Arl8/ARL-8 functions in apoptotic cell removal by mediating phagolysosome formation in Caenorhabditis elegans

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ABSTRACT Efficient clearance of apoptotic cells by phagocytes is important for development, tissue homeostasis, and the prevention of autoimmune responses. Phagosomes containing apoptotic cells undergo acidification and mature from Rab5-positive early to Rab7-positive late stages. Phagosomes finally fuse with lysosomes to form phagolysosomes, which degrade apoptotic cells; however, the molecular mechanism underlying phagosome–lysosome fusion is not fully understood. Here we show that the Caenorhabditis elegans Arl-like small GTPase Arl8 (ARL-8) is involved in phagolysosome formation and is required for the efficient removal of apoptotic cells. Loss of function of arl-8 results in the accumulation of apoptotic germ cells. Both the engulfment of the apoptotic cells by surrounding somatic sheath cells and the phagosomal maturation from RAB-5- to RAB-7-positive stages occur in arl-8 mutants. However, the phagosomes fail to fuse with lysosomes in the arl-8 mutants, leading to the accumulation of RAB-7-positive phagosomes and the delayed degradation of apoptotic cells. ARL-8 localizes primarily to lysosomes and physically interacts with the homotypic fusion and protein sorting complex component VPS-41. Collectively our findings reveal that ARL-8 facilitates apoptotic cell removal in vivo by mediating phagosome–lysosome fusion during phagocytosis.

INTRODUCTION Apoptosis, or programmed cell death, is a genetically regulated process of cell suicide. Under normal conditions, apoptotic cells are recognized and degraded by phagocytes (e.g., macrophages) before they release potentially toxic materials. Defective clearance of apoptotic cells can thus lead to various diseases, including autoimmune disorders (Elliott and Ravichandran, 2010). Phagocytic degradation of apoptotic cells is controlled by mechanisms that are well conserved from the nematode Caenorhabditis elegans to humans (Fadeel, 2003). C. elegans is a useful genetic system in which to study the mechanism controlling apoptotic cell phagocytosis (Reddien and Horvitz, 2004; Lettre and Hengartner, 2006). Two conserved signaling pathways have been proposed to mediate the recognition and internalization of apoptotic cells. In one pathway, the engulfment receptor CED-1, together with CED-7 (an ATP-binding cassette transporter), functions upstream of CED-6 (a phosphotyrosine-binding domain-containing adaptor protein) to regulate the recognition of apoptotic cells and transduce engulfing signals (Liu and Hengartner, 1998; Wu and Horvitz, 1998; Zhou et al., 2001). In
the other pathway, the engulfment receptor PSR-1 acts upstream of the signaling proteins CED-2/Crkll, CED-5/Dock180, and CED-12/ELMO1 to activate CED-10/Rac1; this cascade leads to actin remodeling, which drives the growth of membrane pseudopods to enclose apoptotic cells (Fadok et al., 2000; Wang et al., 2003; Kinchen et al., 2005).

