

Small GTPase CDC-42 promotes apoptotic cell corpse clearance in response to PAT-2 and CED-1 in *C. elegans*

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The rapid clearance of dying cells is important for the well-being of multicellular organisms. In *C. elegans*, cell corpse removal is mainly mediated by three parallel engulfment signaling cascades. These pathways include two small GTPases, MIG-2/RhoG and CED-10/Rac1. Here we present the identification and characterization of CDC-42 as a third GTPase involved in the regulation of cell corpse clearance. Genetic analyses performed by both loss of *cdc-42* function and *cdc-42* overexpression place *cdc-42* in parallel to the *ced-2/5/12* signaling module, in parallel to or upstream of the *ced-10* module, and downstream of the *ced-1/6/7* module. CDC-42 accumulates in engulfing cells at membranes surrounding apoptotic corpses. The formation of such halos depends on the integrins PAT-2/PAT-3, UNC-112 and the GEF protein UIG-1, but not on the canonical *ced-1/6/7* or *ced-2/5/12* signaling modules. Together, our results suggest that the small GTPase CDC-42 regulates apoptotic cell engulfment possibly upstream of the canonical Rac GTPase CED-10, by polarizing the engulfing cell toward the apoptotic corpse in response to integrin signaling and *ced-1/6/7* signaling in *C. elegans*.

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During development and in tissue homeostasis, multicellular organisms frequently use apoptosis to eliminate cells that are useless or potentially dangerous. Apoptotic cells are readily recognized, internalized and degraded by neighboring or specialized engulfing cells. Rapid clearance of unwanted cells avoids the release of harmful intracellular contents into the surroundings that can lead to inflammation and autoimmune disease.¹

The nematode *C. elegans* serves as a simple yet powerful genetic model organism to study cell corpse clearance *in vivo*. Many genes involved in recognition, internalization or degradation of apoptotic corpses have been identified through forward and reverse genetic screens in the past two decades.² Loss of engulfment activity not only results in the persistence of cell corpses, but also leads to the survival of some cells destined to die,³ and – in some cases – leads to impaired cell migration.⁴

Phenotypic, genetic and biochemical analyses of the early ‘classical’ *ced* (cell death abnormal) genes led to the identification of three partially redundant signaling cascades that cooperate to regulate cytoskeletal rearrangements and the migration of the engulfing cell around the apoptotic corpse.^{5–9} In the first pathway, the transmembrane protein CED-1/MEGF10 has been proposed to act as a cell corpse receptor¹⁰ that binds to exposed phosphatidylserine (PS), either directly or indirectly through the action of the bridging

molecule TTR-52/TTR.^{11,12} The lipid transporter homolog CED-7 also plays a role at this stage, at least in part by promoting the exposure of PS in the outer leaflet of the doomed cell.¹³ The adaptor protein CED-6/GULP transduces the signal(s) from CED-1 downstream to CED-10/Rac1 and DYN-1/Dynamin to drive cytoskeletal rearrangements and phagosome maturation.^{8,14–16} In the second pathway, activation of CED-10 is promoted by the bipartite GEF (guanine exchange factor) complex composed of CED-12/Elmo–CED-5/Dock180.^{17–20} This GEF complex in turn is regulated by CED-2/CrkII and the small GTPase MIG-2/RhoG. In the third pathway, the cytoskeletal regulator ABL-1/Abl suppresses corpse clearance by inhibiting ABI-1/Abl-interacting protein.²¹ Active GTP-loaded CED-10 promotes the extensive cytoskeletal rearrangements that are essential for proper cell corpse internalization.⁸ This process is negatively regulated by the GTPase-activating protein (GAP) SRGP-1/srGAP1 that facilitates GTP hydrolysis in CED-10.²²

Here we present the identification and characterization of *cdc-42* (*cell division control protein-42*) as an additional mediator of engulfment signaling regulated by SRGP-1 (Slit/Robo GTPase activating protein 1). Our epistatic analyses, performed with *cdc-42(lf)* mutants and *cdc-42* overexpression experiments, suggest that *cdc-42* acts downstream or in parallel to the *ced-1/6/7* and in parallel to the *ced-2/5/12* signaling cascades. Using a functional and rescuing

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Abbreviations: *cdc*, cell division control; *ced*, cell death abnormal; *pat*, paralyzed arrest at twofold; GAP, GTPase-activating protein; GEF, guanosine exchange factor; *gf*, gain of function; *lf*, loss of function; *mig*, migration defective; RNAi, RNA interference; *Srqp-1*, Slit/Robo GTPase activating protein 1; UTR, untranslated region

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GFP::CDC-42 protein, we show that CDC-42 is recruited to the cell membrane surrounding apoptotic corpses, and that this localization requires the integrin- α PAT-2 but not the canonical *ced-1/6/7* or *ced-2/5/12* cascades.

Taken together, our results suggest that the small GTPase CDC-42 regulates apoptotic cell engulfment upstream of the canonical Rac GTPase CED-10, possibly by polarizing the engulfing cell toward the apoptotic corpse in response to integrin signaling. Our data confirm and significantly expand on recent results published by Hsieh *et al.*,²³ who independently identified CDC-42 as an engulfment regulator downstream of integrin- α PAT-2.

Results

Identification of *cdc-42* as a mediator of engulfment signaling in *C. elegans*. We previously reported the identification of SRGP-1 as a negative regulator of corpse engulfment.²² Biochemical and genetic evidence suggested that SRGP-1 acts as a GAP for the Rac protein CED-10. Because loss of *ced-10* only partially eliminated the suppressive activity of *srgp-1* mutations, we surmised that *srgp-1* might also modulate other Rho GTPases important for cell corpse clearance (Figure 1a).

In order to identify these candidate GTPases, we used RNA interference (RNAi) to knock down known Rho family members (including *rac-2*, *crp-1*, *chw-1*, *rho-1*, *cdc-42* and *ced-10*) in a sensitized *ced-5*; *srgp-1* double mutant background. *ced-5* mutants contain many persistent cell corpses in larval L1 heads as compared with wild type. These corpse numbers are significantly reduced in *ced-5*; *srgp-1* double mutants, at least in part because of an increased activity of CED-10/Rac1 (Supplementary Figure S1).²² We found that in this sensitized background, reduction of *cdc-42* function not only fully reverted the *srgp-1* suppressor phenotype of *ced-5* mutants, but also significantly enhanced the corpse persistent phenotype of *ced-5* single mutants (Figure 1a). We therefore concluded that *cdc-42* might be an additional candidate Rho GTPase involved in cell corpse clearance in *C. elegans*.

