Phagocytic receptor signaling regulates clathrin and epsin-mediated cytoskeletal remodeling during apoptotic cell engulfment in C. elegans

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SUMMARY
The engulfment and subsequent degradation of apoptotic cells by phagocytes is an evolutionarily conserved process that efficiently removes dying cells from animal bodies during development. Here, we report that clathrin heavy chain (CHC-1), a membrane coat protein well known for its role in receptor-mediated endocytosis, and its adaptor epsin (EPN-1) play crucial roles in removing apoptotic cells in Caenorhabditis elegans. Inactivating epn-1 or chc-1 disrupts engulfment by impairing actin polymerization. This defect is partially suppressed by inactivating UNC-60, a coflin ortholog and actin server/depolymerization protein, further indicating that EPN-1 and CHC-1 regulate actin assembly during pseudopod extension. CHC-1 is enriched on extending pseudopods together with EPN-1, in an EPN-1-dependent manner. Epistasis analysis places epn-1 and chc-1 in the same cell-corpse engulfment pathway as ced-1, ced-6 and dyn-1. CED-1 signaling is necessary for the pseudopod enrichment of EPN-1 and CHC-1. CED-1, CED-6 and DYNA-1, like EPN-1 and CHC-1, are essential for the assembly and stability of F-actin underneath pseudopods. We propose that in response to CED-1 signaling, CHC-1 is recruited to the phagocytic cup through EPN-1 and acts as a scaffold protein to organize actin remodeling. Our work reveals novel roles of clathrin and epsin in apoptotic-cell internalization, suggests a Hip1/R-independent mechanism linking clathrin to actin assembly, and ties the CED-1 pathway to cytoskeleton remodeling.

KEY WORDS: Actin cytoskeleton, Apoptotic cell engulfment, C. elegans, Clathrin, Epsin, Membrane remodeling

INTRODUCTION
In multicellular organisms, efficient removal of apoptotic cells through an evolutionarily conserved engulfment and degradation process is crucial for sculpting organs and establishing correct body asymmetry, and, furthermore, for preventing inflammatory and autoimmune responses induced by the contents of dying cells (Elliott and Ravichandran, 2010). Engulfing cells use distinct cell-surface receptors to recognize the surface features of apoptotic cells and initiate the extension of pseudopods around their targets (Elliott and Ravichandran, 2010). The fusion of the extending pseudopods and the ensuing scission of the apoptotic cell-containing membrane vacuole (phagosome) from the plasma membrane completes the engulfment process. The assembly and growth of actin filaments underneath the plasma membrane provide the driving force for pseudopod extension around dying cells (Caron, 2001). In addition, plasma membrane expansion at the phagocytic cup region is also essential (Toure et al., 2005). Despite extensive studies, however, how a phagocytic receptor orchestrates both actin assembly and membrane expansion to accomplish apoptotic-cell engulfment is not fully understood.

The Rho family small GTPases are important regulators for actin rearrangement during phagocytosis (Ravichandran and Lorenz, 2007). However, besides the Rho GTPases and their modulators, whether there are additional regulatory pathway(s) for actin polymerization for apoptotic-cell engulfment is not well known. More specifically, how a phagocytic receptor establishes the spatial cue that attracts actin molecules to the region of engulfment needs thorough investigation.

Traditionally, particles less than 0.5 μm in diameter are believed to be internalized through endocytosis, whereas particles of 0.5 μm in diameter or more, such as parasites and apoptotic or senescent host cells, are thought to be internalized through an actin-dependent, clathrin-independent phagocytosis (engulfment) process (Caron, 2001). However, this stereotypical belief that clathrin and actin each acts independently in separate cell internalization events has been seriously challenged. Accumulating evidence has established the important roles of actin-clathrin crosstalk during endocytosis (Kaksonen et al., 2006). Here, we report that apoptotic-cell engulfment requires the functions of clathrin and its adaptor epsin, two endocytic factors, in actin remodeling.

During C. elegans embryogenesis, 113 somatic cells undergo apoptosis; most apoptosis events occur during mid-embryogenesis [200-450 minutes post-first embryonic cell division (first cleavage)] (Sulston et al., 1983). Genetic screens have identified many genes that regulate apoptotic-cell engulfment or degradation or both; in mutant embryos, cell corpses accumulate during mid- and late embryonic stages (Lu and Zhou, 2012). Epistasis analyses have placed genes acting in the engulfment process into two partially redundant and parallel pathways (Reddien and Horvitz, 2004; Mangahas and Zhou, 2005; Yu et al., 2006). One pathway is led by CED-1, a single-pass transmembrane protein and the prototype of a phagocytic receptor family for apoptotic cells, which also includes Drosophila Draper, mouse Jedi and human mEGF10 and mEGF11
Multiple lines of evidence suggest that phoshatidylserine (PS) serves as an ‘eat-me’ signal that directly or indirectly stimulates CED-1 (Zhou et al., 2001b; Venegas and Zhou, 2007; Wang et al., 2010). The intracellular domain of CED-1 bears conserved binding sites for SH2 and PTB domains (Zhou et al., 2001b). CED-6, a PTB-domain protein, is a candidate adaptor for CED-1 (Liu and Hengartner, 1998; Su et al., 2002). CED-1 and CED-6 recruit a downstream mediator DYN-1 (dynamin), a conserved large GTPase, to budding pseudopods (Yu et al., 2006). During phagocytosis, instead of promoting membrane fission as in endocytosis, DYN-1 and mammalian dynamin 2 play unconventional roles in promoting ‘focal exocytosis’, the recruitment and fusion of intracellular vesicles to the plasma membrane, which supports local plasma membrane expansion and the consequential pseudopod extension (Yu et al., 2006; Gold et al., 1999). These findings define membrane expansion as one particular event regulated by CED-1. Here, we further identified actin rearrangement as another CED-1-regulated event.

