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Phagocytosis of Apoptotic Cells Is Regulated by a UNC-73/TRIO-MIG-2/RhoG Signaling Module and Armadillo Repeats of CED-12/ELMO

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Summary

Background: Phagocytosis of cells undergoing apoptosis is essential during development, cellular turnover, and wound healing. Failure to promptly clear apoptotic cells has been linked to autoimmune disorders. *C. elegans* CED-12 and mammalian ELMO are evolutionarily conserved scaffolding proteins that play a critical role in engulfment from worm to human. ELMO functions together with Dock180 (a guanine nucleotide exchange factor for Rac) to mediate Rac-dependent cytoskeletal reorganization during engulfment and cell migration. However, the components upstream of ELMO and Dock180 during engulfment remain elusive.

Results: Here, we define a conserved signaling module involving the small GTPase RhoG and its exchange factor TRIO, which functions upstream of ELMO/Dock180/Rac during engulfment. Complementary studies in *C. elegans* show that MIG-2 (which we identify as the homolog of mammalian RhoG) and UNC-73 (the TRIO homolog) also regulate corpse clearance in vivo, upstream of CED-12. At the molecular level, we identify a novel set of evolutionarily conserved Armadillo (ARM) repeats within CED-12/ELMO that mediate an interaction with activated MIG-2/RhoG; this, in turn, promotes Dock180-mediated Rac activation and cytoskeletal reorganization.

Conclusions: The combination of in vitro and in vivo studies presented here identify two evolutionarily conserved players in engulfment, TRIO/UNC73 and RhoG/MIG-2, and the TRIO → RhoG signaling module is linked by ELMO/CED-12 to Dock180-dependent Rac activation during engulfment. This work also identifies ARM repeats within CED-12/ELMO and their role in linking RhoG and Rac, two GTPases that function in tandem during engulfment.

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Introduction

Engulfment of apoptotic cells is intimately tied to the apoptotic program and is linked to development, cellular homeostasis, and wound healing [1–4]. Phagocytes recognize and engulf dying cells at an early stage in the apoptotic process to prevent leakage of potentially toxic and inflammatory contents from the dying cells [5]. Failure to clear dying cells has been linked to several autoimmune disorders in mice and humans [6–9]. Thus, understanding the specific molecular pathways regulating engulfment is an issue of fundamental importance.

Studies in *C. elegans* and mammalian cells have identified a number of proteins involved in the recognition and uptake of apoptotic cells [1]. To date, at least seven genes have been identified in *C. elegans* and categorized into two functional genetic pathways [1, 10]. In one pathway, two membrane proteins, CED-1 and CED-7 (representing the mammalian homologs LRP and ABCA1, respectively), have been shown to function upstream of the intracellular adaptor protein CED-6/GULP [11–14]. In a second pathway, three cytosolic proteins, CED-2, CED-5, and CED-12 (representing the mammalian homologs CrkII, Dock180, and ELMO, respectively), have been shown to lead to the activation of the small GTPase CED-10/Rac [15–22]. Recently, ELMO and Dock180 have been shown to function as a novel, bipartite nucleotide exchange factor for Rac, thereby leading to the reorganization of the actin cytoskeleton during engulfment [23].

ELMO is represented by a single gene in the worm (*ced-12*) and fly (*dced-12*) and by three genes (*elmo 1, 2*, and *3*) in mammals [18, 19, 22]. ELMO1 encodes a novel, highly evolutionarily conserved protein of 727 amino acids with no obvious catalytic domains. With the exception of a C-terminal PH domain, a predicted leucine zipper motif, and a Proline-rich motif, no other domains or motifs have been recognized within ELMO/CED-12 [18, 19, 22]. The C-terminal ~195 amino acids of ELMO1 (denoted C-term) are necessary and sufficient to interact with Dock180 and for promoting Rac activation and membrane ruffling when overexpressed in cells [24]. The specific role of the N-terminal two-thirds of ELMO1 (~530 amino acids; denoted N-term) is unknown but might play a role in targeting the ELMO1/Dock180 complex to the membrane [24]. The goal of this study

Table 1. Intact ARM 2 Repeat of CED-12/ELMO1 Is Required for DTC Migration and Cell Corpse Removal

ced-12 ARM2 Repeat Is Required for Efficient Corpse Removal

Genotype	Corpse Number	n
Wild-type	0	10
ced-12(k149)	16.8 ± 5.0	20
ced-12(k149);[P_{eft-3}::ced-12(WT)::yfp]	2.8 ± 1.9	10
ced-12(k149);[P_{eft-3}::ced-12N-term::yfp]	17.5 ± 4.2	30
ced-12(k149);[P_{eft-3}::ced-12C-term::yfp]	19.5 ± 4.5	30
ced-12(k149);[P_{eft-3}::ced-12^{ARM1}::yfp]	2.6 ± 2.0	30
ced-12(k149);[P_{eft-3}::ced-12^{ARM2}::yfp]	17.6 ± 5.0	30

ced-12/ELMO ARM2 Mutation Affects DTC Migration

Genotype	Mismigration (%)	n
Wild-type	0	204
ced-12(k149)	36	305
mig-2(mu28)	21	305
ced-12(k149);[P_{eft-3}::ced-12(WT)::yfp]	9	302
ced-12(k149);[P_{eft-3}::ELMO1(WT)::gfp]	24	302
ced-12(k149);[P_{eft-3}::ced-12N-term::yfp]	39	862
ced-12(k149);[P_{eft-3}::ced-12C-term::yfp]	34	1129
ced-12(k149);[P_{eft-3}::ELMO1^{ARM1}:gfp]	22	922
ced-12(k149);[P_{eft-3}::ELMO1^{ARM2}::gfp]	32	919
ced-12(k149);[P_{eft-3}::ced-12^{ARM1}::yfp]	6	930
ced-12(k149);[P_{eft-3}::ced-12^{ARM2}::yfp]	40	926

Scores shown are an average of three independent transgenic lines. When the *ced-12^{ARM2}* mutant was expressed in a wild-type background, it resulted in a weak DTC migration defect of 2.5% mismigration (average of two independent lines). All constructs have the marker *P_{lim-7}::gfp* used to score DTC migration. n is the number of gonad arms scored in the case of DTC migration or the number of worms scored for engulfment.

was to examine whether the N-term of ELMO may link to upstream players during engulfment, to identify such players, and to better delineate the molecular details of pathways involved in corpse clearance.

