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# Autophagy protects *C. elegans* against necrosis during *Pseudomonas aeruginosa* infection

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**Autophagy, a conserved pathway that delivers intracellular materials into lysosomes for degradation, is involved in development, aging, and a variety of diseases. Accumulating evidence demonstrates that autophagy plays a protective role against infectious diseases by diminishing intracellular pathogens, including bacteria, viruses, and parasites. However, the mechanism by which autophagy regulates innate immunity remains largely unknown. Here, we show that autophagy is involved in host defense against a pathogenic bacterium *Pseudomonas aeruginosa* in the metazoan *Caenorhabditis elegans*. *P. aeruginosa* infection induces autophagy via a conserved extracellular signal-regulated kinase (ERK). Intriguingly, impairment of autophagy does not influence the intestinal accumulation of *P. aeruginosa*, but instead induces intestinal necrosis. Inhibition of necrosis results in the survival of autophagy-deficient worms after *P. aeruginosa* infection. These findings reveal a previously unidentified role for autophagy in protection against necrosis triggered by pathogenic bacteria in *C. elegans* and implicate that such a function of autophagy may be conserved through the inflammatory response in diverse organisms.**

Autophagy, a well-conserved lysosomal pathway that involves the degradation of cytoplasmic components, plays important roles in a broad diversity of the biological processes, ranging from development, senescence, and lifespan extension, to cancer (1, 2). In addition, autophagy has a prominent role in resistance to bacterial, viral, and protozoan infection in metazoan organisms (3–6). Autophagy is unique in its capacity to sequester invading bacteria, and target these pathogens for lysosomal degradation, thus providing a mechanism for the elimination of intracellular microorganisms. For example, after the pathogenic bacterium *Streptococcus pyogenes* (group A *Streptococcus*) enters human epithelial cells, the bacterium in the cytoplasm is sequestered in autophagosome-like compartments and degraded upon fusion with lysosomes. In contrast, *Streptococcus* exits freely into the cytoplasm of autophagy-deficient $Atg5^{-/-}$ cells that lack autophagic ability (3). In phagocytic cells, such as macrophages, *Mycobacterium tuberculosis* resides intracellularly in the phagosome and blocks phagolysosome biogenesis (4). Induction of autophagy by physiological or pharmacological factors promotes mycobacterial colocalization with the autophagosomes and results in a decreased viability of intracellular mycobacteria (4, 7). In addition to pathogen clearance, accumulating evidence suggests that autophagy is associated with other aspects of immunity and inflammation (8–11). For instance, lack of autophagy in macrophages results in the accumulation of dysfunctional mitochondria, which, in turn, promotes secretion of proinflammatory factors IL-1β and IL-18 (8, 10), suggesting that autophagy regulates inflammation responses by suppressing the secretion of immune mediators.

The genetically tractable model host *Caenorhabditis elegans* provides a useful tool to study the innate immune system and the mechanism of host–pathogen interactions (12). In *C. elegans*, the autophagy machinery serves as an essential component of the host defense by degrading intracellular pathogens. Inhibition of autophagy in *C. elegans* leads to a significant increase in accumulation of the microsporidian *Nematocida parisii*, an intracellular pathogen of worms, whereas activation of autophagy by knockdown of *let-363*, the worm ortholog of TOR, causes a dramatic decrease in pathogen load (13). When the pathogenic bacterium *Salmonella typhimurium* invades the intestinal epithelial cells of worms, the bacterium is targeted to lysosomes for degradation in wild-type (WT), but not in autophagy-deficient animals (5). Besides *S. typhimurium*, several clinically relevant bacteria, such as *Pseudomonas aeruginosa* (14) and *Staphylococcus aureus* (15), also induce autophagy in *C. elegans*. Although inhibition of autophagy by knockdown of *vps-34*, a gene crucial for early steps of autophagy, reduces the survival of worms after *S. aureus* infection, *vps-34* RNAi does not affect intestinal pathogen load. Thus, the role for autophagy in defense against *S. aureus* is probably not due to clearance of the pathogen, implicating that other mechanisms of host defense might exist.

So far, the following major signaling cascades have been identified as involved in *C. elegans* defense against pathogenic bacteria: the p38 mitogen-activated protein kinase (MAPK) PMK-1 (16), DAF-16 (17), the extracellular signal-regulated kinase (ERK) MAPK MPK-1 (14, 18), the protein kinase D DKF-2 (19), the G protein-coupled receptor FSHR-1 (20), and the G protein Gqα EGL-30 (21). Here, we investigated the function of autophagy in innate immune responses to *P. aeruginosa* PA14 in *C. elegans*. Furthermore, we investigated the role of the signaling pathways related to innate immunity in the activation of autophagy and discovered that the ERK signaling was involved in autophagy during *P. aeruginosa* PA14 infection. Finally, our results show that autophagy protects worms against necrosis-induced organismal death triggered by *P. aeruginosa*.

## Significance

**The autophagy machinery functions as an innate immune defense mechanism to eliminate intracellular pathogens, including bacteria, viruses, and parasites. However, the alternative functions of autophagy in innate immunity remain unknown. Using the metazoan *Caenorhabditis elegans* as a model system, we show that autophagy plays a crucial role in host defense against a pathogenic bacterium *Pseudomonas aeruginosa*. The function of autophagy lies not to directly diminish *P. aeruginosa*, but instead to neutralize necrosis imposed by the pathogen. These findings indicate that autophagy possesses multifaceted functions that modulate infectious and inflammatory diseases.**

Author contributions: C.-G.Z. and K.-Q.Z. designed research; C.-G.Z., Y.-C.M., and L.-L.D. performed research; C.-G.Z., Y.-C.M., L.-L.D., and K.-Q.Z. analyzed data; and C.-G.Z. and K.-Q.Z. wrote the paper.