In the second pathway, CED-5/Dock180 and CED-12/ELMO1 form a bipartite nucleotide exchange factor to activate CED-10/Rac1 GTPase to promote cytoskeleton reorganization (Reddien and Horvitz, 2004). CED-2/CrkII, an SH2-SH3-domain protein, is proposed to connect a phagocytic receptor with the CED-5/CED-12 complex. A previous report proposes that both pathways converge at CED-10 and that CED-10 mediates the actin-reorganization activity of CED-1 (Kinchen et al., 2005). Our results lead to a different conclusion. Here, we have discovered that C. elegans CHC-1 and EPN-1 (epsin) play crucial roles in actin remodeling during apoptotic-cell engulfment, under the regulation of the CED-1 pathway. Our findings identify a new event regulated by CED-1 and a new mechanism that promotes actin remodeling during the engulfment of apoptotic cells.

**MATERIALS AND METHODS**

**Reagents**

C. elegans strains were raised at 20°C as described (Brenner, 1974). The N2 Bristol strain was the reference wild-type strain, whereas the Hawaii strain CB4856 was the single nucleotide polymorphism (SNP)-mapping strain. Mutations and integrated transgenes used are described elsewhere (Riddle et al., 1997), except when otherwise noted (supplementary material Table S1). Transgenic lines are generated using germline transformation protocol (Hengartner et al., 1997), except when otherwise noted (supplementary material Table S1A). We cloned the gene defined by the en47(m–z–) (m, maternal gene product; z, zygotic gene product) homozygous embryos descended from en47(+); heterozygous mothers display relatively modest Ced phenotype, containing 5.1 cell corpses in late fourfold stage embryos (11-13 hours post-first cleavage) and undergo 100% penetrant L1 larval developmental arrest (supplementary material Fig. S2A,B). We constructed a strain that generated homozygous en47(m’z’) progeny that lost both the maternal and zygotic enp-1(+) (supplementary material Fig. S2A). Approximately one-third of en47(m’z’) progeny underwent embryonic developmental arrest, whereas the rest underwent L1 larval arrest (Fig. 1B, supplementary material Fig. S2B,C). Furthermore, the number of persistent cell corpses retained in late fourfold stage en47(m’z’) embryos was over threefold of that retained in en47(m’z’) embryos (Fig. 1B,D; supplementary material Fig. S2A). These results indicate that depleting both the maternal and zygotic enp-1 products resulted in strong loss-of-function phenotypes. Meanwhile, RNAi of enp-1, which is known to inactivate both maternal and zygotic gene activities (Grishok et al., 2000), resulted in a Ced phenotype as severe as that displayed by the en47(m’z’) embryos (Fig. 1D). We thus further followed phenotypes of en47(m’z’) or enp-1(RNAi) embryos.

Expression of enp-1 cDNA in cell types that can function as engulfing cells, under the control of the ced-1 promoter (P_{ced-1}) (Zhou et al., 2001b), resulted in efficient rescue of the Ced phenotype in enp-1 mutants at all embryonic stages (supplementary material Fig. S1B,C), suggesting that EPN-1 acts in engulfing cells to promote cell-corpse removal.

**RESULTS**

**EPRN-1 is important for cell-corpse removal**

In a screen for mutants containing excessive cell corpses in fourfold stage embryos, we isolated two recessive alleles, en47 and en48 (supplementary material Fig. S1). We cloned the gene defined by the en47 and en48 mutations using standard genetic techniques and found it was enp-1 (supplementary material Fig. S1A) (see Materials and methods) because: (1) enp-1 genomic DNA or cDNA rescued the lethal phenotype and cell-corpse removal defective (Ced) phenotype of the en47 and en48 mutants (supplementary material Figs S1, S2); (2) nonsense and missense mutations were identified in the enp-1-coding sequence in en47 and en48 mutants, respectively (Fig. 1A,F); (3) knocking down enp-1 by RNA interference (RNAi) reproduced the Ced phenotype of en47 and en48 mutants (Fig. 1D); and (4) tm3357, an enp-1 deletion allele (National BioResource Project in Japan), displayed en47 and en48 mutant phenotypes (Fig. 1D; supplementary material Fig. S1B).

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**enp-1 encodes a C. elegans homolog of epsin**

enp-1 encodes an epsin family member. Epsins possess a conserved ENTH (Epsin N-terminal homology) domain that displays high affinity for the membrane phosphoinositide species PtdIns(4,5)P2 and is able to induce membrane curvature (Horvath et al., 2007). In addition, epsins contain ubiquitin interacting

**Microscopy**

Both somatic cell corpses in staged embryos and germ-cell corpses in adult gonads under DIC microscope were scored as described (Lu et al., 2009). Time-lapse recording of the DIC morphology of embryonic cell corpses and of C1, C2 and C3 engulfment events using fluorescence markers were performed using the DeltaVision Imaging System (Applied Precision) as described previously (Lu et al., 2009). The recording of C4 and C5 engulfment started at ~220-250 minutes after first cleavage, and lasted 120-140 minutes. Images were deconvolved and analyzed using the SoftWoRx 4.0 software (Lu et al., 2009).
motifs (UIMs), and motifs for binding other endocytic factors, such as clathrin and clathrin adaptors AP2 and Eps15 (Fig. 1A) (De Camilli et al., 2002; Horvath et al., 2007). Epsins contribute to multiple subcellular events that require membrane remodeling, and are best known for acting as clathrin adaptors during endocytosis (Horvath et al., 2007). However, whether any epsin family member(s) contribute to phagocytosis of apoptotic cells has not been explored previously.