The internalized apoptotic cells are enclosed within membrane-bound compartments called phagosomes. Phagosomes undergo a “maturation process” that involves recruitment of early/late endosomal compartments and VATPases for acidification (Desjardins et al., 1994; Henry et al., 2004). The Rab GTPases Rab5 and Rab7 are sequentially recruited to phagosomes to coordinate early-to-late phagosome maturation (Duclos et al., 2000; Roberts et al., 2006; Seto et al., 2011). Rab5 promotes phagosomal fusion with early endosomes and regulates phagosome conversion to a Rab7-positive stage (Veira et al., 2003). Studies in C. elegans identified several Rab GTPases as critical regulators of phagosome maturation (Kinchen et al., 2008; Lu et al., 2008; Mangahas et al., 2008; Yu et al., 2008; Guo et al., 2010; Kinchen and Ravichandran, 2010). The transition of phagosomes from the RAB-5 (Rab5)- to the RAB-7 (Rab7)-positive stage is regulated by the SAND-1 (Mon1)/CCZ-1 (Ccz1) complex (Kinchen and Ravichandran, 2010). GDP-bound Rab5 interacts with the SAND-1/CCZ-1 complex, and the resultant RAB-5/SAND-1/CCZ-1 complex then facilitates Rab7 activation/recruitment by dislodging GDP-bound Rab5 from its complex with the GDP-dissociation inhibitor (GDI). Rab7 is required for phagosome-lysosome fusion, which forms the phagolysosomes in which apoptotic cells are degraded by lysosomal hydrolytic enzymes (Kinchen et al., 2008; Yu et al., 2008). Studies suggested that the homotypic fusion and vacuolar protein sorting (HOPS) complex components may function downstream of Rab5 and be involved in phagosome-lysosome fusion (Kinchen et al., 2008; Yu et al., 2008; Akbar et al., 2011). The HOPS complex, originally characterized in yeast as a factor in vacuolar fusion, consists of six subunits (a core of four subunits, VPS-11, -16, -18, and -33, with two accessory subunits, VPS-39 and -34). In yeast, the HOPS complex is an effector of Ypt7p (yeast Rab7) and binds the vacuolar soluble N-ethylmaleimide–sensitive factor attachment protein receptors (SNAREs) to drive vacuole fusion (Nickerson et al., 2009; Wickner, 2010; Epp et al., 2011).

Arl8 is a small GTPase that is highly conserved among multicellular organisms and localizes primarily to the lysosomes (Bagshaw et al., 2006; Hofmann and Munro, 2006; Nakae et al., 2010). Previous studies showed that Arl8 is involved in lysosomal movement along microtubules (Bagshaw et al., 2006; Hofmann and Munro, 2006) and endocytic traffic to lysosomes (Nakae et al., 2010). Arl8 was shown to interact with Sla1 and kinesin-interacting protein (SKIP), thereby recruiting kinesin-1 to lysosomes to facilitate lysosome migration toward the cell periphery (Rosa-Ferreira and Munro, 2011). In the context of microtubule-dependent membrane transport, C. elegans Arl8 (ARL-8) facilitates the axonal transport of presynaptic cargo neurons (Klassen et al., 2010). A recent article reported an unexpected role of Arl8 in Tobraonius RNA replication in Arabidopsis thaliana (Nishikiori et al., 2011).

In the present study, we identified ARL-8 as a critical component for efficient phagocytic degradation of apoptotic germ cells in C. elegans. We found that an arl-8 deletion mutant exhibited an accumulation of apoptotic germ cells. Apoptotic cells were engulfed by somatic sheath cells (large phagocytic cells) in the arl-8 mutants; however, degradation of the apoptotic cells was severely impaired. In arl-8 mutants, phagosomes containing apoptotic cells were arrested in a Rab7-positive stage. Time-lapse imaging of live animals revealed that the fusion of lysosomes to phagosomes is defective in arl-8 mutants. ARL-8 localized primarily to lysosomes and physically interacted with the HOPS complex component VPS-41. We therefore propose that arl-8 acts to clear apoptotic cells by mediating phagolysosome formation.