Results and Discussion

ARM Repeats of ELMO1 Are Required for Engulfment

To better understand CED-12-mediated signaling in vivo, we performed genetic rescue studies with fragments of CED-12 into CED-12-deficient worms. We tested whether the C-term of CED-12/ELMO is sufficient for function during engulfment in vivo and whether any requirement exists for the N-term (which does not bind CED-5/Dock180). We expressed the C-term or N-term fragments of CED-12 (denoted *C-term::YFP* or *N-term::YFP*) as a transgene in CED-12-deficient worms. However, neither the *C-term::YFP* nor the *N-term::YFP* construct was able to rescue the engulfment defect (Table 1). This suggested that the CED-5 binding via the C-term is insufficient and that features within the N-terminal 550 amino acids of CED-12 are required for engulfment in vivo.

No obvious motifs/domains have been recognized within the N-terminal 550 amino acid region. With secondary structure-based threading programs, we identified several conserved Armadillo (ARM) repeats within CED-12 and ELMO (see Supplemental Experimental Procedures and Figure S1 in the Supplemental Data available with this article online). ARM repeats are 35–50 amino acids in length [25, 26] and are involved in protein:protein interactions in other signaling proteins. In all, 5–7 ARM repeats were predicted within CED-12/ELMO (Figure 1A; Figure S1). To test the functional requirement of the ARM repeats, we individually mutated two of the repeats (Figure 1A). Two residues in each repeat were chosen for mutation, the choice being based upon their high degree of conservation among CED12/ELMO proteins as well as other known ARM repeat-containing proteins [25, 26]. Whereas ELMO1^{wt} was able to functionally synergize with Dock180 in an in vitro phagocytosis assay, individual mutations in the ARM repeats of ELMO (ELMO1^{ARM1} and ELMO1^{ARM2}) failed to efficiently cooperate with Dock180 (Figure 1B). Whereas transient coexpression of ELMO^{wt} and Dock180 in LR73 fibroblasts induced lamellipodia formation and colocalization of the proteins at the ruffles, the ELMO1^{ARM2} mutant was deficient in promoting lamellipodia formation and was not enriched at the cell periphery (Figure 1C). This suggested a role for the ARM repeats of ELMO in Dock180/ELMO1-mediated induction and/or localization of this complex at membrane ruffles. The failure of the ARM mutants to cooperate with Dock180 in engulfment or in the induction of lamellipodia was not due to a defect in Dock180 binding by the ARM mutants because the mutants efficiently coprecipitated Dock180 (Figure 1D). Moreover, in an in vitro GEF assay, where the presence of ELMO increases the Dock180-dependent GEF activity toward Rac, ELMO1^{ARM2} was still able to enhance the Dock180-mediated Rac-GEF activity (Figure S2). Thus, the requirement for the ARM repeats of ELMO during engulfment appeared distinct from its interactions with Dock180.

RhoG Binds to ARM Repeats of ELMO and Promotes Rac-Dependent Engulfment

Recent studies on Rac-like GTPases in mammals have shown that the GTPase RhoG functions upstream of Rac, with a role for RhoG demonstrated in neurite outgrowth and integrin-mediated cell adhesion [27–30]. Interestingly, ELMO2 was identified in a yeast two-hybrid screen for proteins that bind active (GTP bound) RhoG [29, 30]. We hypothesized that ARM repeats of ELMO1 might be involved in binding to activated RhoG and that RhoG, via the ELMO/Dock180 complex, might regulate Rac activation during engulfment. We first confirmed that ELMO1 associated with activated RhoG (RhoG^{Q61L}) (Figure 2A, lanes 5 and 6). Moreover, a trimeric complex of ELMO1:Dock180:RhoG^{Q61L} could be detected when the three proteins were coexpressed (lanes 7 and 8). Consistent with the association of RhoG with the Dock180/ELMO1 complex, RhoG immunoprecipitates contained a Rac-GEF activity, but only when both ELMO1 and Dock180 proteins were coexpressed (Figure S3). ELMO1 was essential for bridging RhoG to Dock180 because the latter two became part of a complex only when ELMO1 was present (Figure 2A, lanes 7–10). These data suggested a link between activated RhoG and ELMO1/Dock180-mediated Rac activation.

