TORC1, BORC, and ARL-8 cycling drive vesiculation of cell corpse phagolysosomes

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Abstract

The dynamics of phagolysosomes leading to cargo clearance are important to provide cells with metabolites and avoid auto-immune responses, but little is known about how phagolysosomes finally resolve cell corpses and cell debris. We previously discovered that polar body phagolysosomes tubulate into small vesicles to facilitate corpse clearance within 1.5 hours in *C. elegans*. Here, we show that vesiculation depends on activating TORC1 through amino acid release by the solute transporter SLC-36.1. Downstream of TORC1, BLOC-1-related complex (BORC) recruits the Arf-like GTPase ARL-8 to the phagolysosome for tubulation. Disrupting the regulated GTP-GDP cycle of ARL-8 reduces tubulation, delays corpse clearance, and mislocalizes ARL-8 away from lysosomes. We also demonstrate that mammalian phagocytes use TORC1, BORC, and ARL8B to promote phagolysosomal degradation, confirming the conserved importance of this pathway. Thus, by observing single phagolysosomes over time, we identified the molecular pathway regulating phagolysosome vesiculation that promotes efficient resolution.

Introduction

Phagocytosis is an essential part of the innate immune response to pathogens as well as a key element for tissue homeostasis through the clearance of dying cells and debris. Billions of cell corpses are removed from the human body every day, which is important to avoid auto-immune responses (Fond and Ravichandran 2016). Many cell types, including undifferentiated cells, use phagocytosis to engulf and eventually degrade phagocytosed cargo (Fond and Ravichandran 2016; Ghose and Wehman 2021). The mechanisms of phagocytic engulfment and phagosome-lysosome fusion are well studied, but there is relatively little known about how later stages of phagolysosomal dynamics influence cargo degradation, which are important for nutrient acquisition and to modulate the immune response (Martinez et al. 2016). The final stage where a phagolysosome resolves, degrading all cargo and recycling its lipid membrane to endolysosomal pathways, is particularly mysterious due to challenges in observing these late stages in phagolysosome resolution. In most experimental systems, it is difficult to predict when a phagocytic event will occur and unambiguously track an individual phagolysosome for long periods. Furthermore, the widely used study of non-degradable phagocytic cargos, such as inorganic beads, has precluded analysis of the ultimate stages of phagocytic clearance.

We have shown that a single cell corpse, the second polar body, is stereotypically phagocytosed by large blastomeres within a 15-minute window in early *C. elegans* embryos (Fazeli et al. 2018). We found that cargo degradation occurs rapidly, ~90 minutes after phagosome-lysosome fusion, allowing us to image phagolysosomal dynamics during resolution *in vivo*. We discovered that phagolysosomes tubulate to form small vesicles that rapidly degrade corpse proteins (Fig. 1A-B, Supplemental Movie 1) (Fazeli et al. 2018). When vesiculation was inhibited, phagolysosomal degradation was delayed by over an hour (Fazeli et al. 2018), revealing that phagolysosomal vesiculation promotes cargo degradation. This process is distinct from lysosome reformation after autophagy or phagocytosis (Yu et al. 2010; Gan et al. 2019), as the phagolysosomal vesicles contain undegraded cargo (Krajcovic et al. 2013; Fazeli et al. 2018; Lancaster et al. 2021).

In our initial study, we found that the mTOR ortholog LET-363 was required for tubulation and vesiculation of the polar body phagolysosome (Fazeli et al. 2018), consistent with observations in cultured mouse macrophages (Krajcovic et al. 2013). Decreased vesiculation of the phagolysosome delayed degradation of its protein content (Fazeli et al. 2018), revealing the importance of subdividing large phagolysosomes to ensure efficient degradation. mTOR is found in two lysosomal complexes, mTORC1 and mTORC2, which are activated by different signals and activate distinct downstream signaling targets (Saxton and Sabatini 2017). However, it is unknown which proteins upstream or downstream of mTOR are involved in vesiculation of the phagolysosome.