RESULTS
arl-8 is required for cell corpse removal in the adult hermaphrodite gonad
In C. elegans, approximately half of the hermaphrodite germ cells undergo apoptosis and are phagocytosed by surrounding somatic sheath cells for degradation by lysosomal enzymes (Gumienny et al., 1999). We previously reported that C. elegans Arl8 localizes primarily to lysosomes and mediates delivery of endocytosed macromolecules to the lysosomes in macrophage-like ceolocytes (Nakae et al., 2010). We found that arl-8(tm2504), a strong loss-of-function mutant (Nakae et al., 2010), exhibited an increased number of cell corpses in the adult gonads (Figure 1A, arrows). We scored cell corpses in one gonad arm of wild-type and arl-8 mutant animals every 12 h after the L4 stage; the number of cell corpses increased progressively in an age-dependent manner in arl-8 mutants (Figure 1B). There was no apparent difference between the wild-type and arl-8 mutant animals in the frequency of cell death in the U-turn region of the gonad (4.4 ± 0.84 and 4.5 ± 0.74 germ cell deaths in 180 min in wild-type and arl-8(tm2504) animals, respectively; n = 4), suggesting that the increase in the number of cell corpses in arl-8(tm2504) animals was not caused by elevated germ cell apoptosis. We next analyzed cell corpse duration to investigate whether arl-8(tm2504) mutants are defective in cell corpse removal. Most cell corpses were degraded within 50 min after their appearance in the wild type, whereas they persisted for more than 100 min in arl-8(tm2504) animals (Figure 1D), indicating that the clearance of cell corpses is impaired in arl-8(tm2504) animals. The arl-8(tm2504) persistent cell corpse phenotype was significantly rescued by expression of arl-8::venus under the ced-1 promoter, which drives gene expression specifically in the engulfing sheath cells (Figure 1C). These results suggest that arl-8 is required for the efficient removal of apoptotic cells by sheath cells. It remains to be determined whether arl-8(tm2504) animals are defective also for the removal of embryonic apoptotic cells, since the homoyzous embryos from arl-8(tm2504) homozygous hermaphrodites arrest in early embryonic stages before programmed cell death is induced (unpublished data).

Phagosomes containing cell corpses are arrested at an RAB7-positive stage in arl-8 mutants
Apoptotic germ cells are engulfed by sheath cells and subsequently degraded inside phagosomes. We first investigated whether the apoptotic cells were engulfed by the sheath cells in arl-8 mutants using acridine orange (AO), which preferentially stains acidic compartments and can be used to label the internalized cell corpses in living cells (Gumienny et al., 1999; Lu et al., 2008; Xiao et al., 2009). Most cell corpses in arl-8 mutants were stained by AO (Figure 2A), indicating that the cell corpses were engulfed by the sheath cells. Phagosomal acidification in arl-8 mutants was also confirmed by LysoTracker Red staining (Figure 2B). We next analyzed the phagosomal maturation of arl-8 mutants by examining the recruitment of Rab GTPases to the phagosomes. Studies showed that the phagosomes recruit Rab5 and Rab7 sequentially to their membranes; whereas Rab5 is recruited transiently to early phagosomes, Rab7 is present on phagosomes at late stages of phagosome maturation (Kinchen et al., 2008; Kinchen and Ravichandran, 2010; Yu et al., 2008). Time-lapse analysis showed that GFP::RAB-5 was recruited to
nascent phagosomes in arl-8(tm2504) animals and detached from the phagosomes similarly to that in the control arl-8(tm2504)/nT1 animals (Figure 2C), although the average duration of RAB-5 at phagosomes of arl-8(tm2504) was slightly longer than that of arl-8(tm2504)/nT1 (39.2 ± 2.33 and 33.2 ± 1.91 min, respectively; n = 11). The number of RAB-5–positive phagosomes in arl-8 mutants was comparable to that observed in the wild type, whereas the number of RAB-7–positive phagosomes increased to a great degree in arl-8 mutants (Figure 2D). Although we cannot rule out the
posibility that loss of arl-8 affects early stages of phagosome maturation, these results suggest that the phagosomes of arl-8 mutants undergo early maturation and arrest at a RAB-7–positive late stage.