We previously showed that the GAP domain of SRGP-1 binds GTP-bound (active) CED-10/Rac1 *in vitro*.²² Using bacterially expressed His-tagged CDC-42 variants that mimic the GTP- and GDP-bound states (Q61L and T17N, respectively), we found that the SRGP-1 GAP domain also specifically interacts with the active form of CDC-42 (Figure 1b). This is consistent with previous findings, where CDC-42 (Cdc42 in mammals) activity is regulated by SRGP-1 (srGAP).²⁴ Based on these findings, we concluded that SRGP-1, in addition to CED-10, might also regulate engulfment signaling through the Rho GTPase family member CDC-42 in *C. elegans*.

cdc-42 is required for embryonic cell corpse clearance.

Multiple *cdc-42* mutants are available for genetic analysis. A large deletion mutation *cdc-42(gk388)* completely eliminates the first coding exon and is likely a null allele (Supplementary Figure S2; also see Welchman *et al.*²⁵). Although CDC-42 has many important functions in the early embryo, homozygous *cdc-42(gk388)* mutants (generated from heterozygous *cdc-42/+* mothers, m^+z^-) develop up to the L3/L4 larval stages

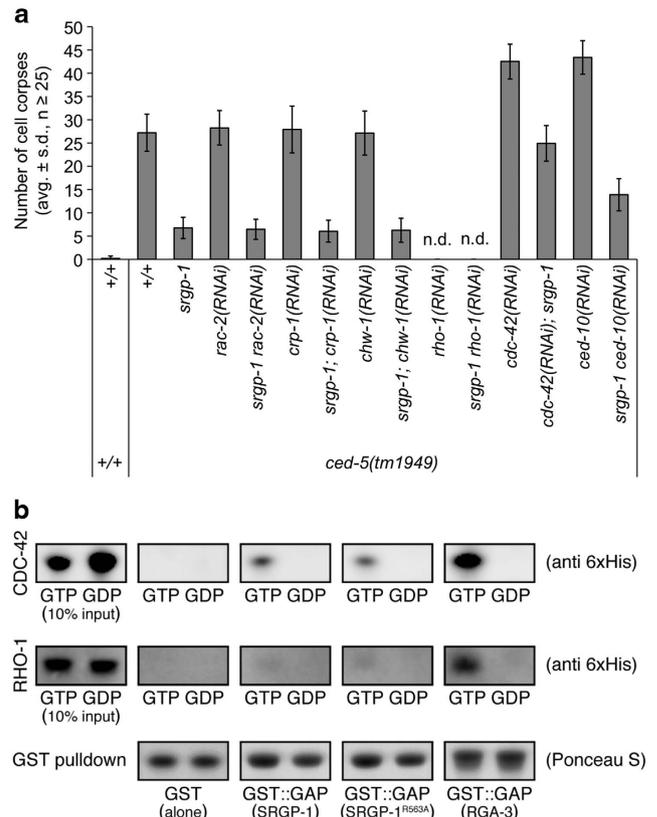


Figure 1 CDC-42 is a candidate SRGP-1 target during engulfment signaling. (a) Knockdown of *cdc-42* and loss of *srgp-1(ok300)* have opposite effects on cell corpse clearance in *C. elegans*. Staged animals (P_0) were grown on RNAi plates and freshly hatched L1 progeny larvae (F_1) were scored for corpse numbers in the head region. All strains carry the *ced-5(tm1949)* mutation in the background. Data are shown as average \pm S.D. ($n \geq 25$). *rho-1(RNAi)* causes embryonic lethality and thus is not done (n.d.). (b) The SRGP-1 GAP domain binds GTP-bound CDC-42 *in vitro*. The GTP- and GDP-bound isoforms of 6xHis-tagged CDC-42 and RHO-1 (CDC-42: Q61L and T17N, RHO-1: Q63L and T19N, respectively) were used for pulldowns. GST-fusions used: GST (alone), the GAP domain of SRGP-1, the GTP hydrolysis-deficient SRGP-1 GAP (R563A) domain²² and the GAP domain of RGA-3, a potential GAP of both RHO-1 and CDC-42 (see Schonegg and Hyman²⁶)

before arresting development because of significant maternal contribution of wild-type *cdc-42* mRNA to the embryo. Thanks to this maternal rescue of early embryos, we could use this allele to score persistent cell corpse numbers in freshly hatched L1 larval heads. Homozygous m^+z^- *cdc-42(gk388)* animals only showed a mild persistent cell corpse phenotype in L1 larval and late embryos, possibly because of the maternally contributed *cdc-42* mRNA (Table 1, Supplementary Figure S4A). Consistent with this hypothesis, progeny from *cdc-42(RNAi)*-treated mothers showed a much stronger engulfment defect (Supplementary Figure S4B).

cdc-42 acts in parallel to the *ced-2*, *ced-5*, *ced-12* and *mig-2* pathway.

To better define the function of CDC-42 in engulfment signaling, we generated double mutants between *cdc-42* and different canonical engulfment mutants. Consistent with our previous *cdc-42(RNAi)* results (Figure 1), *cdc-42(gk388)* significantly enhanced the persistent cell corpse phenotype of *ced-2*, *ced-5*, *ced-12* and *ced-10* mutants

Table 1 Loss of *cdc-42* function enhances *ced-2/5/12*, *ced-10* and *mig-2* persistent cell corpse phenotypes