We then asked whether RhoG would affect engulfment upstream of Rac. We transiently transfected LR73 cells with various GFP-tagged RhoG constructs and tested

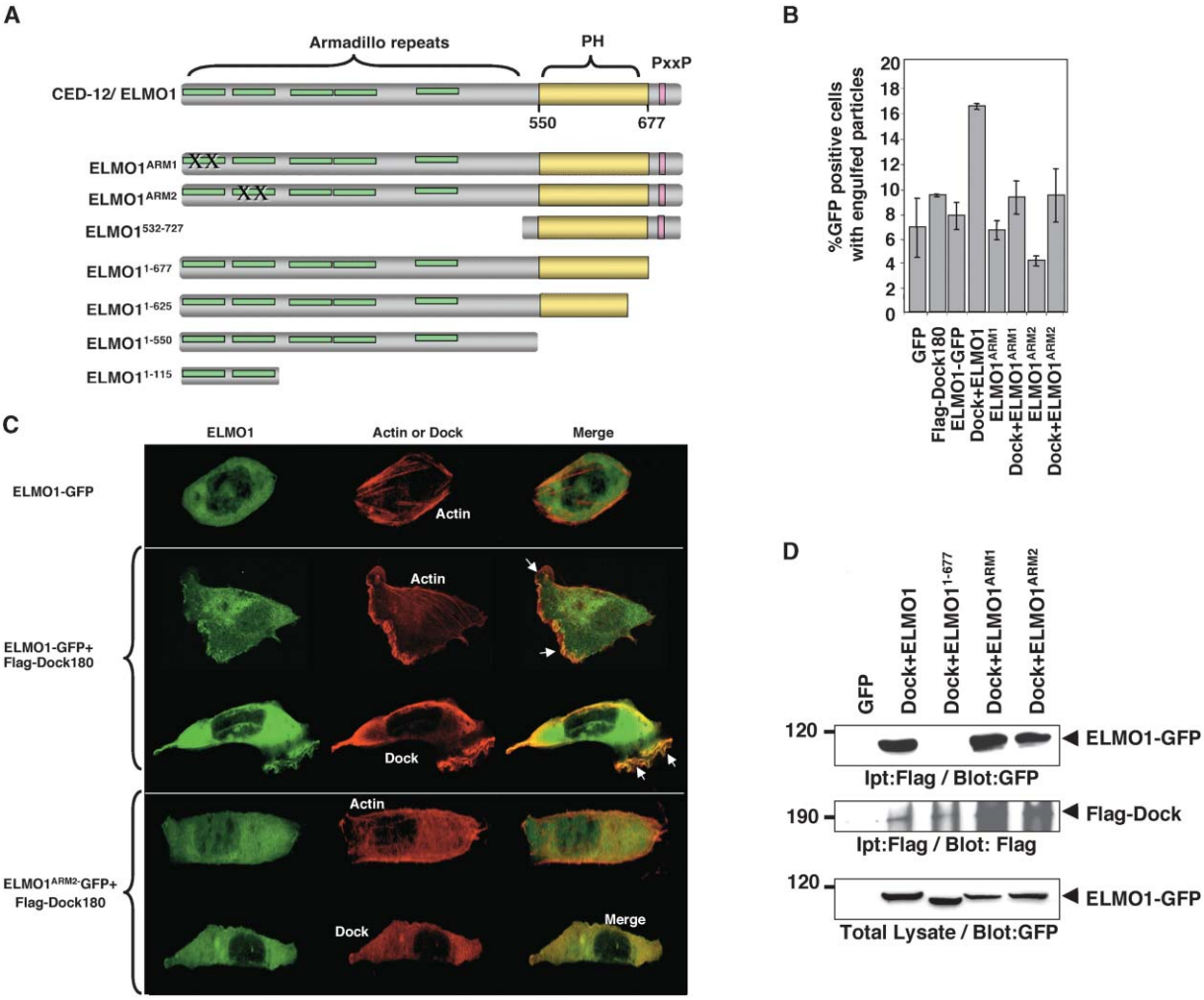

Figure 1. Functional Requirement for the ARM Repeats of ELMO1

(A) Schematic representation of the various ELMO1 constructs.

(B) Requirement for ELMO1 ARM repeats in phagocytosis. GFP or ELMO1-GFP plasmids were transiently transfected into LR73 cells with or without Flag-Dock180, and phagocytosis was measured. Cells with comparable GFP expression were analyzed, and the fraction of GFP-positive cells with engulfed particles is shown (data representative of five independent experiments).

(C) ELMO1^{ARM2} fails to promote ruffling with Dock180 and/or membrane localization. LR73 cells were transiently transfected with the indicated plasmids and analyzed with confocal microscopy. ELMO1-GFP was visualized by its green fluorescence, Dock180 by anti-Flag and labeled secondary antibody, and polymerized actin via phalloidin-rhodamine staining. Quantitation of the cells with ruffles (arrows) in the GFP-positive or GFP-negative untransfected populations were as follows: untransfected cells, 17% (n = 690); ELMO1^{wt}-GFP + Flag-Dock180, 70% (n = 135), and ELMO1^{ARM2}-GFP + Flag-Dock180, 21% (n = 114).

(D) Mutations in ARM repeats do not affect ELMO:Dock180 interaction. LR73 cells were transiently transfected and immunoprecipitated with anti-Flag antibody, and the association of ELMO1 and its mutants with Dock180 was determined by immunoblotting. Immunoblotting of total lysates indicated equal loading. ELMO1-T677 (which does not bind Dock180) served as a negative control.

their effect on phagocytosis. Expression of RhoG^{wt} strongly promoted phagocytosis in multiple independent experiments (fold increase of 2.7 $\pm$ 0.7; $p < 0.0006$; n = 5) (Figure 2B). RhoG^{V12A} or RhoG^{Q61L}, two constitutively active mutants [28], also promoted phagocytosis; however, RhoG mutants deficient in binding to nucleotide (RhoG^{T17N}) or to downstream effectors (RhoG^{F37A}) [28] did not promote uptake (Figure 2B; data not shown). Moreover, coexpression of RhoGIP122, a protein that specifically sequesters GTP bound RhoG [28, 31], potently inhibited the RhoG-mediated engulfment (Figure 2B). Thus, GTP loading of RhoG and the coupling of effectors that bind GTP bound RhoG (such as ELMO1) appeared essential.