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## Results

**Autophagy Mediates Host Defense Against *P. aeruginosa* in *C. elegans*.** We first analyzed induction of autophagy after *P. aeruginosa* PA14 infection by using transgenic worms carrying GFP::LGG-1, because the appearance of GFP::LGG-1-containing puncta has been shown to be a reliable indicator of autophagy (5). We found that GFP::LGG-1 puncta were markedly increased in the seam cells and intestine in young adult WT worms after *P. aeruginosa* PA14 infection (Fig. 1*A*). Meanwhile, we also confirmed autophagy by detecting the ratio of phosphatidylethanolamine (PE)-conjugated LGG-1 (PE-LGG-1::GFP) to LGG-1 by using Western blotting (22). A significant increase in PE-LGG-1-GFP was observed in WT worms after *P. aeruginosa* PA14 infection (Fig. 1*B*). These findings were consistent with the observation that *P. aeruginosa* PA14 infection increases the number of autophagosomes in *C. elegans* as detected by transmission electron microscopy (14). Nutrient deprivation can also induce autophagy in various organisms including worms (22). However, nutrient deprivation is not likely the case because autophagy was also observed in WT worms on a lawn of PA14 mixed with the relatively nonpathogenic food source *Escherichia coli* OP50 (Fig. S1 *A* and *B*). In contrast, heat-killed PA14 did not induce autophagy. We further tested the expression of *Pgst-4::gfp*, which is induced by starvation (23). We found that only starvation, but not OP50, PA14, a mixture of OP50 and PA14, or heat-killed PA14, up-regulated the expression of *Pgst-4::gfp* (Fig. S1*C*). These data suggest that the pathogenicity of *P. aeruginosa*, but not food deficiency, induces autophagic responses.

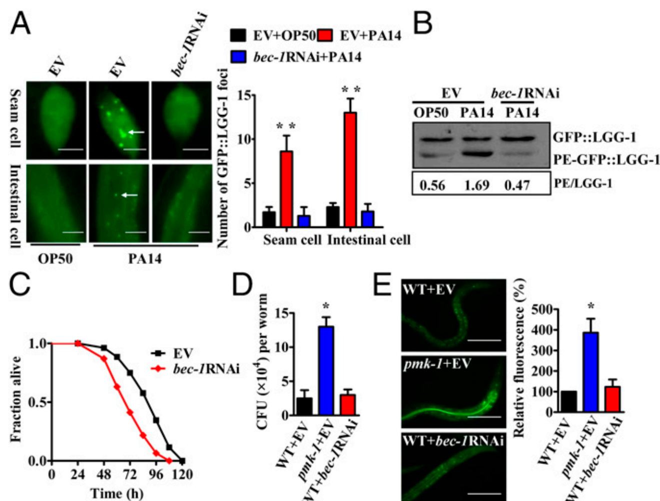


**Fig. 1.** Autophagy mediates host defense against *P. aeruginosa* in *C. elegans*. (*A*) Representative images of autophagosomes (GFP::LGG-1 puncta) in the seam cells and intestinal cells of worms exposed to *P. aeruginosa* PA14 for 12 h. The numbers of GFP::LGG-1 puncta in the seam cells and intestinal cells were counted (*Right*). These results are mean ± SD of three independent experiments performed in triplicate. $^{**}P < 0.01$ versus OP50+empty vector (EV). The arrow denotes a representative autophagosome. (Scale bars: seam cells, 10 μm; intestinal cells, 20 μm.) (*B*) The levels of PE-conjugated LGG-1–GFP and LGG-1–GFP were measured by Western blotting. The blot shown here is typical of three independent experiments. $P < 0.05$, PA14 versus OP50; $P < 0.01$, PA14+*bec-1* RNAi versus PA14+EV. (*C*) *bec-1* RNAi significantly reduced survival of worms exposed to PA14. $P < 0.01$ versus EV. (*D*) Numbers of colony-forming units of PA14 were measured in worms subjected to *bec-1* RNAi. These results are mean ± SD of three independent experiments performed in triplicate. $^{*}P < 0.05$ versus WT+EV. (*E*) Fluorescence of worms exposed to *P. aeruginosa* PA14 expressing GFP for 24 h. The image is representative of three independent experiments. *Right* shows quantification of GFP levels. $^{*}P < 0.05$ versus WT+EV. (Scale bars: 50 μm.)