**ARL-8 is localized to lysosomes and recruited to phagosomes at late stages of phagosome maturation**

To investigate the potential function of ARL-8 in phagosomal maturation, we analyzed the localization of ARL-8::GFP in sheath cells. ARL-8::GFP was detected in the punctate structures (arrows in Figure 3A) and also in the cytoplasm surrounding germ cells (arrowhead in Figure 3A). Consistent with reports showing that Arl8/ARL-8 localizes primarily to lysosomes (Bagshaw et al., 2006; Hofmann and Munro, 2006; Nakae et al., 2010), most ARL-8::GFP-labeled punctate structures colocalized with NUC-1::mCherry (NUC-1: a lysosomal nuclease involved in the degradation of apoptotic cell DNA; Wu et al., 2000; Guo et al., 2010). We observed some ARL-8::GFP-positive structures with little NUC-1::mCherry (open arrowhead in the inset of Figure 3A), suggesting that ARL-8 may also localize to other membrane compartments besides lysosomes (e.g., endosomes).

ARL-8::GFP was also detected at phagosomal membranes (arrow in Figure 3B). To investigate the dynamics of ARL-8 localization to phagosomes, we performed time-lapse imaging of transgenic animals expressing both ARL-8::GFP and mCherry::RAB-5 (an early-stage marker of phagosome maturation). ARL-8::GFP signal was only faint at the RAB-5–labeled phagosomal membranes (0 min in Figure 4A). The ARL-8 signal then proceeded to form a smooth, continuous circle, whereas the RAB-5 signal on phagosomes decreased; phagosomes converted from RAB-5 positive/ARL-8 negative to RAB-5 negative/ARL-8 positive. Next we did time-lapse analysis using transgenic animals expressing both ARL-8::GFP and mCherry::RAB-7 (Figure 4B). Both ARL-8 and RAB-7 persisted on the phagosomes until degradation of phagosomal contents (unpublished data). Collectively these results indicate that ARL-8 was localized to phagosomes at late stages of phagosome maturation.

Because ARL-8::GFP was observed in both lysosomes and phagosomes, we investigated whether phagosomal localization of ARL-8 was simply a consequence of phagosome–lysosome fusion. Time-lapse analysis using transgenic animals expressing both ARL-8::GFP and NUC-1::mCherry showed that ARL-8::GFP was localized to phagosomes in which no apparent incorporation of NUC-1::mCherry was observed (0 min in Figure 4C and Supplemental Movie S1). ARL-8::GFP-labeled phagosomes then gradually fused with NUC-1::mCherry-labeled lysosomes, leading to the formation of phagolysosomes in which NUC-1::mCherry accumulated (24 min in Figure 4C). Given that ARL-8::GFP was detected in...
Localization of ARL-8 on phagosomes requires RAB-7 and the HOPS-complex subunits

Because ARL-8 colocalized with RAB-7 at phagosomal membranes, we investigated whether phagosomal localization of ARL-8 requires RAB-7. We found that ARL-8::GFP failed to localize to phagosomes in rab-7(RNAi) mutants and persisted as punctate fluorescence around phagosomes (Figure 5A). This result indicates that rab-7 is required for the phagosomal localization of ARL-8. Studies suggested that the HOPS complex functions downstream of rab-7 during phagosomal maturation: loss of function of the HOPS-complex component VPS-39 leads to the accumulation of arrested phagosomes at the RAB-7–positive stage (Kinchen et al., 2008; Kinchen and Ravichandr, 2010; Akbar et al., 2011). To determine whether the HOPS complex is involved in the phagosomal localization of ARL-8, we analyzed the ARL-8::GFP localization in the deletion mutants of HOPS-complex subunits VPS-39 and VPS-41. Whereas localization of ARL-8::GFP on the phagosomal membranes in wild-type animals was observed as two fluorescence peaks along the line scan (arrowheads in Figure 5B), no apparent phagosomal localization of ARL-8::GFP was observed in vps-39(ok2422) and vps-41(ok3433) animals (Figure 5B). These results indicate that both rab-7 and the HOPS complex are required for ARL-8 localization to phagosomes. In contrast, ARL-8::GFP colocalized with NUC-1::mCherry in vps-39(ok2422) animals to a similar extent as in wild-type animals (Figure 5D), suggesting that ARL-8::GFP localizes to lysosomes in a HOPS complex–independent manner.