Genotype	Corpse numbers in 4F embryo or L1 head ($n \geq 20$)
Wild type	0.1 ± 0.3
<i>cdc-42(gk388)</i>	0.6 ± 0.7
<i>uig-1(ok884)</i>	0.4 ± 0.4
<i>unc-112(r367); dim-1(gk54)</i>	1.0 ± 0.9
<i>tiam-1(tm1556)</i>	0.4 ± 0.6
<i>cdc-42(gk388); uig-1(ok884)</i>	0.6 ± 0.4
<i>tiam-1(tm1556); cdc-42(gk388)</i>	1.0 ± 0.5
<i>ced-1(e1735)</i>	21.5 ± 1.6
<i>ced-1(e1735); cdc-42(gk388)</i>	21.2 ± 2.1
<i>ced-1(e1735); uig-1(ok884)</i>	24.1 ± 4.0
<i>ced-1(e1735); dim-1(gk54)^</i>	27.8 ± 4.5
<i>ced-1(e1735); unc-112(r367); dim-1(gk54)^</i>	37.6 ± 3.8*
<i>ced-7(n1996)</i>	21.8 ± 1.9
<i>cdc-42(gk388); ced-7(n1996)</i>	22.0 ± 1.8
<i>ced-6(n1813)</i>	22.8 ± 2.9
<i>cdc-42(gk388); ced-6(n1813)</i>	22.5 ± 4.2
<i>ced-6(tm1826)</i>	22.4 ± 3.1
<i>cdc-42(gk388); ced-6(tm1826)</i>	20.5 ± 5.1
<i>ced-2(e1752)</i>	13.5 ± 3.7
<i>cdc-42(gk388); ced-2(e1752)</i>	20.7 ± 5.0*
<i>ced-2(op327)</i>	4.3 ± 2.1
<i>cdc-42(gk388); ced-2(op327)</i>	12.3 ± 3.3*
<i>ced-5(n1812)</i>	24.3 ± 2.6
<i>cdc-42(gk388); ced-5(n1812)</i>	34.1 ± 4.1*
<i>ced-5(n1812); uig-1(ok884)</i>	32.2 ± 4.3*
<i>ced-5(n1812)^</i>	30.5 ± 3.9
<i>ced-5(n1812); dim-1(gk54)^</i>	31.7 ± 5.9
<i>ced-5(n1812); unc-112(r367); dim-1(gk54)^</i>	40.3 ± 4.5*
<i>ced-12(k149)</i>	21.7 ± 2.6
<i>ced-12(k149); cdc-42(gk388)</i>	34.1 ± 3.1*
<i>ced-10(n3246)</i>	20.1 ± 1.4
<i>cdc-42(gk388); ced-10(n3246)</i>	27.1 ± 3.2*
<i>ced-10(n1993)</i>	16.5 ± 1.7
<i>cdc-42(gk388); ced-10(n1993)</i>	23.1 ± 3.9*
<i>tiam-1(tm1556); ced-10(n1993)</i>	21.2 ± 2.1*
<i>tiam-1(tm1556); cdc-42(gk388); ced-10(n1993)</i>	30.2 ± 4.4*
<i>ced-1(e1735); ced-5(n1812)</i>	31.0 ± 6.9
<i>ced-1(e1735); cdc-42(gk388); ced-5(n1812)</i>	36.3 ± 7.2
<i>mig-2(mu28)</i>	0.2 ± 0.4
<i>cdc-42(gk388); mig-2(mu28)</i>	0.8 ± 1.1
<i>ced-2(n1994)</i>	19.9 ± 2.0
<i>ced-2(n1994); mig-2(mu28)</i>	21.4 ± 4.2
<i>cdc-42(gk388); ced-2(n1994)</i>	19.7 ± 2.6
<i>cdc-42(gk388); ced-2(n1994); mig-2(mu28)</i>	28.7 ± 3.9*
<i>ced-1(e1735)^</i>	29.0 ± 3.6
<i>ced-1(e1735); cdc-42(gk388)^</i>	30.6 ± 2.7
<i>ced-1(e1735); mig-2(gm103gf)^</i>	22.4 ± 2.7*
<i>ced-1(e1735); cdc-42(gk388); mig-2(gm103gf)^</i>	20.1 ± 3.3*
Wild type^	0.5 ± 0.5
<i>cdc-42(gk388)^</i>	0.8 ± 0.8
<i>ttr-52(sm211)^</i>	8.3 ± 2.7
<i>cdc-42(gk388); ttr-52(sm211)^</i>	8.6 ± 3.1

Persistent cell corpses were scored in the head of freshly hatched L1 larvae or early fourfold embryos (^) of the indicated genotypes. The *dim-1(gk54)* mutation is used to suppress the slow growth and sickness of the *unc-112(r367)* mutant. Data are shown as average ± S.D., $n \geq 20$. * $P < 0.001$

(Table 1). These results suggest that CDC-42 acts in parallel to the CED-2/5/12 module and its downstream CED-10 GTPase, or possibly upstream of CED-10, as the *ced-10* mutants used here are not null.

We also analyzed the genetic interaction of *cdc-42* with *mig-2*, the other small GTPase involved in cell corpse clearance. *cdc-42(gk388)* significantly enhanced the persistent cell corpse phenotype of *mig-2(mu28)* loss-of-function (lf) mutants in a sensitized *ced-2(n1994)* background. Conversely, the *mig-2(gm103)* gain-of-function (gf) mutation (which leads to activation of the CED-5–CED-12 GEF) decreased corpse numbers in *ced-1; cdc-42* double mutants (Table 1). These results are consistent with a model in which *cdc-42* and *mig-2* act in parallel to each other.

***cdc-42* likely acts downstream of the *ced-1*, *ced-6* and *ced-7* pathway.** Next, we performed double mutant analyses with strong loss-of-function alleles of existing engulfment mutants of the *ced-1/6/7* pathway. In contrast to the analysis described above, mutations in this pathway (*ced-1*, *ced-6*, *ced-7* and *ttr-52*) were not enhanced by loss of *cdc-42* function (Table 1). Importantly, loss of *cdc-42* failed to enhance corpse persistence in *ced-1; ced-5* double mutants. This indicates that an active CED-1/6/7 pathway is required for CDC-42 to promote engulfment in the absence of the CED-5–CED-12 complex. The most likely explanation for these combined observations is that CDC-42 functions in parallel to *ced-5/12*, possibly downstream of the *ced-1/6/7* signaling cascade.

Overexpression of CDC-42 indicates that *cdc-42* acts downstream of the *ced-1/6/7* pathway. To confirm these results, we created three stable transgenic lines that drive the inducible (i.e., heat shock-triggered) expression of GFP-tagged, constitutively active (GTP-bound) CDC-42, CED-10 and RAC-2 GTPases (*gfp::cdc-42(gf)*, *gfp::ced-10(gf)* and *gfp::rac-2(gf)*). We crossed these transgenic lines into the same engulfment-deficient mutants as in our epistatic analyses, and scored L1 head corpse numbers following heat shock treatment (Figure 2 and Supplementary Figure S3). Overexpression of *cdc-42(gf)* resulted in a suppression of *ced-1*, *ced-6* and *ced-7* mutants to almost wild-type levels. We observed a similar yet weaker effect when expressing *gfp::ced-10(gf)*. In contrast, expression of *gfp::rac-2(gf)* failed to suppress such mutants at all. We therefore conclude that *cdc-42*, like *ced-10*, probably acts downstream of the *ced-1/6/7* signaling cascade.

In contrast, overexpression of *cdc-42(gf)* did not change persistent cell corpse numbers in mutants of the *ced-5–ced-12* GEF complex, nor did *cdc-42(gf)* suppress the strong loss-of-function allele *ced-10(n3246)*. Although the weak but viable allele *ced-10(n1993)* was partially suppressed by *cdc-42(gf)*, this suppression was CED-5 dependent (Figure 2b). Moreover, we failed to observe any change in cell corpse numbers upon *cdc-42(gf)* overexpression in a *ced-1; ced-5* or *ced-6; ced-2* mutant background (Figure 2b). Taken together, these results indicate that overexpression of activated CDC-42 can compensate for a defect in the CED-1/6/7 pathway and CED-10, but only in the presence of an active CED-2/5/12 pathway.