*P. aeruginosa*-mediated autophagy was significantly abrogated by RNAi of *bec-1*, the *C. elegans* ortholog of the yeast and mammalian autophagy gene ATG6/VPS30/beclin1 (Fig. 1 *A* and *B*). Meanwhile, worms subjected to *bec-1* RNAi were sensitive to *P. aeruginosa* PA14 infection (Fig. 1*C*), suggesting that autophagy is required for host defense against the pathogen. To determine whether the susceptibility is attributable to accelerated PA14 infection in autophagy-deficient worms, we tested *P. aeruginosa* accumulation in the intestine. However, we found that *bec-1* RNAi had no impact on the colony forming units (CFU) of *P. aeruginosa* PA14 in worms (Fig. 1*D*). Furthermore, the accumulation of *P. aeruginosa* expressing GFP in worms subjected to *bec-1* RNAi was comparable to that observed for WT worms with empty vector (Fig. 1*E*). Consistent with previous observations (16, 24), a mutation in *pmk-1 (km25)*, a conserved p38 MAPK that plays a principal role in innate immunity (16), led to a marked increase in intestinal accumulation of *P. aeruginosa*. These results suggest that the protective role of autophagy in *C. elegans* survival is unlikely the result of direct elimination of *P. aeruginosa*.

**ERK Signaling Is Required for Activation of Autophagy.** The innate immunity in *C. elegans* is triggered by several signaling cascades, including PMK-1, DAF-16, MPK-1, DKF-2, FSHR-1, and EGL-30 (14, 16–21). The active role of autophagy in innate immune responses to *P. aeruginosa* PA14 in *C. elegans* led us to hypothesize that one of these signaling pathways could mediate autophagy. We found that systemic RNAi of *mpk-1*, but not *pmk-1*, *dkf-2*, *egl-30*, *daf-16*, and *fshr-1*, significantly suppressed PA14-induced autophagy (Fig. 2 *A* and *B*). Unlike other signaling pathways, knockdown of *daf-16* in the intestine, but not in the whole body, results in enhanced susceptibility to *P. aeruginosa* PA14 infection (25). However, although DAF-16 in the intestine is required for a protective response against pathogens, intestinal-specific knockdown of *daf-16* failed to affect autophagy induced by PA14 infection (Fig. 2 *A* and *B*). Furthermore, we found that the number of GFP::LGG-1 puncta in the seam cells (Fig. 2*C*) and intestine (Fig. 2*D*) was reduced in *mpk-1(n2521)* mutants after *P. aeruginosa* PA14 infection. The core components of the ERK signaling are the small G protein LET-60 RAS, the MAPK kinase kinase LIN-45 RAF, the MAPK kinase MEK-2, and MPK-1. Mutations in *let-60(n1700)*, *lin-45(sy96)*, or *mek-2(n1989)* led a decrease in the number of GFP::LGG-1 puncta in the seam cells (Fig. 2*C*) and intestine (Fig. 2*D*). In addition to genetic ablation of the ERK signaling, U0126, an inhibitor of MEK-2 (26), significantly blocked autophagy in WT animals after *P. aeruginosa* PA14 infection (Fig. S2 *A* and *B*).

Next, we found that mutations in *let-60(n1700)*, *lin-45(sy96)*, *mek-2(n1989)*, or *mpk-1(n2521)*, enhanced sensitivity of worms to *P. aeruginosa* PA14 infection (Fig. 2*E*). In contrast, a gain-of-function mutation *let-60(n1046)* led to enhanced resistance to the killing by PA14 (Fig. S2*C*). Besides the core components of ERK signaling, we also tested the effect of the regulators of the ERK pathway on innate immunity in response to *P. aeruginosa* PA14 infection. Kinase suppressor of Ras (KSR) functions as a scaffold for RAF, MEK, and ERK, thus positively regulates Ras signaling (27). In *C. elegans*, *ksr* has two isoforms: *ksr-1* and *ksr-2*. The *ksr-1(ku68)*, but not *ksr-2(dx27)* mutants exhibited enhanced susceptibility to *P. aeruginosa*-mediated killing (Fig. S2*D*). The dual specificity phosphatase LIP-1 has been shown to act as a negative regulator of MPK-1 (28). We found that *lip-1 (zh15)* mutants exhibited enhanced resistance to *P. aeruginosa* PA14 infection (Fig. S2*D*). Finally, the numbers of GFP::LGG-1 puncta in the seam cells and intestine were significantly reduced in *ksr-1(ku68)* mutants and markedly increased in *lip-1(zh15)* mutants (Fig. S2 *E* and *F*). Taken together, these results indicate that the ERK signaling is required for activation of autophagy to protect against *P. aeruginosa* PA14 infection.

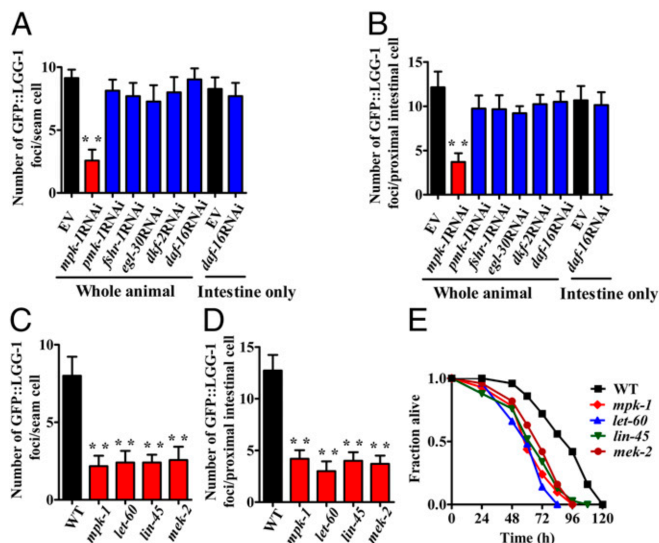

**Fig. 2.** The ERK pathway is required for autophagy during *P. aeruginosa* infection. (*A* and *B*) The numbers of GFP::LGG-1 puncta were counted in the seam cells (*A*) and intestinal cells (*B*) of worms exposed to *P. aeruginosa* PA14. (*C* and *D*) Mutations in the components of the ERK pathway suppressed autophagy in the seam cells (*C*) and intestinal cells (*D*) of worms exposed to PA14. These results are mean ± SD of three independent experiments performed in triplicate. $^{**}P < 0.01$ versus control (EV or WT). (*E*) Mutations in the components of the ERK pathway reduced survival of worms exposed to PA14. $P < 0.01$ versus WT.