RhoG appeared to function upstream of Rac1 because the enhancement of uptake due to RhoG^{V12A} was strongly inhibited by a dominant-negative form of Rac1 (Rac^{T17N}). In contrast, the enhanced uptake due to a constitutively active form of Rac1 (Rac^{V12A}) was still observed when coexpressed with a dominant-negative mutant of RhoG (RhoG^{T17N}) (Figure 2B) [28, 30]. Coexpression of Dock180 and ELMO^{wt} with RhoG modestly (but consistently in multiple experiments) enhanced the uptake over that induced by RhoG alone (Figure 2B). Such a synergy

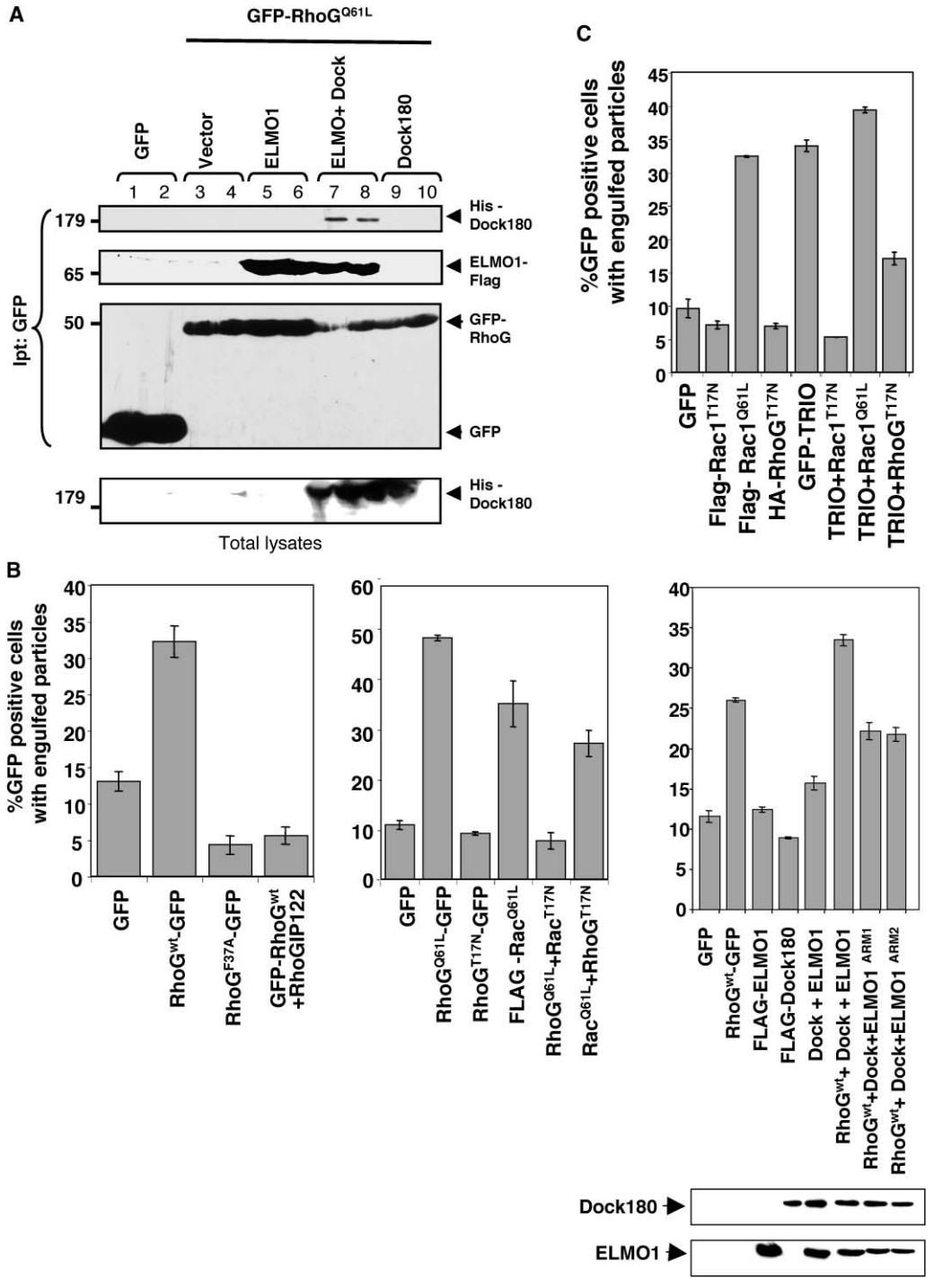

Figure 2. RhoG and TRIO Promote Rac-Dependent Phagocytosis

(A) RhoG^{Q61L} associates with Dock180 via ELMO1. 293T cells were transfected with GFP-RhoG^{Q61L}, His-Dock180, and ELMO1-Flag plasmids as indicated, immunoprecipitated with anti-GFP, and immunoblotted with the indicated antibodies.

(B) RhoG promotes engulfment and synergizes with ELMO and Dock180. LR73 fibroblasts were transiently transfected with plasmids encoding GFP, GFP-RhoG^{wt}, or GFP-RhoG^{F37A} in triplicate with or without coexpression of constitutively active or dominant-negative Rac mutants or the indicated Dock180 and ELMO1 plasmids. The phagocytosis was measured as in Figure 1B. An additional sample was transfected to check protein expression of Flag-tagged Dock180 and ELMO proteins. Data are indicative of at least three independent experiments.

(C) TRIO promotes phagocytosis upstream of Rac1 and RhoG. LR73 cells were transiently transfected with GFP-TRIO alone or with the indicated RhoG or Rac1 mutant plasmids and assayed for phagocytosis (data are representative of three to six independent experiments).