ARL-8 physically interacts with VPS-41 and is required for phagosome–lysosome fusion

We investigated whether ARL-8 can physically interact with the HOPS-complex components. Purified recombinant Flag-tagged ARL-8 proteins prebound to either GTP$_\gamma$S or GDP were incubated with HEK293T cell lysates containing Myc-tagged VPS-41 and then immunoprecipitated with anti-Flag M2 agarose. We found that GTP$_\gamma$S-bound Flag-ARL-8 preferentially associated with Myc-VPS41 (Figure 6A), which is consistent with a report showing that GTP-bound active Arl8b binds to VPS41 in mammalian cells (Garg et al., 2011). These results suggest that VPS-41 may function as an effector protein of ARL-8 in the phagocytic pathway. Finally, we investigated whether arl-8 is required for phagolysosome formation. In wild-type animals, NUC-1::mCherry-labeled lysosomes were observed on the surface of phagosomes, and NUC-1::mCherry fluorescence then

NUC-1::mCherry-negative compartments (Figure 3A), phagosomal localization of ARL-8::GFP may be archived not only via phagosome–lysosome fusion but also via fusion of these compartments with phagosomes before phagolysosome formation.
The engulfment and clearance of apoptotic cells by neighboring cells is crucial for tissue homeostasis and the regulation of immune responses. In the present study, we identified arl-8 as a novel factor essential for the efficient degradation of apoptotic cells in *C. elegans*. Deletion of arl-8 resulted in the accumulation of apoptotic germ cells. The cell corpses were engulfed by phagocytic sheath cells in arl-8 mutants; however, the phagosomes containing cell corpses arrested in RAB-7–positive stages and exhibited impaired cell corpse degradation. ARL-8 localized primarily to lysosomes and physically interacted with the HOPS-complex component VPS-41. Finally, the loss of arl-8 caused defective phagosome–lysosome fusion. These results suggest that ARL-8 mediates phagolysosome formation to promote the degradation of apoptotic cells in vivo.

Phagosomes are dynamic, membrane-bound organelles that degrade a variety of particles, including potentially pathogenic microorganisms and apoptotic cells. After particle internalization, the nascent phagosome undergoes a complex maturation process involving sequential recruitment of endosomal compartments (Vieira et al., 2002; Kinchen and Ravichandran, 2008). Rab5 is transiently recruited to and subsequently removed from nascent phagosomes and is required for phagosomal maturation into a later Rab7-positive stage (Duclos et al., 2000; Vieira et al., 2003; Roberts et al., 2006; Kinchen and Ravichandran, 2010). Although the duration of Rab5 at phagosomes was slightly longer in arl-8 mutants than in wild type, Rab-7 was recruited to the phagosomes, indicating that phagosomes undergo the transition from early- to late-stage maturation in arl-8 mutants. Consistent with this, the phagosomes of arl-8 mutants were stained with acid-tropic dyes (AO and LysoTracker Red), which preferentially stain late-stage, internalized apoptotic cells. These results suggest that phagosomes are arrested at a RAB-7–positive late stage of maturation in arl-8 mutants.

We previously showed that ARL-8 localizes primarily to lysosomes in the macrophage-like coelomocytes (Nakae et al., 2010). Consistent with this, most ARL-8::GFP-labeled structures were positive for the lysosomal enzyme marker NUC-1::mCherry in sheath cells. Note that a fraction of ARL-8::GFP-labeled structures were negative for NUC-1::mCherry. ARL-8 may thus be localized also to other membrane compartments besides lysosomes (e.g., endosomes). Given that early/late endosomal components were recruited to phagosomes during phagosome maturation (Desjardins et al., 1994; Henry et al., 2004) and that ARL-8::GFP was observed at phagosomes before the formation of phagolysosomes (Figure 4C), ARL-8-positive endosomes may fuse with phagosomes to form ARL-8–labeled phagosomes, which subsequently fuse with lysosomes to form phagolysosomes. Further analysis is required to determine whether the preceding association of ARL-8 with phagosomes is involved in the subsequent phagosome–lysosome fusion.