***P. aeruginosa*** **Infection Activates ERK Signaling.** To test whether bacterial infection affected the ERK cascade, we determined the phosphorylation of ERK, which is tightly associated with its activation (28). A substantial increase in the phosphorylation levels of ERK was observed after *P. aeruginosa* PA14 infection (Fig. 3*A*), indicating that the ERK MAPK is activated. The phosphorylation levels of ERK were diminished substantially by mutations in *mek-2(n1989)* or *mpk-1(n2521)*, or treatment with U0126 after *P. aeruginosa* PA14 infection (Fig. 3*A*). The main pathway that activates the Ras/ERK signal transduction cascade is epidermal growth factor (EGF) and its receptor (EGFR). In *C. elegans*, LIN-3, a EGF-like ligand, acts through the worm EGFR ortholog LET-23 to activate the LET-60/MPK-1 signaling (29). We thus examined the mRNA levels of *lin-3* by quantitative PCR and found that *P. aeruginosa* PA14 infection up-regulated the expression of *lin-3* (Fig. 3*B*). Furthermore, we tested the expression of *lin-3* by using the transgenic worms expressing *lin-3::GFP* fusion protein (30). The induction of *lin-3::GFP* in the pharynx was significantly up-regulated after PA14 infection (Fig. 3*C*). In addition, mutations in *lin-3(e1417)* or *let-23(n1045)* significantly inhibited the phosphorylation of ERK induced by *P. aeruginosa* PA14 (Fig. 3*D*). Finally, we tested whether EGF/EGFR is required for autophagy and survival of worms after PA14 infection. *P. aeruginosa*-induced autophagy was markedly abolished in *lin-3(e1417)* or *let-23(n1045)* mutants (Fig. 3*E*). Both *lin-3(e1417)* and *let-23(n1045)* mutants were more sensitive than WT worms to PA14 infection (Fig. 3*F*). Collectively, these results suggest that *P. aeruginosa* PA14 infection activates the ERK pathway through EGF/EGFR.

We found that intestinal-specific RNAi of *let-60*, *lin-45*, *mek-2*, and *mpk-1* significantly reduced the survival of worms after PA14 infection (Fig. S3*A*). In contrast, epidermal- or muscular-specific knockdown of these genes had no such effects (Fig. S3 *B* and *C*). These findings suggest that the ERK pathway functions within the intestine to promote innate immunity against PA14 infection. Because *lin-3* is expressed in the pharynx, these findings imply a cell-nonautonomous role for *lin-3* in the activation of ERK pathway.

**CDC-48.2 Functions Downstream of ERK To Promote Autophagy.** A previous study has revealed that MPK-1 can activate 161 substrates to regulate a variety of biological processes in *C. elegans* (31). These substrates belong to at least 15 functional classes of proteins, which are involved in many aspects of cellular machinery such as DNA replication, ubiquitination, and biogenesis of small RNAs. To identify downstream substrates of ERK upon *P. aeruginosa* PA14 infection, we screened 145 of these genes, which are covered by the Ahringer RNAi library (32). We found that knockdown of 14 genes significantly suppressed the survival of worms after *P. aeruginosa* PA14 infection (Table S1). Of these 14 genes, knockdown of *cdc-48.1* or *cdc-48.2* by RNAi, rather than the other 12 genes, significantly reduced autophagy in worms exposed to *P. aeruginosa* PA14 (Fig. S4*A*). *cdc-48.1* and *cdc-48.2* are *C. elegans* homologs of mammalian p97/VCP and yeast Cdc48p (33), which are members of type II AAA (ATPases associated with diverse cellular activities) ATPases (34). Because of a high degree of similarity (~75%) between the RNAi constructs used for feeding RNAi of *cdc-48.1* and *cdc-48.2*, the RNAi constructs are likely to reduce the expression of both genes simultaneously. To clarify their roles in autophagy, we tested *cdc-48.1(tm544)* and *cdc-48.2(tm659)* mutants individually and found that abrogation of autophagy was only observed in *cdc-48.2(tm659)*, but not in *cdc-48.1(tm544)* mutants (Fig. 4*A*). Moreover, a significant decrease in PE-LGG-1-GFP was observed in *cdc-48.2(tm659)* mutants after PA14 infection (Fig. 4*B*). In addition, *cdc-48.2(tm659)*, but not *cdc-48.1(tm544)* mutants, exhibited enhanced sensitivity to *P. aeruginosa* PA14 infection (Fig. S4*B*).