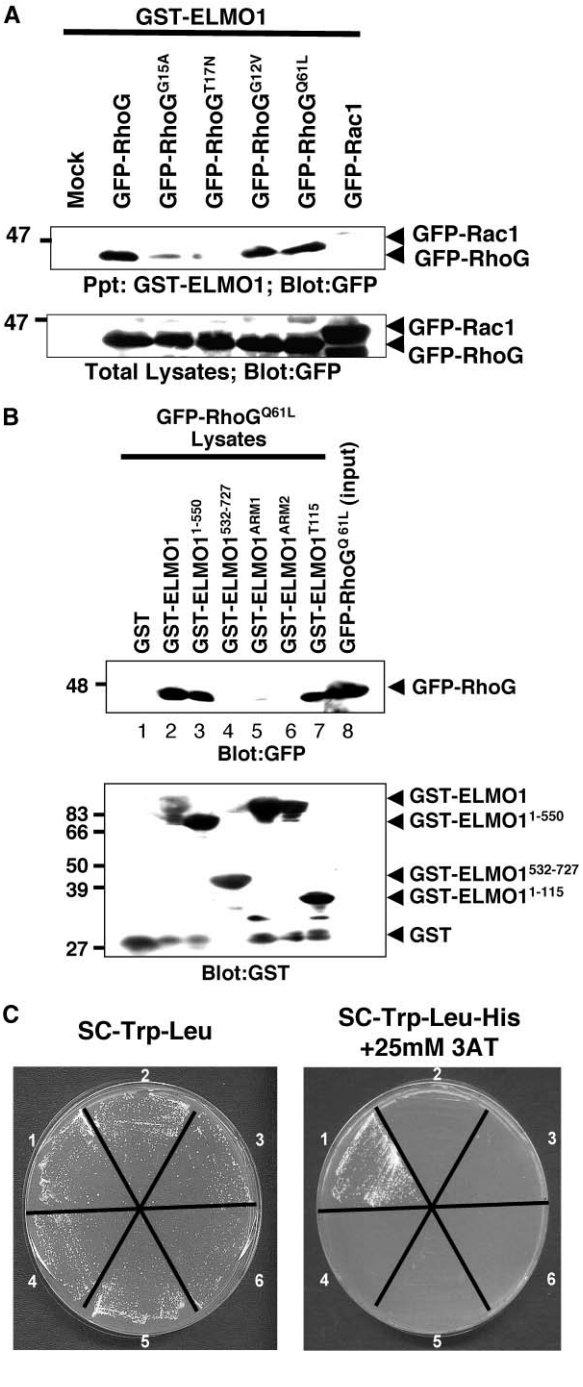

Figure 3. ELMO/CED-12 Specifically Binds Active RhoG/MIG-2 via ARM Repeats

(A) ELMO1 interacts specifically with active RhoG. 293T cells were transiently transfected with the indicated RhoG and Rac plasmids. The lysates were precipitated with GST-ELMO1, and the bound proteins were analyzed by anti-GFP immunoblotting. The intensity of the binding to RhoG^{wt} (presumably GTP bound) was variable between experiments, whereas the binding to RhoG^{Q61L} was consistent.

(B) Intact ARM repeats are required for ELMO/RhoG interaction. 293T cells were transiently transfected with constitutively active RhoG^{Q61L}, and the lysates were precipitated with the indicated bacterially produced wild-type and mutant GST-ELMO1 proteins. The presence of the different GST proteins was confirmed (bottom).

(C) CED-12 specifically interacts with active MIG-2, but not CED-10, in a yeast two-hybrid assay. Constructs expressing the Gal4 DNA binding domain (DB) or DB-fusion proteins (DB-CED-2 and DB-CED-12) and the Gal4 transcription activation domain (AD) or AD-fusion proteins (AD-MIG-2, AD-MIG-2^{G16V}, AD-CED-10, and AD-CED-10^{G12V}) were transformed into yeast, and the transformants were tested for growth in the absence (permissive) or the presence of 3AT (selecting condition). (1) CED-12+ activated MIG-2^{G16V}, (2) CED-12 +MIG-2^{wt}, (3) CED-2 + MIG-2^{G16V}, (4) CED-12 + CED-10^{G12V}, (5) CED-12 + CED-10, and (6) DB + AD (negative control).

was not observed when ELMO^{ARM} mutants were tested (Figure 2B). The enhancement with ELMO^{wt} was not due to the direct nucleotide exchange on RhoG by Dock180 because the Dock180/ELMO complex displayed little detectable in vitro GEF activity toward RhoG (Figure S4).

Further biochemical studies revealed that ELMO1 bound specifically to constitutively active forms of RhoG (Figure 3A). This interaction with RhoG occurred via the N-term of ELMO1, but not the C-term (Figure 3B, lanes 3 and 4). Importantly, an N-terminal 115 amino acid ELMO1 fragment comprising only the ARM repeats 1 and 2 (see schematic in Figure 1A) was able to bind RhoG^{Q61L} (Figure 3B, lane 7); furthermore, ELMO^{ARM1} and ELMO^{ARM2} mutants in the context of full-length ELMO1 failed to interact with RhoG^{Q61L} (Figure 3B, lanes 5 and 6). Taken together, the intact ARM repeats of ELMO1 are required for binding GTP-RhoG, and this correlates with the failure of the ARM mutants to function in phagocytosis.