Previous studies showed that Rab-7–positive phagosomes finally fuse with lysosomes to form the phagolysosomes in which cell corpses are degraded. The molecular mechanism underlying this process is poorly understood. Studies suggested that the HOPS complex functions downstream of Rab-7 to mediate phagosome–lysosome fusion (Kinchen et al., 2008; Akbar et al., 2011). Given that the yeast HOPS complex is recruited to Ypt7p/Rab7 on vacuolar membranes and interacts with SNAREs required for membrane fusion, the HOPS complex may be recruited to phagosomes via Rab-7 in sheath cells and involved in phagosome–lysosome fusion. Of interest, a recent report showed that human Arl8b interacts with the HOPS-complex subunit VPS41 and is involved in the recruitment of the HOPS components to Arl8b–positive compartments (Garg et al., 2011). Given that ARL-8 localized primarily to lysosomes and physically interacted with VPS-41, the HOPS complex may be recruited to
both RAB-7-positive phagosomes and ARL-8-positive lysosomes, thereby promoting fusion between phagosomes and lysosomes. Alternatively, given that phagosomal localization of ARL-8 preceded phagolysosome formation and required the HOPS-complex components, the HOPS complex may be involved in the fusion of ARL-8-positive endosomal compartments with phagosomes to promote phagosomal maturation required for the subsequent fusion with lysosomes. To test these possibilities, we tried to determine the subcellular localization of the HOPS complex in sheath cells; however, various attempts to create transgenic animals that express sufficient levels of GFP- or mCherry-fusion proteins of the HOPS components have been unsuccessful. Future studies are thus needed to clarify the functional relationships among ARL-8, RAB-7, and the HOPS complex in the phagocytic pathway.

We previously reported that ARL-8 is required for endocytic traffic to lysosomes in macrophage-like coelomocytes in C. elegans (Nakae et al., 2010). The present study suggests that ARL-8 plays an important role in the delivery of phagocytosed cargo to lysosomes. Consistent with these findings, mammalian Arl8b was shown to be involved in the transport of various cargoes to lysosomes in macrophages (Garg et al., 2011). In contrast, Arl8b was reported to be dispensable for the trafficking of endocytosed BSA to lysosomes in HeLa cells (Kaniuk et al., 2011). The requirement of Arl8b for cargo trafficking to lysosomes may thus depend on cell type and/or cellular status. Uncovering the lysosomal delivery regulatory mechanism mediated by Arl8b for ARL-8 may help to elucidate the spatiotemporal control of degradation of cargo molecules in lysosomes.