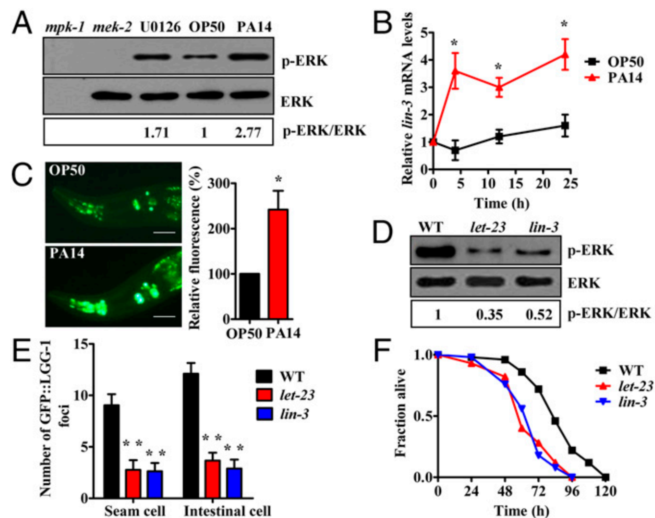

**Fig. 3.** The ERK pathway is activated after *P. aeruginosa* infection. (*A*) The phosphorylation of ERK was elevated in WT worms, but not in *mek-2(n1989)* and *mpk-1(n2521)* mutants, after *P. aeruginosa* PA14 infection for 12 h. U0126 (25 μM), an inhibitor of MEK-2, attenuated the phosphorylation of ERK. The blot is typical of three independent experiments. $P < 0.01$, PA14 versus OP50; $P < 0.05$, U0126+PA14 versus PA14. (*B*) The *lin-3* mRNA levels were up-regulated in WT worms exposed to PA14. These results are mean ± SD of three independent experiments performed in triplicate. $^{*}P < 0.05$ versus OP50. (*C*) Expression of *lin-3::GFP* was up-regulated in WT worms exposed to PA14. *Right* shows quantification of GFP levels. $^{*}P < 0.05$ versus OP50. (Scale bars: 20 μm.) (*D*) Mutations in *lin-3* and *let-23* attenuated the phosphorylation of ERK after PA14 infection for 12 h. The blot is typical of three experiments. $P < 0.05$ versus WT. (*E*) Mutations in *lin-3* and *let-23* suppressed autophagy in worms exposed to PA14. These results are mean ± SD of three independent experiments performed in triplicate. $^{**}P < 0.01$ versus WT. (*F*) Mutations in *lin-3* and *let-23* reduced survival of worms exposed to PA14. $P < 0.01$ versus WT.

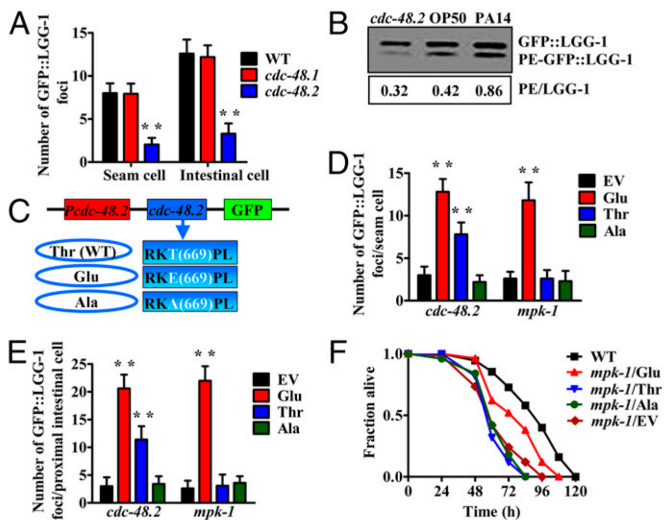


**Fig. 4.** CDC-48.2 is a downstream effector of ERK. (*A*) A mutation in *cdc-48.2(tm659)*, but not *cdc-48.1(tm544)*, suppressed autophagy induced by *P. aeruginosa* PA14 infection. These results are mean ± SD of three independent experiments performed in triplicate. $^{**}P < 0.01$ versus WT. (*B*) The ratio of PE-conjugated LGG-1–GFP and LGG-1–GFP was reduced in *cdc-48.2(tm659)* mutants after PA14 infection. The blot shown here is typical of three independent experiments. $P < 0.05$, *cdc-48.2(tm659)*+PA14 versus WT+PA14. (*C*) Schematic representation of the generated CDC-48.2 site-directed mutants. (*D* and *E*) Expression of CDC-48.2(Thr699Glu) protein rescued *P. aeruginosa* infection-mediated autophagy in the seam cells (*D*) and intestinal cells (*E*) in *cdc-48.2(tm659)* and *mpk-1(n2521)* mutants. These results are mean ± SD of three independent experiments performed in triplicate. $^{**}P < 0.01$ versus EV. (*F*) Expression of CDC-48.2(Thr699Glu) protein partially rescued the immune-deficient phenotype of *mpk-1(n2521)* mutants. $P < 0.01$ versus *mpk-1(n2521)*+EV.