TRIO Promotes RhoG- and Rac-Dependent Engulfment

We then sought components of this pathway that may function upstream of RhoG. The multidomain protein TRIO has been shown to function as a GEF for RhoG within cells [28, 32–34] (Figure S4). We examined whether TRIO would affect engulfment via RhoG activation. Transient transfection of a GFP-TRIO construct [34] into LR73 cells strongly increased phagocytosis compared to GFP-transfected cells in multiple experiments (fold increase of 3.1 $\pm$ 0.4; $p < 0.0009$; $n = 6$) (Figure 2C). However, a construct with a GEF D1 domain point mutation that abolishes the GEF activity of TRIO (GFP-TRIO-AEP) or a deletion mutant lacking the GEF D1 domain (GFP-TRIO 1-1203) [34] failed to enhance engulfment; instead, they partially inhibited the basal engulfment (see Figure S4). The GFP-TRIO-mediated uptake was inhibited by a dominant-negative form of RhoG (RhoG^{T17N}) (Figure 2C) and by RhoGIP122 (data not shown). Moreover, the TRIO-RhoG-mediated phagocytosis was inhibited by dominant-negative Rac^{T17N}, but not the constitutively active Rac^{Q61L} (Figure 2C). Taken together, these data suggest that a signaling module of TRIO → RhoG can function during phagocytosis and may do so at a step upstream of Rac activation.

Requirement for CED-12 ARM Repeats during Engulfment in *C. elegans*

We then determined the evolutionary significance of the ARM repeats of CED-12 as well as the role of worm homologs of RhoG and TRIO in corpse clearance in *C. elegans*. We engineered independent mutations in ARM1 and ARM2 repeats of CED-12 (analogous to the ELMO1 mutants) and tested their ability to rescue the

engulfment defects in *ced-12* null animals. Whereas *ced-12*^{wt} and *ced-12*^{ARM1} were able to efficiently rescue the engulfment defect, *ced-12*^{ARM2} was unable to do so (Table 1). Notably, the residues in the ARM1 repeat are relatively less conserved between CED-12 and ELMO, whereas the ARM2 repeat was highly conserved (Figure S1). It is likely that the ARM1 repeat of CED-12 may not play a requisite role in *C. elegans*.

MIG-2 and UNC-73 Influence Corpse Clearance In Vivo Upstream of CED-12

Whereas the *unc-73* gene in *C. elegans* has been identified as the *trio* homolog [35, 36], the homolog of RhoG has not been defined. Among the three Rac-like genes in the worm (*ced-10, mig-2*, and *rac-2*), we examined whether *mig-2* might represent *rhoG*; this stems from studies suggesting *ced-10* as the Rac1 homolog and *mig-2* being placed genetically in the same pathway as *ced-5/dock180* during several migration events [20]. In yeast two-hybrid assays, CED-12 specifically interacted with the constitutively active (GTP bound) form of MIG-2 (MIG-2^{G12V}) but not wild-type MIG-2 (Figure 3C). The failure of MIG-2^{wt} to bind CED-12 is likely due to the absence of GEFs that could exchange nucleotides on wild-type MIG-2 in the yeast (as reported previously; [37]). The specific binding of CED-12 to active MIG-2^{G12V} again placed CED-12 as a downstream "effector" of GTP bound MIG-2. It is noteworthy that UNC-73 [38] has been previously shown to have in vitro GEF activity toward MIG-2 and likely acts as a GEF for MIG-2 in *C. elegans*. Importantly, CED-12 did not interact with a constitutively active form of CED-10, consistent with a role of CED-12 upstream of CED-10 activation (Figure 3C).

We then assessed whether *mig-2* or *unc-73* could play a role in engulfment in vivo. Although worms deficient in *mig-2* or *unc-73* alone showed no obvious defects in corpse clearance, a role for both of these genes was revealed in double mutants. *ced-12;mig-2* and *ced-12;unc-73* double-mutant worms showed a significantly higher number of unengulfed corpses compared to the *ced-12* single mutant (Table 2). Interestingly, absence of *mig-2* or *unc-73* augmented the number of persistent corpses due to a weak allele of *ced-12 (tp2)*, but the phenotype due to a strong allele of *ced-12 (n3261)* was not further enhanced, suggesting that *mig-2* and *unc-73* likely function in the same pathway as *ced-12*, rather than being in a parallel pathway (Table 2).

We tested whether *unc-73* or *mig-2* mutations would also enhance the corpse clearance defects in mutants of *ced-2, ced-5*, or *ced-10*, three other members of the same genetic pathway as *ced-12* [18, 19, 22]. Loss of *unc-73* significantly enhanced persistent corpses in the *ced-2* ($p < 0.0001$), *ced-10* ($p < 0.0001$), and *ced-5* ($p < 0.02$) backgrounds (Table 2). Again, the strong alleles of *ced-2* and *ced-5* did not show a greater number of corpses due to loss of *unc-73* in the double mutants. In a similar double-mutant analysis, the *mig-2* mutation also significantly increased corpse number in weak *ced-2, ced-5*, or *ced-10* backgrounds, but not in the strong *ced-2* or *ced-5* mutants (Table 2; [20]). It is noteworthy that the *ced-10* mutant used above is not a null mutant but a partial loss-of-function mutant (because a null mutant of *ced-10* is embryonic lethal) (J.K. and M.O.H., unpublished data). Thus, consistent with the enhanced engulfment due to RhoG and TRIO in mammalian phagocytosis, *mig-2* and *unc-73* do play a role in corpse clearance at the level of a whole organism, and they genetically link to the same pathway as *ced-12, ced-2, ced-5*, or *ced-10*. These data also revealed a novel insight on the two Rac homologs *ced-10* and *mig-2*, in that they can function in the same genetic pathway as *ced-12* during engulfment but appear to do so at distinct steps.