We also discovered that the small ARF-like GTPase ARL-8 is required for phagolysosome tubulation and cargo degradation (Fazeli et al. 2018). Similar to other small GTPases, ARL-8 cycles between a GDP-bound and GTP-bound state (Khatter et al. 2015), and GTP-bound ARL-8 can interact with kinesin motor proteins to mediate axonal transport of synaptic vesicles (Klassen et al. 2010; Niwa et al. 2016). Intriguingly, mammalian ARL8 localizes to lysosomes and promotes their anterograde transport by kinesins (Rosa-Ferreira and Munro 2011). Therefore, we hypothesized that ARL-8 links the phagolysosome membrane to motor proteins on microtubules to extend tubules and release vesicles (Fazeli et al. 2018), however it was unclear whether GTP-bound ARL-8 was sufficient to drive phagolysosomal tubulation or whether TOR and ARL-8 were part of the same pathway regulating tubulation.

During organelle transport, ARL8 has been connected to the BLOC-1-related complex BORC (Pu et al. 2015; Farias et al. 2017; Niwa et al. 2017; De Pace et al. 2020). BORC is a multi-subunit complex that localizes on the lysosomal membrane via the myristoyl group of its mammalian subunit Myrlysin (Pu et al. 2015). The Myrlysin ortholog SAM-4 binds to nucleotide free ARL-8, recruiting ARL-8 to the lysosome, and is thought to act as a guanosine exchange factor (GEF) (Niwa et al. 2017). ARL8 recruitment to lysosomes also depends on the BORC subunit Lyspersin (Filipek et al. 2017; Pu et al. 2017), which interacts with LAMTOR/Ragulator, a lysosomal complex that recruits and activates mTOR (Filipek et al. 2017; Pu et al. 2017)

(reviewed in (Colaco and Jaattela 2017)). However, it was unknown whether BORC could act as a link between mTOR and ARL8 to regulate phagolysosome tubulation.

Here, we report that phagolysosome tubulation occurs after amino acid release by solute carriers, which in turn activate TORC1. Downstream of TORC1, BORC activates ARL-8 for tubulation of the phagolysosome. Both GDP- and GTP-locked mutants of *arl-8* show reduced tubulation of the phagolysosome and disrupt ARL-8 localization, revealing that ARL-8 needs to cycle to promote tubulation on phagolysosomes. We further confirmed that these proteins are required to resolve phagolysosomes in mammalian cells. Thus, using an *in vivo* model to observe resolution of a single phagolysosome, we have identified a conserved molecular pathway regulating tubulation and vesiculation to promote degradation of phagolysosome cargos.

Results

Phagolysosome vesiculation requires amino acid release and TORC1 activation

To determine which TOR complex is involved in phagolysosome tubulation in *C. elegans*, we depleted TORC1 and TORC2 subunits that define the substrate specificity of each complex, specifically the Raptor homolog DAF-15 for TORC1 and the Rictor homolog RICT-1 for TORC2 and examined polar body phagolysosome dynamics. Similar to the two-fold decrease in phagolysosome fission events we saw after depleting TOR (Fazeli et al. 2018), treatment with *daf-15* RNAi resulted in a two-fold decrease in fission events (Fig. 1C-D). We also saw that phagolysosomes remained large and that the degradation of polar body protein cargo was delayed over 1 hour after depleting TOR (Fazeli et al. 2018), as well as DAF-15 (Fig. 1C, F). In contrast, *rict-1* RNAi did not significantly affect vesiculation (Fig. 1D) but did lead to a half hour delay in phagolysosomal degradation (Fig. 1F). These findings suggest that only TORC1 is required for phagolysosome vesiculation, but both TORC1 and TORC2 play important roles in phagolysosomal degradation.