In *C. elegans*, MPK-1 phosphorylates CDC-48.2 at Thr669 (31). To investigate whether the phosphorylation site was critical for CDC-48.2-mediated autophagy, we tested animals expressing *Pcdc-48.2::cdc-48.2*(Ala669), in which the Thr669 residue was substituted by Ala to block phosphorylation, and *Pcdc-48.2::cdc-48.2*(Glu669), in which the Thr669 residue was replaced by Glu to mimic phosphothreonine (Fig. 4*C*). Using *cdc-48.2(tm659)* mutants carrying *Pcdc-48.2::cdc-48.2(Thr669)*, we observed that *cdc-48.2* was expressed in the intestine (Fig. S4*C*). However, CDC-48.2-GFP did not aggregate in worms during PA14 infection (Fig. S4*C*). As expected, expression of *cdc-48.2* by using its promoter [*Pcdc-48.2::cdc-48.2*(Thr669)] significantly rescued autophagy induced by PA14 in *cdc-48.2(tm659)* worms (Fig. 4 *D* and *E*). In contrast, expression of *Pcdc-48.2::cdc-48.2*(Ala669) failed to restore autophagy in *cdc-48.2(tm659)* mutants, whereas expression of *Pcdc-48.2::cdc-48.2*(Glu669) restored autophagy in either *cdc-48.2(tm659)* or *mpk-1(n2521)* mutants after *P. aeruginosa* PA14 infection (Fig. 4 *D* and *E*). Meanwhile, expression of *Pcdc-48.2::cdc-48.2*(Glu669), rather than *Pcdc-48.2::cdc-48.2*(Thr669) or *Pcdc-48.2::cdc-48.2*(Ala669), partially restored the immune-deficient phenotype of *mpk-1(n2521)* mutants (Fig. 4*F*). We found that *bec-1* RNAi suppressed GFP::LGG-1 puncta in *cdc-48.2(tm659)* mutants expressing *Pcdc-48.2::cdc-48.2(Glu669)* (Fig. S4*D*). Similar to WT worms, *cdc-48.2(tm659)* mutants expressing *Pcdc-48.2::cdc-48.2(Glu669)* by *bec-1* RNAi were sensitive to PA14 infection (Fig. S4*E*). These results suggest that the ERK signaling promotes autophagy through its substrate CDC-48.2, conferring the host defense against PA14 infection.

Mutations in *lin-45(sy96)*, *mek-2(n1989)*, *mpk-1(n2521)*, or *cdc-48.2(tm659)* shortened the lifespan of *C. elegans* on plates containing heat-killed *E. coli* OP50 (Fig. S5*A*), consistent with the observations reported by Okuyama et al. (35). Meanwhile, the lifespan of these mutants grown on heat-killed *E. coli* OP50 was comparable to that of these mutants grown on heat-killed PA14 (Fig. S5*B*). All of the mutants were alive at 9 d on heat-killed *E. coli* OP50 or PA14. However, all of the worms died within 4 d on live PA14 (Fig. 2*E*). Hansen et al. (36) also has reported that *bec-1* RNAi had no impact on lifespan in WT worms grown on *E. coli* OP50. Taken together, these results indicate that lifespan is not a major factor to determine pathogen susceptibility in these mutants.

**Inhibition of Autophagy Leads to Necrosis During *P. aeruginosa* Infection.** A previous study has demonstrated that necrosis occurs in worms after exposure to pathogenic bacteria *Erwinia carotovora* or *Photorhabdus luminescens* by detecting expression of the *asp-3* and *asp-4* reporter genes (37). Interestingly, we observed an enlarged vacuolar morphology characteristic of necrotic cells in the intestine in *mpk-1(n2521)* mutants, but not in WT worms, upon exposure to *P. aeruginosa* PA14, by differential interference contrast (DIC) images (Fig. 5*A*). Next, we examined the lysosomal injury by using acridine orange, an acidophilic dye that stains lysosomes (38). We found that acridine orange granules were lysed in the intestine in *mpk-1(n2521)*, but not in WT worms, after PA14 infection (Fig. 5*A*). Similar results were obtained from staining analysis by using the dye uranine (Fig. S6*A*), a commonly used indicator for loss of lysosomal membrane integrity (39). These results suggest that disintegration of lysosomes in the intestine occurs in *mpk-1(n2521)* mutants after PA14 infection. To confirm the release of cathepsins, the worms were labeled with the general fluorescent cysteine peptidase substrate, $(Z\text{-FR})_2$-R110 (38) (Fig. 5*A*). We observed an increase in the intensity of cytoplasmic $(Z\text{-FR})_2$-R110 fluorescence in *mpk-1(n2521)* mutants, but not in WT worms, after PA14 infection, indicating the leakage of cysteine peptidases into the