CDC-42 is broadly expressed and accumulates around apoptotic cell corpses. Localization and function of CDC-42 have been mainly studied in one- and two-cell embryos, where CDC-42 participates in the regulation of cell polarity

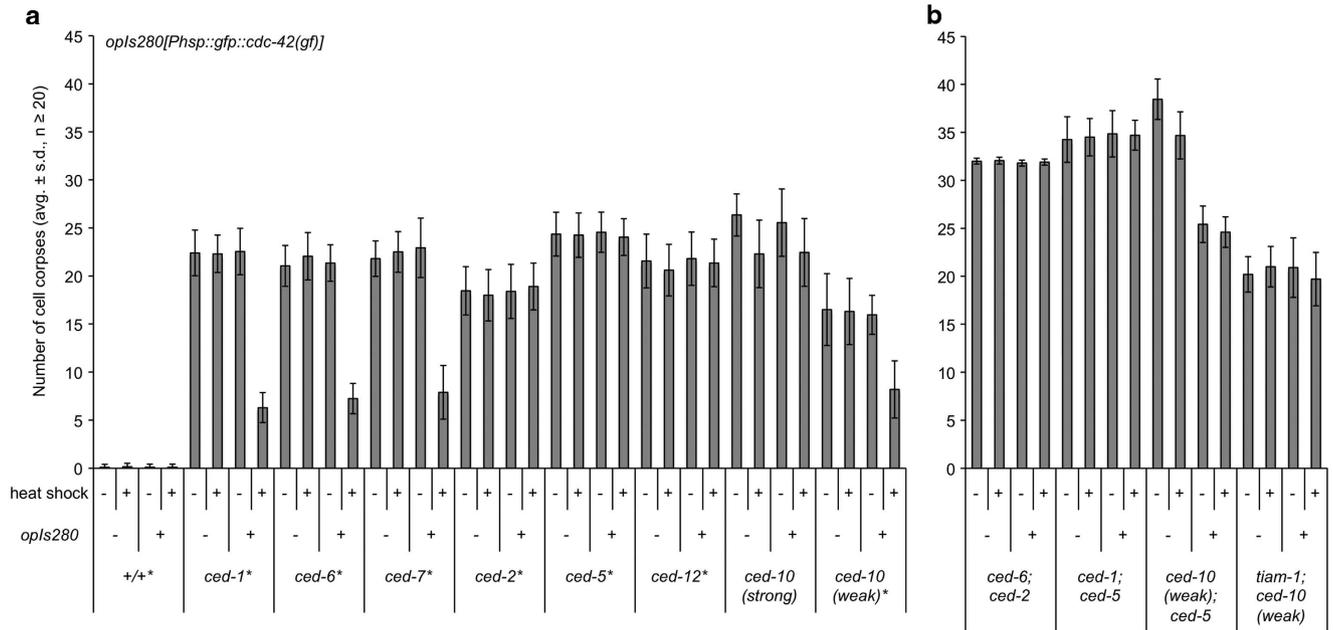


Figure 2 Overexpression of *cdc-42* suppresses the engulfment defect of *ced-1/6/7* mutants. **(a)** The engulfment defect of *ced-1/6/7* but not *ced-2/5/12* mutants is suppressed by *cdc-42* overexpression. **(b)** *cdc-42* overexpression requires a functional *ced-2/5/12* pathway for *ced-1/6/7* suppression. Worms were heat shocked for 90 min at 33 °C, followed by an incubation at 20 °C for 5 h to allow for expression of GFP::CDC-42 (gain of function). Persistent cell corpses were scored in the head of freshly hatched L1 larvae of the indicated genotypes. Alleles used: *opls280*[*P_{hsp-16.41}::gfp::cdc-42(Q61L, gf)*]; *unc-119(+)*; *tiam-1(tm1556)*; *ced-1(e1735)*; *ced-6(n1813)*; *ced-7(n1996)*; *ced-2(n1994)*; *ced-5(n1812)*; *ced-12(k149)*; *ced-10(n3246)* (strong loss of function) and *ced-10(n1993)* (weak loss of function). The *opls280* transgenic strains marked with an asterisk (*) also carry the *unc-119(ed3)* mutation that was used to follow (and is rescued by) the *opls280* transgene

and spindle orientation.^{26,27} In order to address the expression pattern of CDC-42 at later developmental stages, we created a transgene containing the genomic *cdc-42* coding sequence under its endogenous 5' and 3' regulatory sequences fused N-terminally to GFP (*opls295*[*P_{cdc-42}::gfp::cdc-42::3'UTR_{cdc-42}*]; Figure 3a). The *opls295* transgene fully rescues the *cdc-42(gk388)* lethality and fertility defects. Furthermore, GFP::CDC-42 localization in the one-cell embryo resembles previously described expression patterns^{27,28} (Figure 3b). We thus conclude that *opls295* is likely a functional reporter line.

We next used the *opls295* reporter to assess CDC-42 localization during embryonic development. In 2-cell to 32-cell embryos, we found CDC-42 mainly localized to cortexes at cell–cell contacts (Figures 3b and c). At late stages of embryonic development, we also observed a widespread CDC-42 expression in neuronal tissues such as the nerve ring, supporting the involvement of CDC-42 in neuronal processes²⁴ (Figures 3d and e). During larval development and in adults, CDC-42 localized to cortexes in all cells, for example, in the vulva and in the tail (Supplementary Figures S5A–C). We therefore concluded that CDC-42 is very broadly expressed in *C. elegans*.

Previous work has shown that CED-1 and CED-6 in engulfing cells are selectively recruited to the plasma membrane surrounding apoptotic cells, generating a ‘halo’ pattern around the corpses.^{8,10} We observed a similar accumulation of GFP::CDC-42 around apoptotic cell corpses during all developmental stages (Figures 3d–f, Supplementary Figures S5D and S6A). Importantly, CDC-42 was significantly enriched at membranes surrounding

apoptotic cell corpses compared with cortical basal as well as background levels (Figures 3g and h). This was confirmed by GFP intensity quantification using confocal laser microscopy to measure background signal (b_n), the signals at common membrane cortexes (m_n) and at membranes surrounding cell corpses (c_n), respectively (Figure 3i).