**MATERIALS AND METHODS**

**Strains and genetics**

Worm cultures and genetic crosses were typically performed according to standard protocols (Brenner, 1974). The N2 Bristol strain was used as the wild-type strain. Other strains used in this study are as follows: CB3203 ced-1(e1735) I; YB153 arl-8(tm2504) IV/nT[qls51][IV]; VC2542 vps-39(ok2442) V/nT[qls51][IV]; VC2784 +/szT1[lon-2(e678)] I; vps-41(ok3433)/szT1 X; YB1439 arl-8(tm2504) IV/nT[qls51][IV]; qxls58[ced-1::lmp-1::Cherry]; tdEx955 [P::arl-8::gfp::rab-5, rol-6(su1006)]; YB2187 arl-8(tm2504) IV/nT[qls51][IV]; tdEx1504[P::arl-8::gfp::rab-5, P::nuc-1::mCherry, rol-6(su1006)]; YB1468 arl-8(tm2504) IV/nT[qls51][IV]; qxls58[ced-1::lmp-1::Cherry]; tdEx974 [P::arl-8::gfp::rab-5, rol-6(su1006)]; YB1471 arl-8(tm2504) IV/nT[qls51][IV]; qxls58[ced-1::lmp-1::Cherry]; tdEx977 [P::arl-8::gfp::rab-7, rol-6(su1006)]; YB2034 arl-8(tm2504) IV/nT[qls51][IV]; tdEx1385[P::nuc-1::mCherry, rol-6(su1006)]; YB2003 tdEx1361[P::nuc-1::mCherry, rol-6(su1006)]; YB2036 tdls27[arl-8::gfp::rab-5, rol-6(su1006)]; tdEx1387[P::nuc-1::mCherry, rol-6(su1006)]; YB1966 tdls27[arl-8::gfp::rab-5, rol-6(su1006)]; tdEx1332[ced-1::mCherry, rol-6(su1006)]; tdls27[arl-8::gfp::rab-5, rol-6(su1006)]; tdEx1418[arl-8::gfp::rab-7, rol-6(su1006)]; WB2098 vps-39(ok2442) V/nT[qls51][IV]; tdls27[arl-8::gfp::rab-8, rol-6(su1006)]; WB2270 +/szT1[lon-2(e678)] I; vps-41(ok3433)/szT1 X; tdls27[arl-8::gfp::rab-8, rol-6(su1006)]; WB2221 vps-39(ok2442) V/nT[qls51][IV]; tdls27[arl-8::gfp::rab-8, rol-6(su1006)]; tdEx1534[ced-1::lmp-1::Cherry].

Plasmid constructs

To generate P::arl-8::venus, a 5-kb fragment of the upstream promoter region of the ced-1 gene was amplified from genomic DNA by PCR using the primers 5′-GGCGGCCGTGGCCGGCGTCAAAAAACAGG-3′ and 5′-GGATCCCTGGATTAGTCACTACCTCGT-3′ and subsequently cloned into the venus-fusion expression vector pFX-arl-8::venus, which contains the entire coding region of the arl-8 gene. To generate P::arl-8::gfp::rab-7, the entire coding region of the rab-7 gene was amplified from genomic DNA by PCR using the primers 5′-GGCGATCAATTGCGGAAACCCAAAAGAAG-3′ and 5′-GGCCGGATCTTTGAACAACCTTGTGAC-3′ and subsequently cloned into the GFP-fusion expression vector pKK04; this insertion was followed by the insertion of the genomic DNA fragment containing the promoter region of the ced-1 gene. A similar strategy was used to generate P::arl-8::gfp::rab-5 using the primer sets 5′-GGCGGCCGTGGCCGGCGTCAAAAAACAGG-3′ and 5′-GGATCCCTGGATTAGTCACTACCTCGT-3′. To generate P::arl-8::nuc-1::mCherry, the entire coding region of the nuc-1 gene was amplified from genomic DNA by PCR using the primers 5′-GGCGCGATGCGTGGTCCTCTCGGC-3′ and 5′-GGCGGGCCGATGCAAAATTGTTGTGCAAA-3′ and subsequently cloned into the mCherry-fusion expression vector pFX-P::arl-8::mCherry, which contains the promoter region of the ced-1 gene. To generate P::arl-8::mCherry::rab-5 and P::arl-8::mCherry::rab-7, the entire coding regions of rab-5 and rab-7 were prepared by BamHI digestion of P::arl-8::gfp::rab-5 and P::arl-8::gfp::rab-7, respectively, and the resultant DNA fragments were subcloned into the BamHI site of the mCherry-fusion expression vector pKK06, which contains the promoter region of the ced-1 gene.

Quantification of cell corpses

Germ cell corpses were scored using Nomarski optics. Cell corpses in the germline meiotic region of one gonad arm were scored 12, 24, 36, and 48 h after the L4 stage. To record the duration of the cell corpses, adult animals (48 h post L4 stage) were mounted on 4% agarose pads with 2 mM levamisole in M9 medium. The gonad arm was observed under Nomarski optics manually every 5 min for 180 min. The temperature was maintained at 18–22°C during the scoring.

