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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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 cell corpses have been identified through forward and reverse genetic screens in the past two decades. 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, and – in some cases – leads to impaired cell migration.

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.22 This process is negatively regulated by the GTPase-activating protein (GAP) SRGP-1/srGAP1 that facilitates GTP hydrolysis in CED-10.22,23

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; If, loss of function; mig, migration defective; RNAi, RNA interference; Srgp-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., 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. 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 srgp-1 mutations, we surmised that srgp-1 might also modulate other Rho GTPases important for cell corpse clearance (Figure 1a).

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(RNAi) significantly enhances the persistent cell corpse phenotype of ced-2, ced-5, ced-12 and ced-10 mutants 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"⁺" 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).

**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 (F1) 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 ± S.D. (n ≥ 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 6×His-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 GST-GAP domain [S4, 25].

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

Persistent cell corpses were scored in the head of freshly hatched L1 larvae or early fourfold embryo (s) 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 ≥ 20.

*P < 0.001

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

We next used the \textit{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 processes\textsuperscript{24} (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 \textit{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.\textsuperscript{8,10} We observed a similar accumulation of GFP::CDC-42 around apoptotic cell corpses during all developmental stages (Figures 3d–f, Supplementary Figures SSD 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 (\textit{b}\textsubscript{0}), the signals at common membrane cortexes (\textit{m}\textsubscript{n}), and at membranes surrounding cell corpses (\textit{c}\textsubscript{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 \textit{et al.}\textsuperscript{23} recently described the CDC-42 GEF UIG-1 that promotes engulfment.\textsuperscript{23} UIG-1 is an UNC-112-interacting protein and colocalizes with UNC-112.\textsuperscript{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 \textit{ced-5}, but not \textit{ced-1} mutants (Table 1). Because strong \textit{unc-112} mutants are very sick and develop extremely slowly, we performed our \textit{unc-112} epistatic studies in a \textit{dim-1} mutant background\textsuperscript{30} that suppresses the developmental delay of \textit{unc-112} mutants but has no visible effect by itself on engulfment (Table 1). Loss of \textit{unc-112} resulted in an engulfment defect in embryos, RNAi against \textit{cdc-42} failed to further enhance the engulfment defect of \textit{unc-112} mutant (Figure 4 and Supplementary Table S1). In contrast, persistent cell corpse numbers were significantly increased both in \textit{ced-1}; \textit{unc-112} and \textit{ced-5}; \textit{unc-112} double mutants (Table 1). These data are consistent with a model in which...
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. recently reported that integrin-α PAT-2 can activate a CDC-42-dependent engulfment signaling pathway in embryonic muscle cells. 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. 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
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**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. 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. 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 matura-

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*, *ug-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*(n367), *dim-1*(gk54), *ug-1* (ok884), *pat-2* (ek2148), *pat-3* (s664), *ina-1* (gm39) and *opl-295* [*p(~):gfp::cdc-42(genomic);3'UTR::gfp::::unc-119(+)] Scale bar, 10 μm

engulfment defect of *cdc-42(II)* 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. 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 *Pn.aap: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). 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*.

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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, 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 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 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 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(m1826), ced-7(n1996), pat-2(k2148), pat-3(st564), ina-1(g389), ltr-32(m211) and unc-119(ed3); LGIV: ced-2(e1725), ced-2(n1994), ced-2(op327), ced-10(n1993), ced-10(l1875), ced-5(n1812), ced-5(m1949) and srgp-1(k3030); LGV: upg-1(pk884) and unc-112(r267); LGX: mig-2(mz28), mig-2(gm103gf) and dim-1(gk54). Unless otherwise noted, all alleles are described (http://www.wormbase.org/). Integrated arrays were: opIs286[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs135[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs296[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs296[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs260[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::gfp::ced-42[genomic];3’UTR::rps-6::gfp]; unc-119[+]; II, opIs297[pec-10::
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 previously with the following modifications: NAG-agarose plates containing 2 mM IPTG were seeded with 250 μl 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, cpr-1 and che-1 RNAi, staged animals (P0) were grown on RNAi plates and freshly hatched L1 progeny larvae (P1) 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. The dry total RNA was resuspended in 50 μl 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 opl alleles) were generated by microinjection in a Bolistic Delivery System (PDS-1000, Bio-Rad, Hercules, CA, USA) as described previously. As a transformation marker, unc-119(ed3) was used.

In vitro GTPase pulldowns. In vitro pulldowns were performed as described previously. Briefly, C. elegans GTP- and GDP-binding GTPase isoform (OXXL and TXNX) expressing plasmids were transformed into BL21(DE3)pLysS and similar amounts used for cDNA synthesis according to the manufacturer’s instructions (Super Script III, Invitrogen, Carlsbad, CA, USA).