PAT-2/PAT-3 integrin, UNC-112 and the CDC-42 GEF UIG-1 drive CDC-42 recruitment to membranes surrounding cell corpses. We next wanted to know what signaling pathway directed CDC-42 membrane recruitment. Hsieh *et al.*²³ recently described the CDC-42 GEF UIG-1 that promotes engulfment.²³ UIG-1 is an UNC-112-interacting protein and colocalizes with UNC-112.²⁹ Therefore, we asked whether *uig-1* and *unc-112* were involved in the regulation of engulfment. Loss of *uig-1* function led at best to a mild increase, if any, of persistent cell corpses on its own (Figure 4 and Supplementary Table S1). As was the case for *cdc-42*, loss of *uig-1* markedly enhanced the engulfment defect of *ced-5*, but not *ced-1* mutants (Table 1). Because strong *unc-112* mutants are very sick and develop extremely slowly, we performed our *unc-112* epistatic studies in a *dim-1* mutant background³⁰ that suppresses the developmental delay of *unc-112* mutants but has no visible effect by itself on engulfment (Table 1). Loss of *unc-112* resulted in an engulfment defect in embryos. RNAi against *cdc-42* failed to further enhance the engulfment defect of *unc-112* mutant (Figure 4 and Supplementary Table S1). In contrast, persistent cell corpse numbers were significantly increased both in *ced-1*; *unc-112* and *ced-5*; *unc-112* double mutants (Table 1). These data are consistent with a model in which

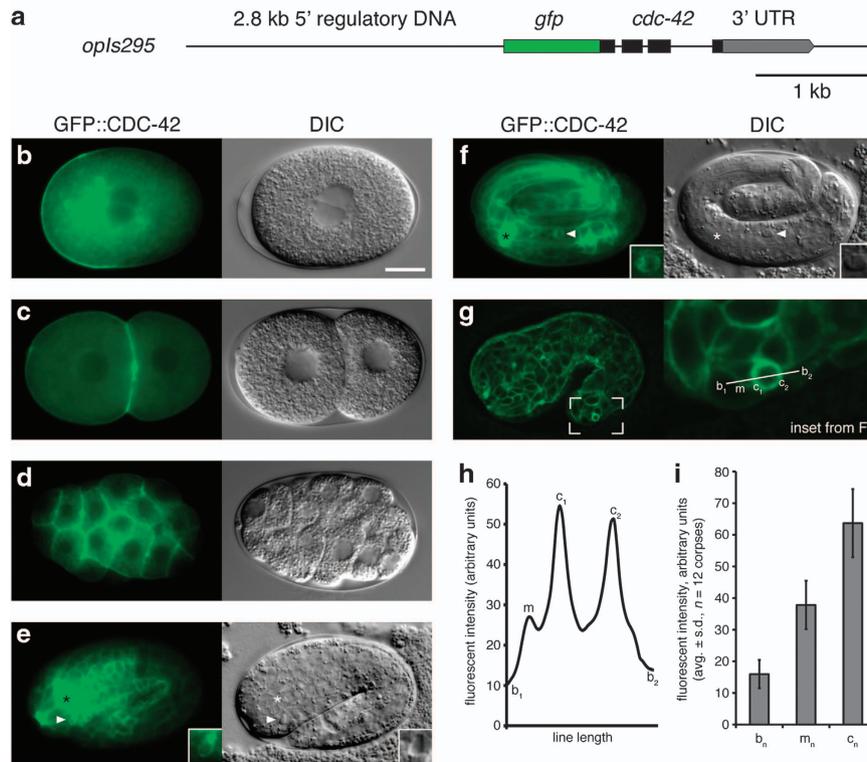


Figure 3 CDC-42 is membrane associated and accumulates around apoptotic cell corpses in embryos. (a) Schematic of the rescue construct *opls295*. A 2.8-kb sequence upstream of the predicted start codon was used as a promoter to drive the expression of an N-terminal *gfp::cdc-42*(genomic) fusion product, followed by a 1-kb 3' fragment that includes the *cdc-42* 3' UTR. (b) Localization of CDC-42 to the anterior cortex in one-cell embryos. (c) In two-cell embryos, CDC-42 is enriched at the cortex of AB/P cell boundary. (d–f) CDC-42 expression pattern in early 24-cell (d, 80 min post fertilization (m.p.f.)), early twofold (e, 440 m.p.f.) and fourfold (f, 600 m.p.f.) embryos. CDC-42 is mainly localized to the cell cortexes and the cytosol. Representative apoptotic cell corpses are shown in magnified insets (e and f, arrowheads). Asterisk indicates nerve ring (e and f). Fluorescence channel (left), DIC (right). (g–i) CDC-42 accumulates at membranes around apoptotic cell corpses. (g) Confocal image of a 1.5-fold embryo with indicated area magnified to the right. (h) Plotted signal intensities along the line shown in (g), indicated by b_n , m_n and c_n (background, membranes and membranes next to cell corpse, respectively). (i) Quantification of CDC-42 signal intensities. In different embryos, 12 different apoptotic cell corpses were analyzed. Data are shown as average \pm S.D. Genotype used: *cdc-42(gk388) opls295[P_{cdc-42}::gfp::cdc-42]*. In all images, anterior is to the left, dorsal on top. Scale bar, 10 μ m

CDC-42 acts downstream of UNC-112 and UIG-1 in the clearance of apoptotic cell corpses.

In wild-type embryos, approximately one-quarter of cell corpses visible by DIC microscopy (Leica DM6000 B, Mannheim, Germany) are surrounded by a CDC-42::GFP halo (Figure 4). Mutations in the *ced-1/6/7* and *ced-2/5/12* pathways increased the number of cell corpses visible under DIC optics, but did not greatly affect the fraction of halo-positive corpses (25–35%; Figure 4). In contrast, loss of *unc-112* and *uig-1* function greatly reduced the number of halo-positive corpses (8%; Figure 4). Hsieh *et al.*²³ recently reported that integrin- α PAT-2 can activate a CDC-42-dependent engulfment signaling pathway in embryonic muscle cells.²³ Consistent with these observations, we found that CDC-42::GFP halos were also greatly reduced in *pat-2* (α -integrin) and *pat-3* (β -integrin) mutants (10% and 8%, respectively).

The presence of CDC-42 halos in internalization-defective mutants suggests that CDC-42 recruitment is an early process during corpse recognition. Consistent with this hypothesis, kinetic studies revealed that CDC-42::GFP accumulates significantly more frequently around early apoptotic corpses than around late corpses (Supplementary Figure S6B). We also noticed that a similar fraction of corpses in the L1 stage, at which point all corpses would be considered

as ‘late’, as in embryos were labeled by CDC-42::GFP in *ced-1* mutants (Figures 4c and d).

Taken together, our findings suggest that upon recognition of a neighboring dying cell, engulfing cells use a PAT-2/PAT-3-dependent signaling pathway, which includes UNC-112 and UIG-1, to recruit CDC-42 to the plasma membrane surrounding the corpse, possibly to help polarize the engulfing cell toward its prey.

The RacGEF TIAM-1 promotes cell corpse engulfment.