EPN-1 possesses all of the above domains and motifs (Fig. 1A). In EPN-1, the nine α-helices that are crucial for the tertiary structure of the ENTH domain and residues that are crucial for binding to PtdIns(4,5)P₂ are all highly conserved (Fig. 1F) (Ford et al., 2002; Itoh et al., 2001). The en47 allele alters residue Q94 to a stop codon and creates a presumptive null mutation (Fig. 1F). en48 carries a missense mutation, converting an invariant residue (R63) that is crucial for PtdIns(4,5)P₂-binding to alanine (Fig. 1F). The Ced phenotypes displayed by the en48 and en47 mutants are quantitatively similar, indicating that PtdIns(4,5)P₂-binding is likely to be essential for EPN-1 function (Fig. 1D).

Inactivating C. elegans clathrin heavy chain causes cell-corpse accumulation

We further examined whether chc-1, the only C. elegans clathrin heavy chain (CHC-1)-coding gene (Fig. 1C), was involved in
apoptotic cell removal. *chc-1*(RNAi) resulted in severe defects in the uptake of yolk by oocytes (supplementary material Fig. S3A) and in embryonic development (Fig. 1E), as previously reported (Grant and Hirsh, 1999). Although embryonic elongation was arrested, the head appeared to develop normally, allowing us to visualize developmental apoptosis events (Fig. 1E). *chc-1*(RNAi) caused cell-corpse accumulation in embryos (Fig. 1D,E). The Ced and lethal phenotypes were also observed from *epn-1*(bc376)(m 3') deletion mutant embryos (Fig. 1C-E). In adult hermaphrodite gonads, where apoptotic germ cells should be swiftly removed by gonadal sheath cells, *chc-1*(RNAi) also resulted in the accumulation of germ-cell corpses (supplementary material Fig. S3B,C).

*ced-3(n717)*, a loss-of-function mutation of the *C. elegans* CED-3 caspase, blocks all apoptosis (Yuan et al., 1993). In *ced-3(n717)*, *chc-1*(RNAi) embryos, no cell-corpse-like objects were observed (Fig. 1D), indicating that the button-shaped objects observed from *chc-1*(RNAi) embryos (Fig. 1E) were indeed cell corpses.

**The cell-corpse removal activity of EPN-1 is unique among clathrin adaptors**

The functions of clathrin as a coat protein in different sorting events are specified by distinct adaptors (Robinson, 2004). Major clathrin adaptors include the AP (adaptor protein) 1 and 3 complexes for dependent endocytosis, and several monomeric clathrin adaptors, including epsins and AP180 (Robinson, 2004). To examine whether any additional clathrin adaptors regulate cell-corpse removal, we scored the Ced phenotype in embryos in which *C. elegans* homologs of particular adaptors were individually inactivated. We found that inactivation of subunits of AP-1, AP-2 and AP-3 complexes or AP180 (Grant and Hirsh, 1999; Boehm and Bonifacino, 2001; Nonet et al., 1999; Gu et al., 2008) by either RNAi or loss-of-function mutations failed to cause abnormal accumulation of cell corpses (Fig. 2A). RNAi of *apb-1* and *apb-1* both resulted in highly penetrant embryonic arrest as previously reported (Fig. 2B) (Grant and Hirsh, 1999; Simmer et al., 2003; Sönnichsen et al., 2005), indicating effective gene inactivation by RNAi. Interestingly, inactivating AP-1, AP-2 or AP-3 complex subunits or *epn-1* failed to affect germ cell-corpse removal (supplementary material Fig. S3B). These results suggest that among the clathrin adaptors examined, EPN-1 is uniquely involved in cell-corpse removal in embryos.

Previously, through comparative analysis of two distinct kinds of DYN-1 mutations, we learned that endocytosis and cell-corpse engulfment were independent events (Yu et al., 2006). Inactivating each of the AP2 subunits APA-2, APB-1, DPY-23 or APS-2 individually is known to impair endocytosis (Grant and Hirsh, 1999; Gu et al., 2008; Gu et al., 2013), but not cell-corpse removal (Fig. 2A). Likewise, inactivating yolk receptor gene *rme-2* resulted in severe yolk endocytosis defect and embryonic lethality (Grant and Hirsh, 1999) (Fig. 2Bd) but did not affect cell-corpse removal (Fig. 2A). Our observation that inactivating AP2 subunits or *rme-2* does not result in Ced phenotype (Fig. 2) again confirms that impairing clathrin-mediated endocytosis does not necessarily inactivate cell-corpse removal; they further underline the unique function of EPN-1 in apoptotic-cell removal.