There are at least two possibilities for the lack of an engulfment phenotype in single mutants of UNC-73 and MIG-2. First, there are multiple (and what often appear redundant) engulfment receptors operational in mammals, and homologs for many of these receptors also exist in worms. Because the precise receptor(s) upstream of UNC-73 has not yet been defined, it is possible that only some of them go through the UNC-73/MIG-2 module, whereas some others may directly recruit the CED-12/CED-5/CED-10 pathway. Alternatively, recent studies suggest that the CED-1/CED-7/CED-6 pathway of engulfment also can signal to CED-10, although how this recruitment occurs is unclear (J.K. and M.O.H., unpublished data). Thus, either of these possibilities could

Table 2. *unc-73* and *mig-2* Enhance Persistent Cell Corpses in *ced* Mutants

Strain	Corpse Number	Change[a]	t test
Wild-type	0.0 ± 0.0		
unc-73(e936)	0.0 ± 0.0		
mig-2(mu28)	0.0 ± 0.0		
unc-73(e936);mig-2(mu28)	0.0 ± 0.0		
ced-10(n3246)	24.7 ± 5.3		
ced-10(n3246);unc-73(e936)	31.7 ± 3.0	↑	$p < 0.0001$
ced-12(tp2)	17.3 ± 2.4		
unc-73(e936);ced-12(tp2)	23.8 ± 3.5	↑	$p < 0.0001$
ced-12(n3261)	27.9 ± 3.9		
unc-73(e936);ced-12(n3261)	26.6 ± 4.2	—	$p = 0.133$
ced-5(n2002)	37.1 ± 5.9		
unc-73(e936);ced-5(n2002)	41.1 ± 4.1	↑	$p = 0.019$
ced-5(n1812)	41.9 ± 3.7		
unc-73(e936);ced-5(n1812)	40.1 ± 5.3	—	$p = 0.232$
ced-2(e1752)	12.6 ± 4.0		
unc-73(e936);ced-2(e1752)	32.1 ± 6.3	↑	$p < 0.0001$
ced-2(n1994)	35.2 ± 4.4		
unc-73(e936);ced-2(n1994)	33.4 ± 6.4	—	$p = 0.087$
ced-2(e1752)	12.6 ± 4.0		
ced-2(e1752);mig-2(mu28)	43.1 ± 5.4	↑	$p < 0.0001$
ced-2(n1994)	35.3 ± 4.4		
ced-2(n1994);mig-2(mu28)	41.5 ± 4.2	↑	$p < 0.0001$
ced-5(n2002)	37.7 ± 5.9		
mig-2(mu28);ced-5(n2002);	46.4 ± 7.4	↑	$p < 0.0001$
ced-5(n1812)	42.8 ± 4.5		
mig-2(mu28);ced-5(n1812)	44.4 ± 5.9	—	$p = 0.377$
ced-12(tp2)	17.9 ± 3.0		
mig-2(mu28);ced-12(tp2)	30.5 ± 5.0	↑	$p < 0.0001$
ced-12(n3261)	27.9 ± 3.9		
mig-2(mu28);ced-12(n3261)	30.0 ± 3.2	—	$p = 0.052$
ced-10(n1993)	18.3 ± 3.0		
mig-2(mu28);ced-10(n1993)	23.6 ± 5.0	↑	$p < 0.0001$

Number of cell corpses in the head regions of twenty L1 larvae within 30 min of hatching was scored with Nomarski optics as described [40].

[a] ↑ indicates significant increase of cell corpse number in double mutants when compared to corresponding *ced* single mutants ($p < 0.05$); — indicates no significant change ($p > 0.05$).