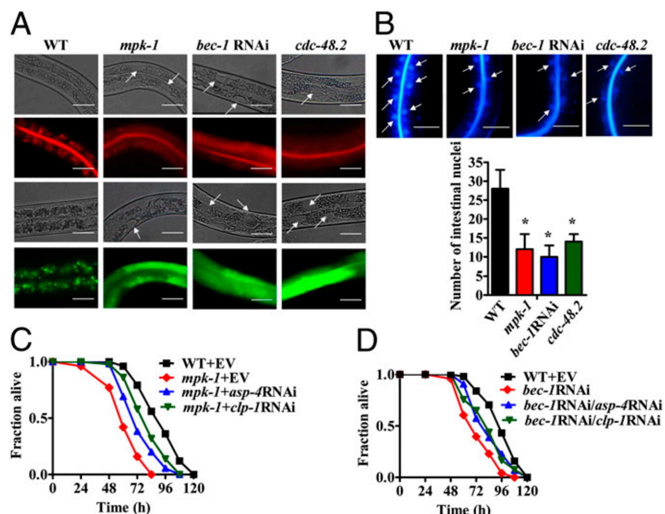


**Fig. 5.** Necrosis is induced in autophagy-deficient worms. (*A*) Necrosis in the intestine occurred in *mpk-1(n2521)*, *cdc-48.2(tm659)* mutants, or worms subjected to *bec-1* RNAi in the presence of *P. aeruginosa* PA14. DIC images of worms and fluorescence microscopy of acridine orange (AO)- (*Upper*) and $(Z\text{-FR})_2$-R110– (*Lower*) labeled intestine of worms are shown. Enlarged vacuoles are indicated by arrows. (B) DAPI-stained nuclei of intestine were reduced after PA14 infection. The lower panel shows quantification of the number of nuclei. These results are mean ± SD of three independent experiments performed in triplicate. $^{*}P < 0.05$ versus WT. Nuclei are indicated by arrows. (*C* and *D*) Knockdown of necrosis-related genes (*asp-4* and *clp-1*) by RNAi partially inhibited the immune-deficient phenotype of *mpk-1(n2521)* mutants (*C*) or worms subjected to *bec-1* RNAi (*D*) after PA14 infection. $^{*}P < 0.05$ versus *mpk-1(n2521)* or *bec-1* RNAi. (Scale bars: *A*, 20 μm; *B*, 25 μm.)

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surrounding cytosol (Fig. 5*A*). To quantify intestinal pathology, intestinal nuclei were counted by using 4′,6-diamidino-2-phenylindole (DAPI). A significant reduction in intestinal nuclei was observed in *mpk-1(n2521)*, but not in WT worms, after PA14 infection (Fig. 5*B*). Similarly, necrosis was also observed in *cdc-48.2(tm659)* mutants, and worms were subjected to *bec-1* RNAi upon exposure to *P. aeruginosa* PA14 (Fig. 5 *A* and *B*). However, necrosis was not detected in *mpk-1(n2521)*, *bec-1* RNAi, and *cdc-48.2(tm659)* worms when propagated on *E. coli* OP50 (Fig. S6 *B* and *C*). These data suggest that in autophagy-deficient animals, necrosis is induced by *P. aeruginosa* PA14. A previous study has demonstrated that autophagy is involved in necrotic cell death induced by a mutation in *mec-4* or prolonged hypoxia in *C. elegans* (40). These results suggest that autophagy plays a distinct role under different pathophysiological conditions.

If necrosis is responsible for enhanced organismal death in *mpk-1(n2521)* worms, inhibition of the necrosis pathway, such as aspartic (*asp-1* to *asp-6*) and cysteine (calpains, *clp-1*, *clp-3*, *tra-3*/*clp-5*, *clp-6*, and *clp-7*) peptidases (41), could promote survival of *mpk-1(n2521)* mutants after PA14 infection. Indeed, knockdown of *asp-4* and *clp-1* (Fig. 5*C*), but not other genes (Table S2), by RNAi protected *mpk-1(n2521)* animals from *P. aeruginosa* PA14 infection. Moreover, knockdown of *asp-4* and *clp-1* by RNAi markedly promoted the survival of worms subjected to *bec-1* RNAi (Fig. 5*D*), but not WT worms (Fig. S6*D*) or *pmk-1(km25)* mutants (Fig. S6*E*), after *P. aeruginosa* PA14 infection. It should be noted that double RNAi of *bec-1* and *asp-4* or double RNAi of *bec-1* and *clp-1* led to a significant down-regulation of expression of *bec-1* and *asp-4* or *bec-1* and *clp-1* (Fig. S6*F*). These results suggest that necrosis causes hypersensitivity of worms to *P. aeruginosa* infection when autophagy is inhibited.

Inhibition of autophagy probably induces apoptosis, resulting in enhanced susceptibility to *P. aeruginosa* infection. However, using the SYTO 12 dye staining against the apoptotic germ cells (42), we found no apparent accumulation of apoptotic germ cells in *mpk-1(n2521)* worms after PA14 infection (Fig. S7*A*). In addition, knockdown of *ced-4*, a homolog to mammalian Apaf-1 that is required for apoptosis, failed to inhibit mortality in *mpk-1(n2521)* mutants after PA14 infection (Fig. S7*B*). Therefore, our results suggest that apoptosis is not involved in worm death after autophagy is suppressed.

## Discussion

Our findings demonstrate that autophagy plays an important role in host defense against the bacterial pathogen *P. aeruginosa* in *C. elegans*. *P. aeruginosa* infection activates the ERK pathway, which, in turn, contributes to the activation of autophagy through its substrate CDC-48.2. Autophagy then functions to ameliorate necrosis to enhance the survival of infected *C. elegans*.

Autophagy plays a conserved role in host defense against pathogens by diminishing intracellular bacteria in mammals, *Drosophila*, and worms (3–6). In *C. elegans*, genetic inactivation of the autophagy pathway markedly increases accumulation of pathogens including *S. typhimurium* (5) and *Nematocida parisii* (13). In contrast, inhibition of autophagy does not influence the intestinal colony counts of *P. aeruginosa* PA14 or *S. aureus* (15), indicating that the primary protective role of autophagy is not to directly eliminate these pathogens. Instead, our results show that autophagy inhibits necrosis by an unknown mechanism, supporting the hypothesis that autophagy may limit the damage to promote the host's ability to survive large burdens of pathogens (15). The fact that defects in autophagy aggravate systemic inflammation and induce organ injury has been observed in patients and animal models (10, 43–45). A striking example is Crohn's disease, a chronic inflammatory disorder with segmental lesions in the gastrointestinal tract that is associated with genetic variants of two autophagy genes, ATG16L1 and IRGM (43, 44). Patients with chronic granulomatous disease who have a mutated NADPH complex develop severe colitis, which is due to defective autophagy (45). In addition, mice genetically deficient in autophagy genes such as *ATG16L1* exhibit enhanced susceptibility to a murine model of colitis, which is attenuated by anti–IL-18 therapy (46). Thus, an ancient role for autophagy in inflammatory responses may be to facilitate the maintenance of cellular homeostasis to alleviate the inflammatory insults and prevent the death of cell populations.