**Inactivating *chc-1* or *epn-1* specifically impairs cell-corpse engulfment**

We first found that *chc-1*(RNAi) did not induce any extra apoptosis events (supplementary material Fig. S4A). By contrast, frequent delays in cell-corpse removal were observed from *chc-1*(RNAi) embryos (supplementary material Fig. S4B). These observations indicate that the Ced phenotype observed in *chc-1*(RNAi) animals is caused by impairing cell-corpse removal rather than excessive cell deaths.

An engulfing cell-specific CED-1ΔC::GFP reporter, in which the intracellular domain of CED-1 is replaced by GFP, allows us to track both the engulfment and degradation of cell corpses, because this transmembrane reporter is capable of recognizing neighboring apoptotic cells and is enriched on extending pseudopods; moreover, it is subsequently retained on the surface of a phagosome until total degradation (Zhou et al., 2001b) (N.L. and Z.Z., unpublished). We scored the number of engulfed (labeled with a CED-1ΔC::GFP circle) and unengulfed (not labeled) cell corpses in embryos 11-13 hours post-first cleavage. Data are mean±s.d. n, number of embryos scored. (B) DIC images of 11- to 13-hour-old embryos that develop normally (a) or undergo elongation arrest (b-d). Arrows indicate pharyngeal lumen. Scale bars: 10 μm.

**Fig. 2. Inactivating multiple clathrin adaptors other than EPN-1 or the actin-clathrin coupling protein HPR1 failed to cause cell-corpse removal defects in embryos.** (A) Number of cell corpses in embryos 11-13 hours post-first cleavage. Data are mean±s.d. n, number of embryos scored. (B) DIC images of 11- to 13-hour-old embryos that develop normally (a) or undergo elongation arrest (b-d). Arrows indicate pharyngeal lumen. Scale bars: 10 μm.
In wild-type embryos, the engulfment process, starting with the budding of pseudopods and ending when the phagocytic cup is fully closed, took ~7 minutes (Fig. 3E,Fa-e). In chc-1(b1025) mutants raised at the restrictive temperature and in epn-1(en47)(m–z–) embryos, the initiation of pseudopod budding towards C1, C2 and C3 occurred at normal time points (data not shown), yet pseudopod extension took a significantly longer time to complete (Fig. 3E,Fh-x). By contrast, phagosomes containing C1, C2 or C3, once formed, took relatively normal time to degrade the contents in chc-1 or epn-1 mutants (Fig. 3E,F). These results revealed that pseudopod extension, but not phagosome maturation, is specifically affected by chc-1 or epn-1.

epn-1 and chc-1 both act in the ced-1 pathway

To determine whether epn-1 and chc-1 act in either one of the two known cell-corpse engulfment pathways (see Introduction), we performed epistasis analysis, following the same principle that governs the original analyses placing the engulfment genes into two pathways (Mangahas and Zhou, 2005). We found that chc-1(RNAi) in ced-5(n1812) null or ced-12(n3261) strong loss-of-function mutants (Wu and Horvitz, 1998b; Zhou et al., 2001a), which belong to the ced-10 pathway, increased the number of persistent cell corpses by 32% and 49%, respectively (Fig. 4Aa). By contrast, chc-1(RNAi) in ced-6(n2095) strong loss-of-function mutants (Yu et al., 2006), and in ced-6(n2095) strong loss-of-

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protocol (Lu et al., 2009). In wild-type embryos, the engulfment process, starting with the budding of pseudopods and ending when the phagocytic cup is fully closed, took ~7 minutes (Fig. 3E,Fa-e). In chc-1(b1025) mutants raised at the restrictive temperature and in epn-1(en47)(m–z–) embryos, the initiation of pseudopod budding towards C1, C2 and C3 occurred at normal time points (data not shown), yet pseudopod extension took a significantly longer time to complete (Fig. 3E,Fh-x). By contrast, phagosomes containing C1, C2 or C3, once formed, took relatively normal time to degrade the contents in chc-1 or epn-1 mutants (Fig. 3E,F). These results revealed that pseudopod extension, but not phagosome maturation, is specifically affected by chc-1 or epn-1.
function mutants (Liu and Hengartner, 1998), which all belong to the ced-1 pathway, did not significantly enhance the Ced phenotype of any single mutants (Fig. 4Aa).

We further constructed double mutants in which the epn-1(en47)(m–z–) allele was coupled to six ced mutants, three from each pathway. epn-1(en47) enhanced the Ced phenotype of ced-5, ced-12 or ced-10 mutants by 47%, 69% and 122%, respectively, but failed to significantly enhance the ced-1, ced-6 or ced-7 single mutant phenotypes (Fig. 4Ab). chc-1(RNAi) did not significantly enhance the Ced phenotype of epn-1(en47)(m–z–) (Fig. 4Aa) either, indicating that they act in the same pathway. Together, the above results indicate that both chc-1 and epn-1 act in the ced-1,
**DEVELOPMENT**

*Development 140 (15)*

**CED-1 pathway-dependent manner**

We detected a broad expression pattern of P$_{egm}$::epn-1::gfp, which is functional in rescuing the epn-1(en47) mutant phenotype (supplementary material Fig. S1B) in many cell types, including cells that can act as engulfing cells (supplementary material Fig. S5). In adults, EPN-1::GFP was observed in the pharynx, the nerve ring, body wall muscles, the intestine, vulva, hypodermis and coelomocytes (supplementary material Fig. S5c-j) (data not shown). In embryos, EPN-1::GFP was observed in most cells, in particular in hypodermal and intestinal cells, major embryonic engulfing cell types (supplementary material Fig. S5a-b). EPN-1::GFP, regardless expressed from P$_{epn-1}$ or P$_{ced-1}$, is localized to both the cytoplasm and plasma membrane (Fig. 4B; supplementary material Fig. S5). In the cytoplasm, EPN-1::GFP is either evenly distributed or associated with puncta that likely represent intracellular organelles (Fig. 4Bb,d).