To confirm that the TORC1 pathway is involved in vesiculation, we next disrupted upstream regulators of TORC1. TORC1 is activated by amino acid release by solute carriers,

including SLC-36.1 (Gan et al. 2019), and negatively regulated by AMP Kinase (Kim et al. 2011). Knockdown of *slc-36.1* decreased the number of fission events by half and delayed degradation of the phagolysosome over 40 minutes (Fig. 1C-D, F), consistent with amino acid release promoting vesiculation. In contrast, deleting the AMPK catalytic subunit AAK-2 resulted in a 60% increase in vesiculation in *aak-2(ok524)* mutants (Fig. 1D-E), consistent with AAK-2 negatively regulating TORC1 in phagolysosome vesiculation. Interestingly, increased vesiculation did not lead to faster clearance of polar body contents (Fig. 1F), suggesting that other lysosomal factors are limiting for protein degradation. Thus, activation and inhibition of TORC1 activity modulates vesiculation and degradation of phagolysosomal cargos.

BORC is required for phagolysosome tubulation and resolution

As mTOR activates ARL8 via the lysosome-resident BORC complex during organelle transport (Pu et al. 2015; Niwa et al. 2017), we asked whether BORC subunits play a role in phagolysosome vesiculation, starting with the Myrlysin homolog SAM-4 (Niwa et al. 2017). In *sam-4(tm3828)* mutants, phagolysosomes remained large (Fig. 2A). We observed a five-fold decrease in phagolysosome vesiculation (Fig. 2B), and degradation of the phagolysosome contents was delayed over an hour (Fig. 2C). Loss-of-function mutants for BORC subunits *BORCS1/blos-1(ok3707)*, *BORCS2/blos-2(js1351)*, and *Lyspersin/blos-7(wy1159)* also reduced vesiculation by five-fold and delayed degradation over an hour (Fig. 2A-C, Supplemental Movie 2). These results demonstrate that the major subunits of BORC are required for phagolysosome vesiculation and the timely degradation of a cell corpse.

BORC also contains several small subunits that have disparate roles in synaptic vesicle or lysosome transport: only KXD1 and Diaskedin were required for lysosome transport (Pu et al. 2015), while only the MEF2BNB ortholog BLOS-9 was required for synaptic vesicle transport (Niwa et al. 2017). We found that phagolysosome tubulation and degradation occurred normally in mutants for the Diaskedin homolog *blos-8(js1354)* and for *blos-9(js1352)* (Fig. 2A-C), while degradation of the phagolysosome was delayed almost an hour in *kxd-1(js1356)* mutants (Fig. 2C). These findings suggest that the small BORC subunits have

separable functions in synaptic vesicle transport, lysosome transport, or phagolysosomal degradation. Interestingly, *kxd-1(js1356)* mutants showed a bimodal vesiculation phenotype, with half normal and half disrupted (Fig. 2B), leaving open the possibility that the small subunits also play redundant roles in phagolysosomal vesiculation. To test this, we generated *blos-8(js1354); kxd-1(js1356)* double mutant embryos and discovered that phagolysosome vesiculation was reduced five-fold and cargo degradation was delayed over an hour, similar to the major BORC subunits (Fig. 2A-C). These data indicate that the small BORC subunits have distinct, but partially overlapping functions and implicate the subunits involved in lysosome transport in phagolysosome degradation.

BLOS-1 and BLOS-2 also act in a related complex BLOC-1, which is involved in the biogenesis of lysosome-related organelles (Langemeyer and Ungermann 2015). Therefore, we tested whether the BLOC-1-specific subunit Pallidin/GLO-2 has a role in phagolysosome tubulation. Vesiculation and degradation occurred normally in strong loss-of-function *glo-2(zu455)* mutants (Fig. 2A-C), suggesting that BLOS-1 and BLOS-2 act as part of the BORC complex in phagolysosome resolution.