How does CDC-42 influence cell corpse engulfment? Recently, Demarco *et al.*³¹ reported that the Rac GEF protein TIAM-1 acts downstream of CDC-42 and upstream of CED-10/Rac1 in neuronal protrusion and axon guidance, offering a concrete way for CDC-42 to influence cytoskeletal rearrangement. Several lines of observations suggest that TIAM-1 also plays a role in cell corpse engulfment. First, whereas *tiam-1* single mutants did not show any persistent cell corpses (Supplementary Table S1), loss of *tiam-1* function increased the engulfment defect of weak *ced-10* mutants (Table 1). Second, we found that in the absence of TIAM-1 activity, overexpression of CDC-42 failed to suppress the engulfment defect in *ced-10* mutants (Figure 2). Finally, we observed that whereas loss of *tiam-1* did not increase the

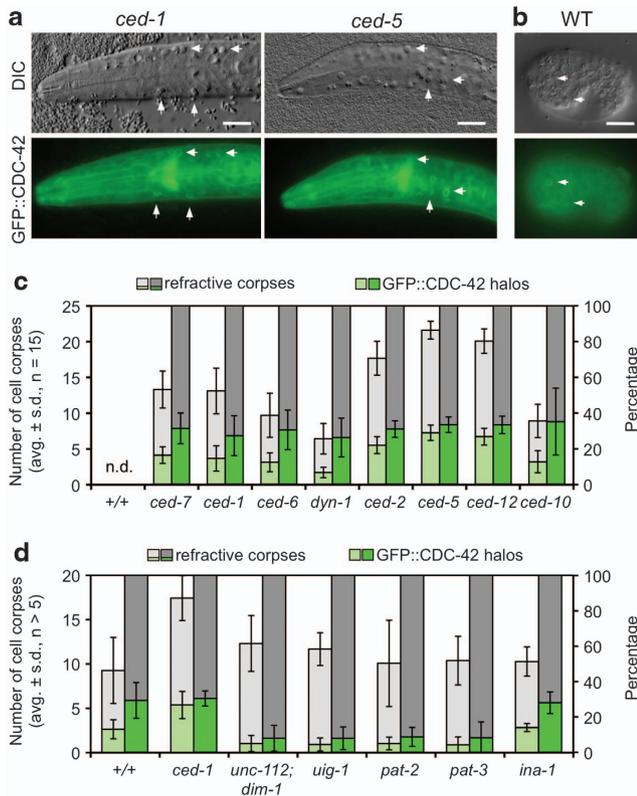


Figure 4 CDC-42 accumulation around apoptotic cell corpses depends on PAT-2 signaling. **(a and c)** Visualization and quantification of CDC-42 around somatic cell corpses in freshly hatched L1 larvae. **(a)** GFP::CDC-42 accumulates efficiently around cell corpses in L1 heads of *ced-1* and *ced-5* mutants. Representative apoptotic cell corpses are shown in magnified insets. **(c)** CDC-42 accumulation around apoptotic corpses does not depend on the CED-2/5/12 or CED-1/6/7 pathways. **(b and d)** Visualization and quantification of CDC-42 around somatic cell corpses in 1.5-fold stage embryos. **(b)** GFP::CDC-42 accumulates efficiently around cell corpses in 1.5-fold embryos in wild-type animals. **(d)** CDC-42 accumulation around apoptotic corpses depends on *unc-112*, *uig-1*, *pat-2* and *pat-3* signaling. Quantification of cell corpse numbers and GFP::CDC-42 coverage, shown in %, calculated as [GFP::CDC-42 halos]/[total corpse numbers] × 100. The absolute numbers of corpses are shown by light color on the left side, and the percentages are shown by dark color on the right side. Error bars on the right are only for the GFP::CDC-42 halos. Alleles used: *ced-1(e1735)*, *ced-2(n1994)*, *ced-5(n1812)*, *ced-6(n1813)*, *ced-7(n1996)*, *ced-10(n1993)*, *ced-12(k149)*, *dyn-1(n4039)*, *unc-112(r367)*, *dim-1(gk54)*, *uig-1(ok884)*, *pat-2(ok2148)*, *pat-3(st564)*, *ina-1(gm39)* and *opls295* [*P_{cdc-42::gfp::cdc-42(genomic)::3'UTR_{cdc-42}}*; *unc-119(+)*]. Scale bar, 10 μm

engulfment defect of *cdc-42(lf)* or *cdc-42(RNAi)* animals, *tiam-1*; *cdc-42*; *ced-10* triple mutant animals contained more corpses than both *tiam-1*; *ced-10* and *cdc-42*; *ced-10* double mutants (Table 1 and Supplementary Table S1). These results are consistent with a role of TIAM-1 either in parallel to or downstream of CDC-42 in cell corpse clearance.

***cdc-42* promotes cell killing.** We and others have previously shown that engulfment can also kill cells on the verge of death.^{3,22,32,33} To test whether CDC-42 can also promote the removal of living cells, we measured P1, P2 and P9–12 *Pn.aap* cell survival in the ventral nerve cord of L3 larvae. In wild-type animals, these cells, which can be visualized with the *P_{lin-11::gfp}* reporter *nls96*, undergo programmed cell death

during early larval development. In engulfment-deficient mutants, a significant fraction of those cells survive and remain alive during adulthood (Supplementary Figure S7).^{32,34} As is the case for other engulfment mutants such as *ced-1*, *ced-5* and *ced-10*, the *cdc-42(gk388)* animals showed extra *Pn.aap* surviving cells compared with wild-type controls (Supplementary Figure S7). This effect was particularly pronounced in a sensitized *ced-3(n2438)* reduction-of-function background. These observations support an involvement of *cdc-42* in the recognition and elimination of subviable cells in *C. elegans*.

Discussion

Rho GTPase family members are known to drive cytoskeletal rearrangements in several processes, such as phagocytosis, cell migration and invasion, integrin-mediated cell adhesion and spreading.^{35–37} In *C. elegans*, two Rho GTPase family members, CED-10/Rac1 and MIG-2/RhoG, have previously been shown to regulate apoptotic corpse clearance. Here, we show that a third small GTPase, CDC-42, also plays an important role in this process. Our genetic analysis suggests that *cdc-42* acts in parallel to or downstream of the *ced-1/6/7* module and in parallel to the *ced-2/5/12* module, possibly upstream or in parallel to *ced-10* (Figure 2, Table 1 and Supplementary Table S1). Recruitment of CDC-42 to the plasma membrane facing apoptotic cells depends on PAT-2/PAT-3 integrin, UNC-112 and the CDC-42 GEF UIG-1, but not the canonical *ced-1/6/7* or *ced-2/5/12* modules (Figure 4). Our observations confirm and greatly expand on a recent publication by Hsieh *et al.*²³ that showed that integrin-α PAT-2 might recognize exposed PS on apoptotic cells and regulate corpse engulfment through UIG-1 and CDC-42 in *C. elegans* muscle cells.