In embryos, EPN-1::GFP is enriched on extending pseudopods and remains on the surfaces of nascent phagosomes for 4-6 minutes before disappearing (Fig. 4Cb; supplementary material Fig. S5a-b). PtdIns(4,5)P$_2$, which binds to many clathrin adaptors [including epsin (Itoh et al., 2001)], is a plasma membrane-enriched lipid essential for actin cytoskeleton remodeling in many cellular events, including phagocytosis (Botelho et al., 2004). We constructed a reporter for PtdIns(4,5)P$_2$ by tagging the pleckstrin homology (PH) domain of human phospholipase C$_{δ1}$, a high-affinity PtdIns(4,5)P$_2$-binding domain, with GFP, and expressed it in engulfing cells (P$_{ced-1}$). In embryos, PH::GFP is transiently enriched on extending pseudopods during engulfment and disappears once a nascent phagosome forms (Fig. 4Ca). The temporal enrichment patterns of EPN-1::GFP and PtdIns(4,5)P$_2$ are almost identical (Fig. 4Ca,b), suggesting that PtdIns(4,5)P$_2$ might be a key factor recruiting EPN-1.

When the point mutation en48(R63A) was introduced into the EPN-1::GFP reporter, EPN-1(R63A)::GFP was neither localized to the plasma membrane nor enriched on phagocytic cups or phagosomes (Fig. 4Cc), indicating that PtdIns(4,5)P$_2$ plays an important role in attracting EPN-1 to pseudopods. In *ced-1*, *ced-5*, *ced-6* and *ced-12* mutants, the speed of pseudopod extension was severely reduced; however, in *ced-1* and *ced-6* mutants, but not in *ced-5* or *ced-12* mutants, EPN-1::GFP failed to enrich on pseudopods (Fig. 4Ccd-g). Interestingly, in *ced-5* and *ced-12* mutants, EPN-1::GFP persisted on pseudopods whose extension was blocked (Fig. 4Cd,e). These results indicate that the recruitment of EPN-1 to pseudopods is dependent on *ced-1* and *ced-6* but not on *ced-5* or *ced-12*. By contrast, the dynamic clustering of CED-1::GFP on extending pseudopods is normal in *epn-1(mz*) mutants (Fig. 4D). Together, our studies indicate that EPN-1 acts downstream of the CED-1 signaling pathway.

**EPN-1 recruits CHC-1 to extending pseudopods in response to CED-1 signaling**

A CHC-1::GFP reporter expressed in embryos from P$_{ced-1}$ is localized to the plasma membrane, the cytoplasm and the perinuclear region (Fig. 5A). Part of CHC-1::GFP was enriched on cytoplasmic puncta (Fig. 5A), which might correspond to early endosomes and/or Trans-Golgi network. CHC-1::GFP is transiently enriched on pseudopods and nascent phagosomes (Fig. 5A-C), frequently appearing as puncta (Fig. 5B), indicating that CHC-1 might form either traditional clathrin-coated pits or flat patches through self-oligomerization (Brodsky, 2012). In embryos co-expressing CHC-1::GFP and EPN-1::mRFP in engulfing cells, they exhibit colocalization on extending pseudopods and nascent phagosomes, with significant co-enrichment on puncta (Fig. 5B), indicating that EPN-1 is associated with oligomerized clathrin coating specific regions of the pseudopod membrane.

In *epn-1*(RNAi) embryos, the recruitment of CHC-1::YFP to pseudopods and phagosomes was defective (Fig. 5D,E). In cells lacking enrichment of CHC-1::GFP on pseudopods (Fig. 5De), a phagosome was distinguishable as a dark sphere embedded inside the green engulfing cell cytoplasm.] By contrast, the enrichment pattern of EPN-1::GFP on pseudopods and phagosomes remained relatively normal in *chc-1*(RNAi) embryos (Fig. 4Ch). These results indicate that enrichment of CHC-1 to pseudopods and nascent phagosomes relies on EPN-1, but not vice versa.

Like EPN-1::GFP, in *ced-5*, *ced-10* and *ced-12* mutants, CHC-1::YFP was persistently enriched on pseudopods and nascent phagosomes (Fig. 5C,E), indicating that the *ced-10* pathway is not involved in recruiting CHC-1 to pseudopods. By contrast, in *ced-1*, *ced-6* or *dyn-2* mutants, the enrichment of CHC-1 to pseudopods was severely reduced or completely blocked (Fig. 5Cb,c,Dd,E), indicating that the *CED-1* pathway recruits CHC-1 to the engulfment site. The enrichment of DYN-1::GFP to pseudopods and nascent phagosomes remained normal when *epn-1* or *che-1* was inactivated (supplementary material Fig. S6), again suggesting that DYN-1 acts upstream of both EPN-1 and CHC-1.