4D microscopy. The 4D microscopy was performed as described previously. Briefly, young embryos were isolated and mounted on agarose slides in M9 and sealed by vaseline. The 4D microscopy was performed as previously described. In vitro GTPase pulldowns were preformed as previously described. As a transformation marker, unc-119(ed3) was used.

Conflict of Interest

The authors declare no conflict of interest.

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1. Nagata S, Hanayama R, Kawane K. Autoimmunity and the clearance of dead cells. Cell 2010; 140: 619–630.
2. Fullard JF, Kale A, Baker NE. Clearance of apoptotic corpses. Apoptosis 2009; 14: 1029–1037.
3. Hengartner MO, Schnabel R. Engulfment genes cooperate with ced-3 to promote cell death in Caenorhabditis elegans. Nature 2001; 412: 202–206.
4. Schwartz HT. A protocol describing pharynx counts and a review of other assays of apoptotic cell death in the nematode worm Caenorhabditis elegans. Nat Protoc 2007; 2: 705–714.
5. Ellis RE, Horvitz HR. Two C. elegans genes control the programmed deaths of specific cells in the pharynx. Development 1991; 112: 591–603.
6. Zhou Z, Cao N, Hartwig E, Hall A, Horvitz HR. The C. elegans PH domain protein CED-12 regulates cytoskeletal reorganization via a Rho/Rac GTPase signaling pathway. Dev Cell 2001; 1: 477–489.
7. Gumienny TL, Hengartner MO. How the worm removes corpses: the nematode C. elegans as a model system to study engulfment. Cell Death Differ 2001; 8: 564–568.
8. Kinchen JM, Cabello J, Klinegole D, Wong K, Feichtinger R, Schnabel H et al. Two pathways converge at CED-10 to mediate actin rearrangement and corpse removal in C. elegans. Nature 2005; 434: 93–99.
9. Pinto SM, Hengartner MO. Clearing up the mess: cell corpse clearance in Caenorhabditis elegans. Curr Opin Cell Biol 2008; 20: 881–889.
10. Zhou Z, Hartwig E, Horvitz H. CED-1 is a transmembrane receptor that mediates cell corpse engulfment in C. elegans. Cell 2001; 104: 43–56.
11. Wang X, Li W, Zhao D, Liu B, Shi Y, Chen B et al. Caenorhabditis elegans transhyretin-like protein TTN-52 mediates recognition of apoptotic cells by the CED-1 phagocyte receptor. Nat Cell Biol 2010; 12: 655–664.
12. Wang X, Wang J, Gengyo-Ando K, Gu L, Sun CL, Yang C et al. C. elegans mitochondrial factor WAH-1 promotes phosphatidylinerine externalization in apoptotic cells through phospholipid scramblase SCR-1. Nat Cell Biol 2007; 9: 541–549.
13. Wu YC, Horvitz HR. The C. elegans cell corpse engulfment gene ced-7 encodes a protein similar to ABC transporters. Genes Cells 1998; 3: 951–966.
14. Liu QA, Hengartner MO. Candidate adaptor protein CED-6 promotes the engulfment of apoptotic cells in C. elegans. Cell 1998; 93: 961–972.
15. Yu X, Oeda S, Chuang S-H, Lu N, Zhou Z. C. elegans Dynamin mediates the signaling of phagocytic receptor CED-1 for the engulfment and degradation of apoptotic cells. Dev Cell 2006; 10: 743–757.
16. Yu X, Lu N, Zhou Z. Phagocytic receptor CED-1 initiates a signaling pathway for degrading engulfed apoptotic cells. PLoS Biol 2008; 6: e61.
17. Redden PW, Horvitz HR. CED-2/Crml and CED-10/Rac control phagocytosis and cell migration in Caenorhabditis elegans. Nat Cell Biol 2000; 2: 131–136.
18. Wu YC, Horvitz HR. C. elegans phagocytosis and cell-migration protein CED-5 is similar to human DOK180. Nature 1996; 382: 501–504.
19. Wu YC, Tsai MC, Cheng LC, Chou CJ, Weng NY. C. elegans CED-12 acts in the conserved crml/DOCK180/Rac pathway to control cell migration and cell corpse engulfment. Dev Cell 2001; 1: 491–502.
20. Aikawa S, Kar B, Singh S, Cho L, Tibrewal N, Sanokawa-Akakura R et al. Crml domain of CrkII regulates the assembly and function of the DOCK180/ELMO Rac-GEF. J Cell Physiol 2005; 204: 344–351.
21. Horwitz ME, Vanderzalm PJ, Bloom L, Goldman J, Garriga G, Horvitz HR. Abl kinase inhibits the engulfment of apoptotic [corrected] cells in Caenorhabditis elegans. PLoS Biol 2009; 7: e99.