What is the function of CDC-42 in apoptotic cell removal? Corpse clearance in *C. elegans* is commonly divided into three main steps: recognition of the neighboring apoptotic cell, corpse internalization/engulfment and phagosome maturation.⁹ Rac GTPase CED-10 promotes the rearrangement of the cytoskeleton that is required to drive formation of the phagocytic cup and corpse internalization. Previous studies in *C. elegans* showed that CDC-42 plays an important role in the establishment and maintenance of cell polarity in early embryos.^{27,38–40} In mammals, Cdc42 participates in the regulation of various processes including cell polarity, cell migration, phagocytosis and endomembrane trafficking.^{41–44} Cdc42 has also been implicated in the integrin and Fc receptor-mediated internalization of apoptotic corpses by macrophages.^{45,46}

Our genetic analysis and the role of CDC-42 in cell polarity suggest a model in which corpse internalization includes two distinct engulfment activities: in this model an early polarization toward the apoptotic corpse is later followed by cytoskeletal remodeling activities that drive phagocytic cup formation. Our data suggest that apoptotic cells are recognized via at least two receptor pathways (Figure 5): PAT-2/3 integrins, which recruit CDC-42 to the plasma membrane via UNC-112 and UIG-1, and CED-1, which is also enriched around apoptotic corpses independently of CDC-42 (data not shown). Based on the known role of CDC-42 in the control of

cell polarity in other processes, we postulate that active CDC-42, in response to CED-1/6/7 pathway activation, drives the subsequent establishment of a dynamic asymmetry in the distribution or activation of the cytoskeletal rearrangement machinery, including the CED-5–CED-12 GEF and its target CED-10/Rac1, ultimately leading to phagocytic cup formation around the recognized corpse (Figure 5). Malfunctioning polarization (i.e., in *ced-1/6/7* mutants) still allows cytoskeletal remodeling to take place, but likely in a less efficient/directional way. An impaired cytoskeletal remodeling machinery (i.e., in *ced-2/5/12* mutants) leads to an engulfing cell that still polarizes toward the corpse but engulfs it only poorly (probably through a less-efficient back-up signaling cascade acting in parallel). Finally, interfering with both polarity and cytoskeletal remodeling results in an additive phenotype.

Such a model can explain many of our observations. First, overexpression of CDC-42 might make its activation independent of the *ced-1/6/7* pathway, explaining why such an overexpression rescues *ced-1/6/7* pathway mutants. In contrast, overexpression of CDC-42 cannot efficiently drive phagocytic cup formation, and hence cannot suppress *ced-2/5/12* mutants. Second, *cdc-42(lf)* would not enhance *ced-1/6/7* pathway mutants (as CDC-42 activation and cell polarization is already disrupted in these mutants) but would enhance mutants involved in phagocytic cup formation (*ced-2/5/12*). Finally, hyperactivation of the pathway leading to phagocytic cup formation could be expected to compensate for defects in cell polarization, whereas the opposite would not be true – which is indeed what is experimentally observed. For example, we found that *mig-2(gf)* mutants fail to suppress mutants of the *ced-5–ced-12* GEF complex, but can rescue *ced-1* or *ced-1;cdc-42* double mutants. Finally, overexpression of *ced-10* has been shown to rescue engulfment mutants in both *ced-1/6/7* and *ced-2/5/12* pathways.

An alternative function for CDC-42 in phagocytosis has recently been suggested by Mohammadi and Isberg,⁴⁷ who showed that in mammalian cells, Cdc42 is required to drive the exocytosis of recycling vesicles, likely to generate the additional plasma membrane required for phagocytic cup

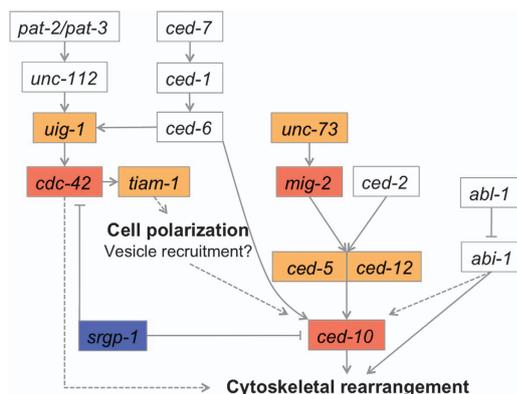


Figure 5 *cdc-42* regulates engulfment in response to PAT-2/CED-1–UIG-1 signaling. Genetic model of cell corpse clearance in *C. elegans*. *cdc-42* acts downstream of *pat-2/3*, downstream of or in parallel to the *ced-1/6/7* pathway. See text for details. Components involved in phagosome maturation are not shown. GTPases, GEFs and GAP are shown in gray, orange and blue boxes, respectively. Dashed arrows indicate potential regulation

formation. Overexpression of the small GTPase Rab11 could rescue the large particle uptake defect in *Cdc42*-depleted cells, identifying RAB-11 as a potential downstream regulator of *CDC-42*.⁴⁷

Our model however also leaves several questions unanswered. For example, how *CED-1/6/7* might mediate *UIG-1*-dependent membrane recruitment and activation of *CDC-42* remains to be determined. Downstream targets of *CDC-42* beyond *TIAM-1* also remain to be identified. Finally, it remains unclear why loss of *CDC-42* function on its own only leads to a mild engulfment defect. One possibility is that *CDC-42* function is required only in a subset of engulfing cells. Consistent with this hypothesis, Hsu and colleagues^{23,48} reported the existence of a second integral signaling pathway, activated by *INA-1*, that acts in parallel to *PAT-2/3* and activates the *ced-2/-5/-12/-10* signaling module via the tyrosine kinase *SRC-1*. Different cell types might thus use different integrins, leading to the activation of distinct signaling pathways that trigger similar cellular processes.