Our data reveal that the ERK signaling node modulates the autophagic response to the pathogen *P. aeruginosa*. In *C. elegans*, the ERK signaling can be activated in response to a variety of extracellular stimuli, such as infection of pathogenic bacteria *Microbacterium nematophilum* (18), ionizing radiation (47), and chemotherapeutic agents (48). Our results demonstrate that *P. aeruginosa* infection leads to the activation of ERK signaling by up-regulating the expression of *lin-3*, which encodes an EGF-like ligand (29). However, the mechanism underlying PA14 infection-mediated induction of *lin-3* remains unclear and needs to be investigated further in light of our current results.

We identified 14 ERK substrates that are involved in innate immune responses to *P. aeruginosa* infection. Of these 14 genes, only genetic inactivation of *cdc-48.2* results in inhibition of autophagy. Cdc48/p97 is a highly conserved member of the AAA-ATPases family that is involved in a variety of cellular functions, including protein degradation, cell cycle progression, membrane fusion, and autophagy (34). In mammals, Cdc48/p97 is required for autophagosome-lysosome fusion (49, 50). However, Cdc48/p97 has been shown to be involved in autophagosome biogenesis in the budding yeast (51). Whether the two distinct functions of Cdc48/p97 in mammals and yeast are due to their evolutionary divergence remains unclear. It has been speculated that in yeast, Ccd48 interacts with Shp1/p47 to extract Atg8-PE prior delipidation and regulates autophagosome biogenesis (51). Genetic inactivation of *cdc-48.2* leads to no accumulation of GFP::LGG-1-positive autophagosomes and a significant decrease in PE-LGG-1-GFP, implicating that *cdc-48.2* in *C. elegans* appears to have a similar function to Cdc48 in yeast. In addition, our data demonstrate that Thr669 in CDC-48.2 is a critical phosphorylation site for its function. A mutation of the Thr669 residue to Ala that blocks phosphorylation fails to restore autophagy in *cdc-48.2(tm659)* mutants, whereas a mutation of the Thr669 residue to Glu that mimics phosphorylation state rescues autophagy in either *cdc-48.2(tm659)* or *mpk-1(n2521)* mutants after *P. aeruginosa* infection. Although a potential threonine phosphorylation site of Cdc48 is conserved in yeast, whether the phosphorylation of the site is required for Cdc48-mediated autophagy needs to be clarified.

It has been shown that the ERK signaling in *C. elegans* is required for the rectal epithelial cell swelling response to pathogenic bacteria *M. nematophilum* (18) and *S. aureus* (14). Consistent with this observation, our results demonstrate that mutations in the core components of the ERK signaling lead to enhanced susceptibility to *P. aeruginosa* PA14 infection. These results suggest that the ERK signaling is required for immune responses to bacterial pathogens in general. The fact that, when one of immune response pathways is lost but other pathways still work, the worms are still susceptible to pathogens, remains a mystery. Unlike the conserved p38 MAPK pathway that fights off infection by up-regulating secreted immune response genes, including C-type lectins, lysozymes, and antimicrobial peptides (52), the main function of the ERK signaling node is probably to promote autophagy, which, in turn, protects a host against organismal insults induced by pathogens. Our results provide a striking example that innate immune pathways that act in parallel may play distinct roles in the pathogenesis of infectious diseases.

## Materials and Methods

Details of materials and methods are available in *SI Materials and Methods*.

**Nematode Strains.** Mutated and transgenic strains used in this study include the following: DA2123(lgg-1p::gfp::lgg-1), SyIS107[unc-119(+)+lin-3(delta-pes-10)::gfp], mpk-1(n2521), let-60(n1700), lin-45(sy96), mek-2(n1989), lin-3 (e1417), let-23(n1045), ksr-1(ku68), ksr-2(dx27), lip-1(zh15), cdc-48.1(tm544), cdc-48.2(tm659), pmk-1(km25), dvIs19(pgst-4::gfp::nls); let-60(n1046 gf), NR222 (rde-1(ne219); kzIs9[pKK1260(plin-12::nls::gfp), pKK1253(plin-26::rde-1), rol-6]; and NR350 (rde-1(ne219); kzIs20[pDM#715(phlh-1::rde-1) were obtained from the Caenorhabditis Genetics Center, University of Minnesota, St. Paul. The strain for intestinal-specific RNAi [sid-1(qt9); Is(vha-6pr::sid-1); Is(sur-5pr:: gfp::nls)] was provided by Gary Ruvkun, Massachusetts General Hospital, Harvard Medical School, Boston. All strains were maintained on nematode growth media agar plates and fed *E. coli* strain OP50. Standard conditions were used for *C. elegans* growth at 20 °C (53).

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