**EPN-1 and CHC-1 promote actin polymerization during pseudopod extension**

Actin rearrangement underneath the phagocytic cup provides a mechanical driving force for pseudopod extension (Caron, 2001). Using a GFP-tagged actin-binding domain of *Drosophila* moesin as a reporter for polymerized actin filaments (F-actin) expressed in engulfing cells (Lu et al., 2011), we monitored the dynamics of actin polymerization during pseudopod extension around *C1*, *C2* and *C3*. In wild-type embryos, F-actin first appeared at the site where pseudopods budded (Fig. 6A, 0 minutes), and subsequently extended around the cell corpse in a zipper-like manner until a phagocytic cup was closed, in a period lasting 4-6 minutes (Fig. 6A,D). Afterwards, F-actin disassembled asymmetrically from the base of a phagosome, completing within ~6 minutes (Fig. 6A, 6-9 minutes). Furthermore, GFP::moesin was simultaneously co-enriched with EPN-1::mRFP on extending pseudopods and nascent phagosomes, frequently on patches and puncta that might be actin organizing centers (Fig. 6A).

When *epn-1* or *che-1* was RNAi inactivated, the extension of F-actin was often slowed down (Fig. 6B, 0-8 minutes and 6C, 0-16 minutes). Furthermore, we observed repeated extension, retraction and re-extension of actin filaments (Fig. 6B, 8-14 minutes and 6C, 16-26 minutes and 28-34 minutes). As a consequence, engulfment took two to four times as long as in wild-type embryos (Fig. 6D). These phenotypes indicate that EPN-1 and CHC-1 are essential for the extension and stability of F-actin underneath pseudopods.

To explore further the variety of actin rearrangement defects in engulfing cells of different identities, we monitored the actin behavior during the engulfment of two additional dying cells, *C4* and *C5*, by their sister cells ABplppapa and ABprpppapa, respectively, during early embryogenesis (Fig. 3A,B) (Sulston et al., 1983). Unlike *C1*, *C2* or *C3*, which lacked detectable GFP::moesin expression, *C4* and *C5* inherited a faint GFP::moesin signal from their mother cells (Fig. 6F, 0 minutes). However, the
extension of engulfing cell F-actin around them could still be easily recognized because of the further enriched GFP signal within phagocytic cups (Fig. 6F; supplementary material Movie 1). In wild-type embryos, the time needed for engulfing C4 and C5 varied over a bigger range than for engulfing C1, C2 and C3 (Fig. 6E,F), perhaps influenced by factors such as the different identities of the corresponding engulfing cells. In epn-1(RNAi) and chc-1(RNAi) embryos, delayed formation and closure of phagocytic cups occurred frequently and over a large time span (Fig. 6E). In certain extreme cases, despite the attempt of engulfing cells to extend actin filaments around them, C4 and C5 were detached from their engulfing cells and eventually from the embryo (Fig. 6G; supplementary material Movie 2). This detachment phenotype, which was not observed from C1, C2 or C3, suggests defects in another actin-related event: cell adhesion (Cougoule et al., 2004).

**Impairing actin depolymerization partially suppresses epn-1 and chc-1 phenotypes**

*C. elegans* unc-60 encodes two isoforms that are members of the ADF/cofilin family of actin-depolymerization factors (McKim et al., 1994; Ono and Benian, 1998). These isoforms exhibit differential actin-remodeling activities (Ono et al., 2008). UNC-60A, a non-muscle isoform, strongly inhibits actin polymerization in vitro (Ono and Benian, 1998). Inactivation of unc-60 results in actin organization defects in multiple tissues (Ono et al., 2003; Ono et al., 2008). We found that inactivating unc-60, through either gk239, a null mutation,
or RNAi, reduced the number of persistent cell corpses generated by epn-1 (RNAi) or chc-1 (RNAi) (Fig. 7A). We further monitored engulfment in unc-60 single RNAi and unc-60/epn-1 and unc-60/chc-1 double RNAi embryos using GFP::moesin (Fig. 7B-F). Inactivation of unc-60 partially reverted the delayed actin filament extension phenotype caused by inactivating epn-1 or chc-1 (Fig. 7C,D). As a result, engulfment took much shorter time than in epn-1 or chc-1 single RNAi backgrounds (Fig. 7E,F). In unc-60(RNAi)-treated adult hermaphrodites, instead of forming long filaments, actin formed short aggregates and puncta in gonadal sheath cells (supplementary material Fig. S7), similar to a previous report (Ono et al., 2008). This phenotype indicates the important function of UNC-60 in establishing F-actin structures in multiple cell types. Interestingly, unc-60(RNAi) alone resulted in a modest delay of pseudopod extension (Fig. 7E), whereas in wild-type embryos, the engulfment of C1, C2 and C3 was finished within 6 minutes in 100% of cases, and in unc-60(RNAi) embryos, 27% of engulfment events lasted 7-10 minutes. Together, our results thus indicate that the organization and extension of actin filaments along the engulfment targets requires the balanced action of dynamic actin polymerization and depolymerization. They further suggest that inactivation of epn-1 and chc-1 directly influences the extension and stability of actin filaments.