22. Neukomm LJ, Frei AP, Cabral J, Kinchen JM, Frei AP, Cabello J, et al. Identification of a conserved crkII/DOCK180/Rac pathway to control cell migration and cell corpse engulfment. Cell 2001; 107: 201–210.
23. Hsieh H-H, Hsu T-Y, Jiang H-W, Wu Y-C. Integrin-mediated apoptosis of apoptotic cells in C. elegans. Dev Cell 2007; 140: 881–888.
24. Motegi F, Sugimoto A. Sequential functioning of the ECT-2 RhoGEF, RHO-1 and CDC-42 promotes engulfment in C. elegans. Dev Cell 2007; 13: 3507–3516.
25. Kunfer KT, Cook SJ, Squirrel JM, Elicerki KW, Peel N, O’Connell KF et al. CED-1 and CHIN-1 regulate CDC-42 activity during asymmetric division in the Caenorhabditis elegans embryo. Mol Biol Cell 2010; 21: 266–277.
26. Motteau F, Sugimoto A. Sequential functioning of the ECT-2 RhoGEF, RHO-1 and CDC-42 establishes cell polarity in Caenorhabditis elegans embryos. Nat Cell Biol 2006; 8: 978–985.
27. Hikita T, Sadota H, Tsuboi D, Taya S, Moerman DG, Kaibuchi K et al. Identification of a novel C4D2 GEF that is localized to the PAT-3-mediated adhesive structure. Biochem Biophys Res Commun 2005; 335: 139–145.
30. Rogalski TM, Gilbert MM, Devenport D, Norman KR, Moerman DG. DIM-1, a novel immunoglobulin superfamily protein in Caenorhabditis elegans, is necessary for maintaining bodywall muscle integrity. Genetics 2003; 163: 905–915.
31. Demarco RS, Strudhoff EC, Lundquist EA. The Rac GTP exchange factor TIAM-1 acts with CDC-42 and the guidance receptor UNC-40/DCC in neuronal protrusion and axon guidance. PLoS Genet 2012; 8: e1002665.
32. Reddien PW, Cameron S, Horvitz HR. Phagocytosis promotes programmed cell death in C. elegans. Nature 2001; 412: 198–202.
33. Galvin BD, Kim S, Horvitz HR. Caenorhabditis elegans genes required for the engulfment of apoptotic corpses function in the cytotoxic cell deaths induced by mutations in lin-24 and lin-33. Genetics 2008; 179: 403–417.
34. Sulston JE, Horvitz HR. Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Dev Biol 1977; 56: 110–156.
35. Price LS, Leng J, Schwartz MA, Bokoch GM. Activation of Rac and Cdc42 by integrins mediates cell spreading. Mol Biol Cell 1998; 9: 1863–1871.
36. Niedergang F, Chavrier P. Regulation of phagocytosis by Rho GTPases. In: Boquet P, Lemichez E (eds). Bacterial Virulence Factors and Rho GTPases. Springer: Berlin, Heidelberg, 2005, pp 43–60, available at http://link.springer.com/chapter/10.1007/3-540-27511-8_4.
37. Partridge MA, Marcantonio EE. Initiation of attachment and generation of mature focal adhesions by integrin-containing filopodia in cell spreading. Mol Biol Cell 2006; 17: 4237–4248.
38. Anderson DC, Gill JS, Cinalli RM, Nance J. Polarization of the C. elegans embryo by RhoGAP-mediated exclusion of PAR-6 from cell contacts. Science 2008; 320: 1771–1774.
39. Beautiful A, Morton DG, Kamphues K, PAR-2, LGL-1 and the CDC-42 GAP CHN-1 act in distinct pathways to maintain polarity in the C. elegans embryo. Development 2013; 140: 2005–2014.
40. Caron E, Hall A. Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. Science 1998; 282: 1717–1721.
41. Caron E. Phagocytosis: Rac and roll over the corpses. Curr Biol 2000; 10: R489–R491.
42. Hoppe AD, Swanson JA, Cdc42, Rac1, and Rac2 display distinct patterns of activation during phagocytosis. Mol Biol Cell 2004; 15: 3509–3519.
43. Kamath RS, Ahringer J. Genome-wide RNAi screening in Caenorhabditis elegans. Methods 2003; 30: 313–321.
44. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162: 156–159.
45. Neukomm LJ, Nicot AS, Kinchen JM, Almendinger J, Pinto SM, Zeng S et al. The phosphoinositide phosphatase MTM-1 regulates apoptotic cell corpse clearance through CED-5-CED-12 in C. elegans. Development 2011; 138: 2003–2014.

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