The BORC subunit Lyspersin has been shown to bind to LAMTOR2 (Filipek et al. 2017; Pu et al. 2017), part of the Ragulator complex that acts as a GEF for Rag GTPases to recruit mTORC1 to lysosomes (Sancak et al. 2010; Bar-Peled et al. 2012). LAMTOR2 binding to Lyspersin inhibits BORC to prevent ARL8-mediated lysosome transport (Filipek et al. 2017; Pu et al. 2017). Therefore, we asked whether disrupting LMTR-2 would activate BORC and bypass the requirement for TORC1 in phagolysosome vesiculation. In *lmtr-2*(*tm2367*) deletion mutants, phagolysosome vesiculation was reduced over five-fold and degradation was delayed by over an hour (Fig. 1D, F), suggesting that releasing LMTR-2 inhibition of BLOS-7 was not sufficient to allow BORC and ARL-8 to promote phagolysosome resolution.

To test whether BORC acts downstream of the TORC1 pathway in phagolysosome resolution, we asked whether deleting *sam-4* could suppress TORC1 activation caused by disrupting AMPK. The polar body phagolysosome in *sam-4(tm3828); aak-2(ok524)* double mutants showed a five-fold reduction in vesiculation and degradation delayed by over an hour

(Fig. 2A-C), which was significantly different from controls or *aak-2* single mutants ($p<0.001$). These data place BORC downstream of TORC1 activation in regulating phagolysosome vesiculation.

The ARL-8 GTPase must cycle between GDP- and GTP-bound states for phagolysosome tubulation

We next asked whether ARL-8 acts downstream of TORC1 in phagolysosome tubulation using a partial loss-of-function *arl-8(wy271)* mutant lacking 0.2 kb of the promoter, the 5' UTR, and the first two codons of *arl-8* (Fig. 3A) (Klassen et al. 2010). Consistent with *arl-8* knockdown (Fazeli et al. 2018), phagolysosome vesiculation was reduced five-fold and degradation was delayed over an hour in *arl-8(wy271)* deletion mutants (Fig. 3B-D). The *arl-8(wy271); aak-2(ok524)* double mutants showed similarly decreased phagolysosome fission events and delayed degradation (Fig. 3B-D), confirming that both ARL-8 and BORC act downstream of TORC1 activation in phagolysosome resolution.

As BORC recruits nucleotide-free ARL-8 and is thought to promote GTP exchange for ARL-8 (Niwa et al. 2017), we asked how the state of the bound nucleotide dictates the activity of ARL-8 during phagolysosome tubulation. We first generated point mutations in ARL-8 which are predicted to lock the GTPase in either a GDP- or GTP-bound state (Bagshaw et al. 2006). The GDP-bound *arl-8(T34N)* mutant reduced vesiculation five-fold and delayed degradation by over an hour (Fig. 3B-D), consistent with previous reports predicting it to be inactive (Bagshaw et al. 2006). Interestingly, the putative GTP-locked *arl-8(Q75L)* mutant caused a two-fold decrease in vesiculation and also delayed phagolysosome degradation over an hour (Fig. 3B-D), in contrast to previous reports that this allele can rescue synaptic vesicle trafficking defects (Klassen et al. 2010) or embryonic lethality of the strong loss-of-function *arl-8(tm2504)* deletion allele (Nakae et al. 2010). We next examined mutations in the GTPase G4 motif at Aspartate 133, which are predicted to allow nucleotide exchange independent of a GEF (Morris et al. 2018) and have been shown to rescue synaptic vesicle trafficking defects (Niwa et al. 2017). We also observed a five-fold reduction in phagolysosome vesiculation and an hour

delay in cargo degradation with *arl-8(D133A)* and *arl-8(D133N)* mutants (Fig. 3B-D). These data indicate that both GDP- and GTP-locked as well as unlocked alleles disrupt phagolysosome tubulation and suggest that ARL-8 needs to cycle between GDP- and GTP-bound in a controlled fashion to regulate phagolysosome budding.