The involvement of *cdc-42* in apoptotic cell clearance by the phagocytic cell expands the already broad variety of conserved developmental processes such as asymmetric cell division, cell migration, epithelial remodeling and nervous system development in which *CDC-42* plays a key role.³⁹ Given the evolutionarily conserved function of the engulfment machinery, it is likely that *Cdc42* promotes cell corpse clearance in similar ways in mammals and *C. elegans*. Using *C. elegans* to further dissect *cdc-42* signaling mechanisms will provide new insights into the mechanism of apoptotic cell clearance in humans, a process that has been associated with a variety of human diseases.¹

Materials and Methods

Mutations/strains used. *C. elegans* strains were grown at 20°C as previously described. *Wild type*: used was Bristol N2. The alleles used were as follows: LGI: *tiam-1(tm1556)*, *ced-12(k149)* and *ced-1(e1735)*; LGII: *cdc-42(gk388)*; LGIII: *ced-6(n1813)*, *ced-6(tm1826)*, *ced-7(n1996)*, *pat-2(ok2148)*, *pat-3(st564)*, *ina-1(gm39)*, *trr-52(sm211)* and *unc-119(ed3)*; LGIV: *ced-2(e1752)*, *ced-2(n1994)*, *ced-2(op327)*, *ced-10(n1993)*, *ced-10(t1875)*, *ced-5(n1812)*, *ced-5(tm1949)* and *srgp-1(ok300)*; LGV: *uig-1(ok884)* and *unc-112(r367)*; LGX: *mig-2(mu28)*, *mig-2(gm103gf)* and *dim-1(gk54)*. Unless otherwise noted, all alleles are described (<http://www.wormbase.org>). Integrated arrays were: *opls280[P_{hsp-16.41}::gfp::cdc-42(gf)::3' UTR_{let-858}; unc-119(+)]*, *opls295[P_{cdc-42}::gfp::cdc-42(genomic)::3' UTR_{cdc-42}; unc-119(+)]* II, *opls296[P_{cdc-42}::cfp::cdc-42(genomic)::3' UTR_{cdc-42}; unc-119(+)]*, *opls135[P_{ced-1}::ced-1(genomic)::yfp::3' UTR_{ced-1}; unc-69(+)]* and *nls96[P_{lin-11}::gfp]* V. Balancers were as follows: *mln1(dpy-10(e128))*, *mls14[myo-2::gfp; pes-10::gfp]* (II), *hT2[bli-4(e937) let-??? (q782) qIs48(l;III) and nT1[qIs51[myo-2::gfp; pes-10::gfp]]* (IV,V).

Phenotypic analysis. Larval L1 head apoptotic cell corpses: Mixed culture plates were washed off with M9 several times to remove everything except eggs. After 45 min, freshly hatched L1 were mounted on a 3% agar pad, anesthetized (2.5 mM levamisole in M9) and immediately scored for persistent cell corpses in the head region (i.e., anterior from the posterior bulb) using a DIC microscope. Germ cell corpses: More than 20 either 12 or 24 h post L4/adult molts were mounted on a 3% agar slide and anesthetized in a droplet of 5 mM levamisole in M9. Refractive apoptotic germ cell corpses were scored using the same microscope, followed if applicable by fluorescent halo scoring (of strong fluorescent reporters), under the corresponding epifluorescence channel of the microscope described above. Gonads of weak fluorescent germline reporter strains were dissected in PBS and mounted on 3% agar slides. *Pn.aap* cell survival: The number of surviving *Pn.aap* cells (P1, P2, P9–P12.aap) were scored in *nls96[P_{lin-11}::gfp]* L3 or early L4 larvae under an M2Bio epifluorescence dissecting microscope (Zeiss, Hamburg, Germany).³² Heat shock treatment: Mixed culture plates were

heat shocked for 90 min at 33°C. After 5 h, worms were washed off the plates, and 50 min later freshly hatched L1 larvae were scored for persistent cell corpses in the head region.

RNAi by feeding. RNAi was performed as described previously⁴⁹ with the following modifications: NGM-agarose plates containing 2 mM IPTG were seeded with 250 μ l of appropriate bacterial clones 12 h before the addition of worms. Approximately 30 staged L1 larvae of the corresponding genotype were seeded in triplicates on plates and grown at 20°C. For *rac-2*, *ced-10*, *crp-1* and *chw-1* RNAi, staged animals (P₀) were grown on RNAi plates and freshly hatched L1 progeny larvae (F₁) were scored for corpse numbers in the head region. For *cdc-42(RNAi)*, staged P₀ L3 larvae were used instead of L1 larvae. No condition applied for *rho-1(RNAi)* led to viable F₁ larvae.

Total RNA isolation and cDNA synthesis. Mixed worm cultures from two 9 cm plates were washed off with M9, rinsed twice with M9 and total RNA was extracted as described previously.⁵⁰ The dry total RNA was resuspended in 50 μ l ddH₂O and similar amounts used for cDNA synthesis according to the manufacturer's instructions (Super Script III, Invitrogen, Carlsbad, CA, USA).

Generation of transgenic strains. Transgenic worms (*opEx* and *opls* alleles) were generated by microparticle bombardment in a Biolistic Particle Delivery System (PDS-1000, Bio-Rad, Hercules, CA, USA) as described previously.²² As a transformation marker, *unc-119(ed3)* was used.

In vitro GTPase pulldowns. *In vitro* pulldowns were performed as described previously.²² Briefly, *C. elegans* GTP- and GDP-binding GTPase isoform (QXXL and TXXN) expressing plasmids were transformed into BL21(DE3)pLysS (Fisher Scientific, Hampton, NH, USA) and protein expression was induced by 1 mM IPTG at 37°C. All His-tagged GTPase proteins were purified using His-Bind resin (Novagen, Darmstadt, Germany) according to the manufacturer's instructions. All of the buffers contained 2 mM MgCl₂ and 1 mM Tris(Hydroxypropyl)Phosphine (THP). The proteins were dialyzed against 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 2 mM MgCl₂ and 0.1 mM THP, and snap frozen at -80°C. GST and GST::GAP domain isoform containing plasmids were transformed into BL21(Gold) (Fisher Scientific) and their expression products purified and immobilized onto Glutathione Sepharose beads by standard methods. Immobilized GST fusion proteins (30 μ g) were incubated with 10 μ g of His-tagged GTPase proteins in a buffer containing 10 mM Tris-HCl (pH 7.9), 100 mM NaCl, 2 mM MgCl₂ and 1 mM THP, 0.5% NP-40 and 10% glycerol. The incubation was carried out for 2 h at 4°C with agitation. The beads were washed in the same buffer three times and the proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). His-tagged proteins were immunoblotted with rabbit anti-His antibodies (Santa Cruz, Santa Cruz, CA, USA; 1:1000).

4D microscopy. The 4D microscopy was performed as previously described.^{34,51} Briefly, young embryos were isolated and mounted on agarose slides in M9 and sealed by vaseline. The Z-stacks of embryos were taken every minute using DIC microscope as described before during early embryonic development (~4 h). Persistent cell corpses were scored at indicated stages of embryonic development.

Primers and plasmids. Primers and plasmids are listed in Supplementary Table S2. Plasmids were fully sequenced before microparticle bombardment or expression in bacteria. If not mentioned otherwise, the following 'LazyBoy' starting plasmids were used: *pLN022[SbfI promoter Ascl gene FseI reporter PaclI let-858₃UTR ApaI, unc-119(+)]* or *pLN019[SbfI promoter Ascl reporter Ascl gene FseI let-858₃UTR ApaI, unc-119(+)]*.

Conflict of Interest

The authors declare no conflict of interest.

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