Budding yeast Sla2 and its mammalian homolog Hip1R are proteins that couple actin with clathrin-coated pits during endocytosis (Boettner et al., 2012). We examined the involvement of HIPR-1, the only full-length homolog of Hip1R in C. elegans, in engulfment. We found that hipr-1(ok1081), a deletion that eliminates the C-terminal two-thirds of HIPR-1 and is likely a null mutation (www.wormbase.org), does not affect embryonic cell-corpse engulfment (Fig. 2A). This result does not support a role of HIPR-1 in clathrin-mediated engulfment.

ced-1, ced-6 and dyn-1 mutants are defective in promoting actin polymerization for cell-corpse engulfment

If, as indicated by the above results, the CED-1 pathway regulates CHC-1 localization during engulfment through EPN-1, inactivating CED-1, CED-6 or DYN-1 should impair actin rearrangement around cell corpses. In ced-1 mutant embryos, the extension of F-actin along C1, C2 or C3 was often much slower
than in wild-type embryos (Fig. 8A). In addition, along the path of pseudopod extension, F-actin repeatedly retracted and re-grew (Fig. 8A, red boxes). Similar slow extension and repeated retraction phenotypes of F-actin were also observed in ced-6(n2095) and dyn-1(n4039) mutant embryos (Fig. 8B,C). These defects significantly contributed to the overall slow speed of engulfment (Fig. 8D).

During the engulfment of C4 and C5, we also observed retraction of actin filaments around the target in ced-1(e1735) background (Fig. 8F, 0-23 minutes). In addition, persistent C4 and C5 were often seen partially or fully detached from their engulfing cells (Fig. 8F, 47-55 minutes) and remained eventually unengulfed (Fig. 8F, 55-131 minutes). The severe engulfment defects are shared by dyn-1 mutants (Fig. 8E,G; supplementary material Movie 3).

The distinct actin-related defects observed from ced-1, ced-6 and dyn-1 mutants, such as the repeated retraction of actin filaments in the phagocytic cups, and the loss of cell-cell attachment, are similar to that observed in epn-1(RNAi) and chc-1(RNAi) embryos (Figs 6, 8), indicating that the CED-1 pathway regulates actin assembly underneath the growing pseudopods through EPN-1 and CHC-1.

**DISCUSSION**

**Clathrin and EPN-1 form an actin-organizing center to facilitate pseudopod extension**

During endocytosis, clathrin forms triskelions that further assemble into polyhedron cages surrounding endocytic vesicles (Young, 2007). The diameter of a canonical clathrin cage is usually less than 150 nm (Harrison and Kirchhausen, 1983; Fotin et al., 2004). The clathrin coat was thus long thought to be only involved in the internalization of small particles, not large ones such as dying cells. Although, traditionally, endocytosis was regarded as an actin-independent event, recent discoveries have established that actin is recruited to clathrin cages to facilitate vesicle invagination, applying the membrane-bending force generated by F-actin (Mooren et al., 2012).

Here, we reveal the essential functions of clathrin heavy chain and its adaptor epsin in *C. elegans* apoptotic-cell engulfment. These functions establish an engulfment mechanism mediated by clathrin, implicating multiple novel aspects. First, CHC-1::GFP form puncta decorating phagocytic cups. These puncta might represent clusters of clathrin-coated buds as intermediates of endocytosis, or, alternatively, larger lattices that are not subject to being pinched off the plasma membrane. If they represent clathrin-coated buds, inactivating dynamin, a fission factor that pinches off clathrin-coated vesicles (Schmid and Frolov, 2011), should result in their membrane retention. However, inactivating dyn-1 prevents CHC-1 from being recruited to phagocytic cups instead of causing its retention, indicating that the CHC-1-puncta are unlikely to represent endocytic intermediates. By contrast, EPN-1 colocalizes with F-actin underneath pseudopods; furthermore, inactivating chc-1 or epn-1 impairs multiple aspects of actin rearrangement underneath the phagocytic cup, reducing the growth speed and stability of pseudopods and weakening the
engulfing-dying cell adhesion. Based on these observations, we propose that underneath a phagocytic cup, CHC-1 oligomerizes into a scaffolding structure that facilitates actin remodeling. This structure might resemble the flat clathrin patches that coat specific membrane domains on endosomes or the trans-Golgi network (Young, 2007; Williams and Urbé, 2007), or the clathrin plaques that facilitate the entry of bacterial pathogens into non-phagocytic mammalian cells through organizing actin polymerization (Bonazzi et al., 2011). Our observations further suggest that, unlike in endocytosis, where actin primarily facilitates the invagination of clathrin-coated membrane and the generation of relatively small endocytic vesicles, during the engulfment of apoptotic cells, which are much larger (at least 3 μm), the clathrin-actin crosstalk not only induces membrane curvature, but, more importantly, directs actin polymerization and drives pseudopod extension around apoptotic cells.

Further study is needed to determine, during phagocytosis of apoptotic cells or other targets, how a flat clathrin scaffold promotes actin assembly and whether traditional coated pits play any role in actin remodeling.

Second, our study suggests that HIPR-1, the only worm homolog of mammalian actin-clathrin coupling protein Hip1R in endocytosis, is either not involved in clathrin-mediated engulfment, or, alternatively, is involved in a manner completely redundant with another unknown protein. Either way, this finding implies that a yet-to-be-identified non-HIPR-1 protein couples clathrin with actin, and thereby might regulate F-actin organization via a novel mechanism.

Third, this work reveals that DYN-1 regulates clathrin-actin crosstalk in engulfment. DYN-1 acts to recruit clathrin to phagocytic cups through first recruiting EPN-1. This unconventional activity of dynamin might be used in multiple cytoskeleton remodeling events.