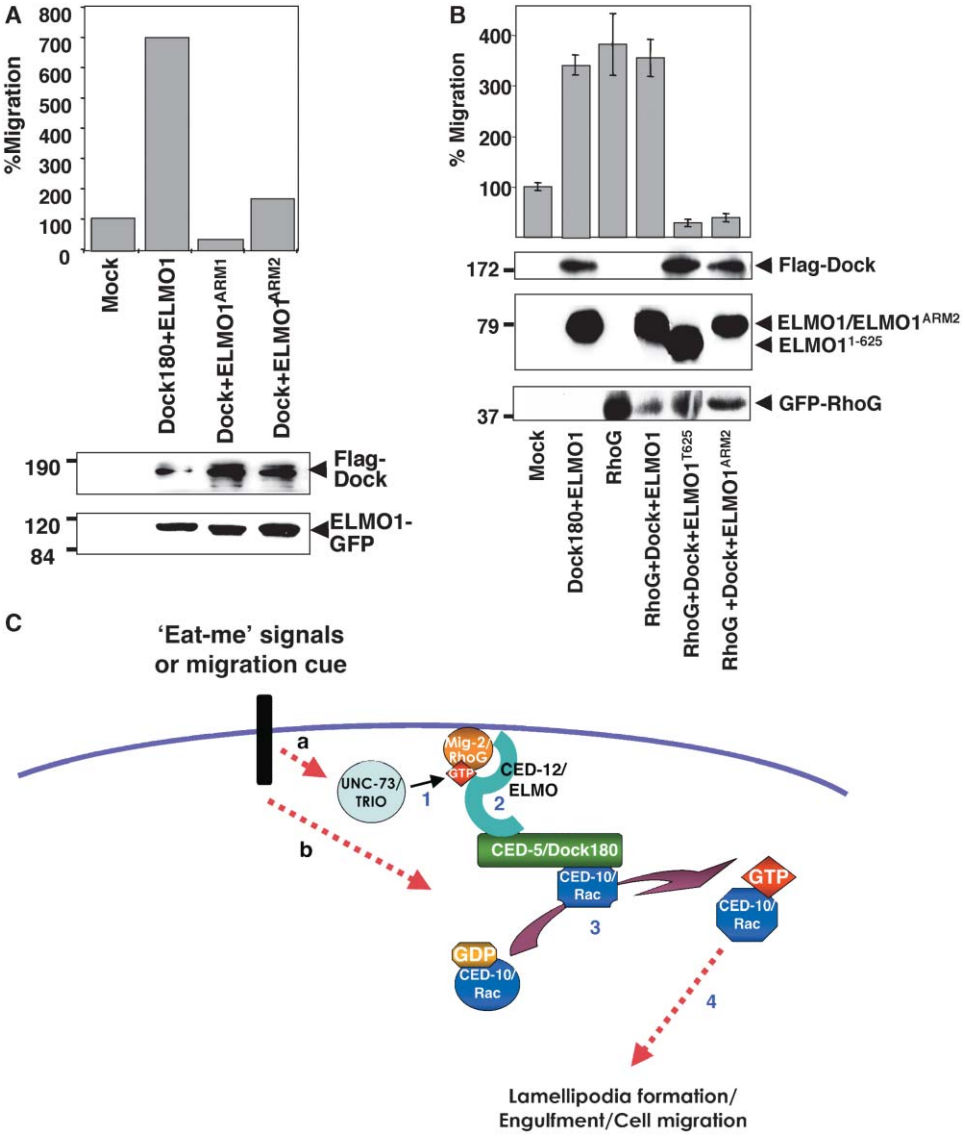

Figure 4. ARM Repeats of ELMO1 Affect In Vitro Cell Migration

(A) ARM mutants of ELMO1 fail to synergize with Dock180 in migration in vitro. LR73 cells were transiently transfected with the indicated plasmids, and the migration of transfected cells through a 24-well Transwell chamber filter was assessed. The luciferase alone control was set at 100%. Aliquots of cells from each transfection were immunoblotted to confirm expression of Dock180 and ELMO mutants.

(B) Intact ARM repeats of ELMO are required for RhoG-mediated in vitro migration. RhoG was expressed alone or together with the indicated ARM mutant ELMO plasmids in LR73 cells, and the migration was assessed as above.

(C) Working model for the TRIO/RhoG signaling module leading to Rac activation via the ELMO/Dock complex. "Eat-me" signals or migration cues via an unidentified receptor(s) promote the activation of RhoG through its GEF, TRIO (1, a). Genetic studies in *C. elegans* suggest the existence of a parallel pathway independent of UNC-73 and MIG-2 (b), but signals through CED-10. GTP bound RhoG/MIG-2 could target ELMO1 to the membrane (2) where the Dock180/ELMO complex becomes recruited/activated and can function as a bipartite GEF for Rac1 (3), in turn leading to lamellipodia formation (4).

lead to UNC-73/MIG-2-independent CED-10/Rac activation to promote engulfment.

ARM Repeats of CED-12/ELMO Are Also Critical for Cell Migration

In addition to the corpse clearance defect, worms deficient in *ced-12* also show defects in migration of the distal tip cells (DTCs), which guide the migration of the hermaphrodite gonad during development in the worm [18, 19, 22]. The expression of the *ced-12*^{ARM2} mutant failed to rescue the DTC migration defect in the *ced-12* null animals (Table 1), suggesting that the second ARM repeat of CED-12 is essential in both engulfment and DTC migration. Similarly, the *elmo*^{ARM2} mutant also failed to rescue the DTC migration defect in *ced-12* null worms, whereas the *elmo*^{wt} (as has been reported previously; [22]) and *elmo*^{ARM1} mutant were able to partially rescue the defect (Table 1). The requirement of the intact ARM

repeats of ELMO was also tested in a mammalian cell migration assay, where coexpression of ELMO1 and Dock180 promotes Transwell migration of LR73 cells [24]. Although wild-type ELMO1 promoted migration when coexpressed with Dock180, none of the three ELMO1 ARM mutants were able to promote migration (Figure 4A).

Because MIG-2 has also been shown to be involved in DTC migration (see also Table 2), we asked whether RhoG could promote mammalian cell migration. Low-level expression of RhoG strongly promoted migration of LR73 cells in the in vitro Transwell migration assay (Figure 4B). This RhoG-mediated migration was potently inhibited by ELMO^{T625}, a mutant that can bind RhoG but cannot couple to Dock180/Rac [23, 30], and by the ELMO^{ARM2} mutant that cannot bind RhoG; in contrast, coexpression of Dock180 and ELMO1^{wt} did not affect the enhanced migration due to RhoG (Figure 4B). These data suggest that increased cell migration due to RhoG requires the intact ARM repeats of ELMO1 as well as the ability of ELMO to bind Dock180 and thereby activate Rac.

Conclusions

Although several molecules involved in engulfment have been identified in recent years, the molecular mechanisms coordinating their function during engulfment are not fully understood [1, 39]. Here, through a combination of in vitro and in vivo approaches, we identify two evolutionarily conserved proteins, UNC-73/TRIO and MIG-2/RhoG, as players upstream of the CED-12/CED-5/CED-2/CED-10 module during engulfment. These data also suggest that CED-12/ELMO functions as a multifunctional adaptor, on the one hand acting as an effector for GTP bound MIG-2/RhoG, while also serving as part of a bipartite GEF with CED-5/Dock180 for CED-10/Rac, and thereby coordinating cytoskeletal reorganization during engulfment and cell migration (Figure 4C). The identification of a series of ARM repeats within CED-12/ELMO proteins, their binding to GTP bound active RhoG, and targeting the ELMO/Dock180 complex fill an important gap in our understanding of signaling via the ELMO/Dock180 proteins during engulfment. In summary, this work presents evidence for a model where two GEFs, UNC-73/TRIO and CED–5/Dock180, and their respective substrates, the small GTPases RhoG/MIG-2 and Rac/CED-10, work in tandem to regulate engulfment, with CED-12/ELMO serving as a link between these two GEFs (Figure 4C).

Supplemental Data

Detailed Experimental Procedures and several supplemental figures are available at http://www.current-biology.com/cgi/content/full/14/24/2208/DC1/.

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