Acridine orange and LysoTracker staining

AO staining was performed as described previously, with minor modifications (Xiao et al., 2009). Adult animals were incubated in 250 μl of M9 medium containing AO (0.14 mg/ml) and OP50 bacteria for 90 min in the dark. The stained animals were transferred to growth medium plates to recover for 1 h and then examined under fluorescence microscopy to score the AO-positive germ cell corpses. LysoTracker staining was performed as described previously, with slight modifications (Guo et al., 2010). Briefly, adult animals were dissected in staining buffer (20 mM Na 4,4-dihydroxyethyl)-1-piperazinethanesulfonic acid, pH 7.4, 60 mM NaCl, 32 mM KCl, 3 mM Na2HPO4, 5 mM MgCl2, 2 mM levamisole, 0.1% tritamine, 1 μM LysoTracker Red DND-99) and examined by fluorescence microscopy.

**Microscopy**

Nomarski and fluorescence images were obtained with a Zeiss Axio Imager M1 microscope system equipped with AxioVision software (Zeiss, Jena, Germany). Confocal images were acquired with a Nikon ECLIPSE TE2000-E (Nikon, Melville, NY) equipped with a CSU10 (Yokogawa, Tokyo, Japan)/IXon DV887 (Andor, Belfast, United Kingdom) confocal scanner unit and processed with Nikon NIS-Elements AR3.2, ImageJ (National Institutes of Health, Bethesda, MD), and
Photoshop (Adobe, San Jose, CA) software. For quantification of the fluorescence intensity of GFP on phagosomes, the area of the limiting phagosomal membrane in each image was selected manually using ImageJ software, and the fluorescence intensity in the selected area was measured.

RNA interference experiments

The C. elegans open reading frame–RNA interference (RNAi) library, version 1.1, was purchased from Open Biosystems (Huntsville, AL). The feeding RNAi protocol was performed as described previously (Kamath et al., 2001; Poteryaev et al., 2007). Briefly, adult animals (P0) were placed on RNAi plates, and F1 adult animals were observed. The animals were maintained at 20°C during RNAi treatment.

In vitro pull-down assay

Flag-tagged ARL-8 (Flag-ARL-8) was produced in the Escherichia coli strain BL21-CodonPlus (DE3)-RIL (Agilent Technologies, Santa Clara, CA) using pCold TF expression vector (Takara Bio, Otsu, Japan). cDNA encoding vps-41 (isoform a) was subcloned into pYcM-CMV5 vector, and the expression vector was transfected into HEK293T cells using Lipofectamine2000 (Life Technologies, Carlsbad, CA). The transfected cells were homogenized 24 h after transfection in buffer A (10 mM triethanolamine-acetic acid, pH 7.6, 250 mM sucrose and 1 mM EDTA) and centrifuged (200,000 × g, 30 min, 4°C). The supernatant was mixed with purified Flag-ARL-8 proteins that had been preloaded with GDP or GTPγS and bound to anti–Flag M2 agarose (Sigma-Aldrich, St. Louis, MO), and the mixture was incubated for 1.5 h at 4°C in buffer B (37 mM Tris-HCl, pH 7.5, 2.6 mM triethanolamine-acetic acid, pH 7.6, 74 mM NaCl, 65 mM sucrose, 3.7 mM MgCl₂, 0.26 mM EDTA, 0.74 mM dithiothreitol [DTT], 2.22 mM dimyristoylphosphatidyl choline [DMPC], 0.37 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, and 3.7 μM GDP or GTPγS). Anti-Flag M2 agarose (Sigma-Aldrich, St. Louis, MO), and the mixture was incubated for 1.5 h at 4°C in buffer B (37 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 3 mM DMPC, and 5 μM GDP or GTPγS), followed by incubation with Flag peptide (200 μg/ml; Sigma-Aldrich) for 30 min at 4°C. The Flag-peptide eluted fraction was subjected to Western blot analysis using anti-Myc and anti-Flag antibodies.

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