Moreover, this work identifies the novel physiological role of EPN-1 in apoptotic-cell engulfment. Epsins are known to recruit clathrin to the plasma membrane during endocytosis and to the trans-Golgi network during vesicle trafficking from the trans-Golgi network to endosomes (Wendland, 2002; Mills et al., 2003). C. elegans EPN-1 was reported to promote the endocytosis of Notch ligands and LRP-1/Megalin, a low-density lipoprotein receptor family member (Tian et al., 2004; Kang et al., 2013). We find that EPN-1 and CHC-1 act in the same engulfment pathway, and, furthermore, EPN-1 promotes the recruitment of CHC-1 to phagocytic cups, supporting the model that EPN-1 serves as a clathrin adaptor (Fig. 9B).

In addition to recruiting clathrin, epsin might also contribute its membrane curvature-inducing activity to promote efficient engulfment. Pseudopod extension around a prey requires continuous...
gen...actin further stabilize the curvature. The cooperative actions of EPN-1, CHC-1 and F-actin thus result in the continuous extension of pseudopods along apoptotic-cell surfaces (Fig. 9B).

**The CED-1 pathway recruits EPN-1 to phagocytic cups**

During apoptotic-cell engulfment, we observe transient enrichment of lipid second messenger PtdIns(4,5)P₂ to extending pseudopods, and further discover that PtdIns(4,5)P₂ is an important signaling molecule recruiting EPN-1 to pseudopods. Similarly, during the phagocytosis of opsonized objects by mammalian phagocytes, PtdIns(4,5)P₂ is transiently enriched to extending pseudopods (Botelho et al., 2004). As CED-1, CED-6 and DYN-1 are all essential for recruiting EPN-1 to pseudopods, we propose that, as a phagocytic receptor, CED-1 might promote the regional burst of a PtdIns(4,5)P₂-synthesis activity through its mediator DYN-1 and an effector PI-kinase(s) (Fig. 9), similar to how it promotes PtdIns(3)P synthesis on nascent phagosomes (Yu et al., 2008; Lu et al., 2012).

Epsins are known to interact with transmembrane receptors through ubiquitin-mediated or ubiquitylation-independent interactions (Horvath et al., 2007; Kang et al., 2013). Currently, it is unknown whether CED-1 is ubiquitylated and whether CED-1 and EPN-1 directly interact. If direct interaction occurs, CED-1 and PtdIns(4,5)P₂ might cooperatively control the transient enrichment of EPN-1 to the site of engulfment initiation (Fig. 9B).

**The CED-1 signaling pathway orchestrates the remodeling of both the plasma membrane and cytoskeleton during apoptotic-cell engulfment**

Previously, we have identified ‘focal exocytosis’ as a membrane fusion event stimulated by CED-1 and mediated through DYN-1 for facilitating membrane expansion around apoptotic cells (Yu et al., 2006). Our current study further reveals F-actin assembly as another crucial pseudopod extension event regulated by CED-1. The similar category of actin-remodeling defects resulted from inactivating ced-1, ced-6, dyn-1, epn-1 or chc-1, together with the essential roles of CED-1, CED-6 and DYN-1 in recruiting EPN-1 and CHC-1, indicate that the CED-1 pathway drives EPN-1 and CHC-1 to regulate specific aspects of actin remodeling underneath pseudopod membrane.

Rat CED6 was reported to interact with clathrin heavy chain (Martins-Silva et al., 2006). Recently, Drosophila CED-6 was found to act as a clathrin adapter that mediated yolk uptake in egg chambers (Jha et al., 2012). During C. elegans apoptotic-cell engulfment, however, ced-6(–); chc-1(–) and ced-6(–); epn-1(–) double mutants display quantitatively similar levels of Ced phenotype as ced-6(–) single mutants (Fig. 4A,B), suggesting that CED-6 and EPN-1 are unlikely to act as two parallel clathrin receptors. Rather, we find CED-6 acts upstream of EPN-1 in a linear pathway (Fig. 4C). Inactivating ced-1, ced-6 or dyn-1 all resulted in engulfment defects stronger than that of epn-1 or chc-1, supporting the model in which actin remodeling is one of the two branches regulated by the CED-1 pathway.

Previously, the two parallel C. elegans engulfment pathways were proposed to converge at the point of CED-10, primarily based on epistasis analysis results (Kinchen et al., 2005). However, the same type of analysis, performed both previously (Mangahas and Zhou, 2005; Yu et al., 2006) and here, indicates that not only do CED-1, CED-6, CED-7 and DYN-1 act in parallel to CED-10, CED-2, CED-5 and CED-12, but CHC-1 and EPN-1 also belong to the CED-1, but not the CED-10, pathway (Fig. 9A). Furthermore, the transient enrichment of EPN-1 and CHC-1 to phagocytic cups is independent of CED-5, CED-12 or CED-10. These results indicate that the CED-1-EPN-1-clathrin pathway regulates actin remodeling in a Rac GTPase-independent manner (Fig. 9B). Identifying the candidate protein(s) that links the CED-1 pathway to actin remodeling will further reveal this Rac-independent mechanism.

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Author contributions
Z.Z., Q.S. and B.H. designed the experiments. Q.S., B.H. and Z.Z. performed the experiments. N.L., B.C. and B.D.G. generated critical reagents and contributed to experimental design and manuscript preparation. Z.Z., Q.S. and B.H. were responsible for manuscript preparation.

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Clathrin and its adaptor in engulfment


