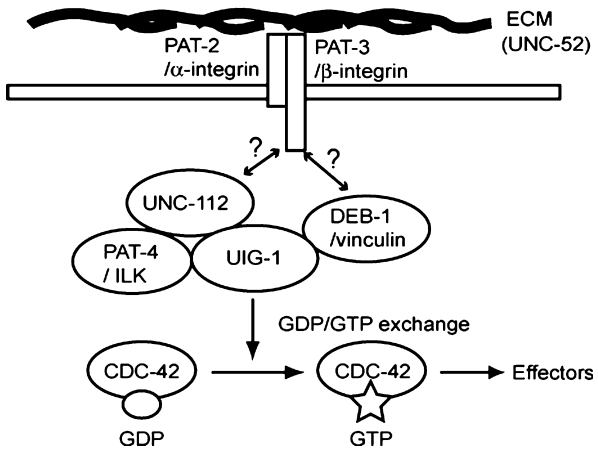

Fig. 6. Model schema to regulate the CDC-42 activation under integrin UNC-112 is thought to work as the adaptor protein that recruits signaling molecules under PAT-3/PAT-2 heterodimer. UIG-1, a CDC-42 specific GEF, interacts with UNC-112 and is localized to the PAT-3/β-integrin-mediated adhesive structure. UIG-1 should be activated by other dense body localized proteins and activates CDC-42. In addition, UIG-1 interacts with both PAT-4/ILK and DEB-1/vinculin, and should work as a linker protein in a PAT-3/β-integrin-mediated structure.

UIG-1 interacted with DEB-1. In addition, UIG-1 interacted with UNC-112, PAT-4, and UNC-97. UIG-1 is the only molecule that interacts with the components of both molecular complexes. These results suggest that UIG-1 works not only as a CDC-42 specific GEF but also as a "linker protein" that links UNC-112/PAT-4 complex and DEB-1 complex at dense bodies (Fig. 6).

Acknowledgments

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