ARL-8 localization depends on its nucleotide state

To understand how ARL-8 localization is affected by its nucleotide state, we first tagged endogenous ARL-8 with mCitrine (Fig. 3A). The ARL-8::mCit knock-in did not alter vesiculation events (Fig. 3C), suggesting that ARL-8::mCit promoted phagolysosome vesiculation normally. However, degradation of polar body phagolysosome content was delayed by half an hour (Fig. 3B, D), suggesting that the fluorescent tag may have a minor effect on another aspect of ARL-8's function in phagolysosomal protein degradation. ARL-8::mCit localized to punctate structures in early embryos (Fig. 4B), which we confirmed were 50 ± 6% lysosomal by colocalization with the lysosome-resident BORC subunit SAM-4 (Fig. 4L, S2F-G). 10 ± 1% of ARL-8::mCit also colocalized with the early endosomal small GTPase mCh::RAB-5 (Fig. 4M, Pearson coefficient 0.0003 ± 0.0119), consistent with overexpression studies showing that ARL-8::GFP mainly localized to lysosomes but was also observed on other structures (Sasaki et al. 2013). ARL-8::mCit-positive vesicles started appearing on the polar body phagosome shortly after engulfment and ARL-8 appeared as a ring around the phagolysosome 6 ± 2 minutes after its engulfment (n=8, Fig. 4A), similar to the timing of LMP-1-positive lysosome recruitment (Fazeli et al. 2018), which suggests that ARL-8 is recruited to the phagosome at lysosome fusion.

We next generated *arl-8* point mutations in the mCit knock-in strain and examined their localization. GDP-bound ARL-8(T34N)::mCit appeared largely dispersed (Fig. 4C), consistent with the known role of GTP binding in promoting membrane association of GTPases (Cherfils and Zeghouf 2013). GTP-locked ARL-8(Q75L)::mCit localized on enlarged tubulovesicular structures, the plasma membrane and the ER (Fig. 4D, Fig. S1A), but was not observed on discrete puncta indicative of lysosomes. In contrast, unlocked ARL-8(D133N)::mCit and ARL-

8(D133A)::mCit localized to large tubulovesicular structures and other unidentified structures (Fig. 4E-F), but not the plasma membrane or ER (Fig. 4E-F, S1B). The large tubulovesicular structures colocalized with mCh::RAB-5 in *arl-8(D133N)* mutants (Fig. 4N), revealing that interfering with the regulated GDP/GTP cycle traps ARL-8 on early endosomes and alters endosomal morphology. These findings show that ARL-8 can associate to different membranes depending on its nucleotide state (Fig. 4K). Thus, a regulated cycle between the GDP- and GTP-bound states is required for ARL-8 lysosome localization and phagolysosome tubulation.

BORC but not TORC1 is required for ARL-8 localization to lysosomes and the phagolysosome

As Myrlysin is required for ARL8 localization to lysosomes in HeLa cells (Pu et al. 2015), we asked whether SAM-4 and BORC are required for ARL-8 localization to the polar body phagolysosome. ARL-8::mCit was not observed on the polar body phagolysosome in *sam-4(tm3828)* mutants (Fig. 5A). Instead, ARL-8::mCit associated with large tubulovesicular networks in *sam-4(tm3828)* mutants (Fig. 4H), which we confirmed to be early endosomes by colocalization with mCh::RAB-5 (Fig. 5B) and not lysosomes by LMP-1 staining (Fig. 5C). ARL-8::mCit similarly associated with enlarged tubulovesicular networks in *blos-1(ok3707)* and *blos-7(wy1159)* mutants (Fig. 4I-J). Thus, BORC is required for both the lysosomal and phagolysosomal localization of ARL-8, suggesting that the phagolysosome resolution defects in BORC mutants are likely to be caused by altering ARL-8 localization.

Next, we asked whether TORC1 activity also alters ARL-8 localization. We saw no gross changes to ARL-8::mCit localization after disrupting TORC1 with *TOR/let-363* or *daf-15* RNAi (Fig. 4G, S2B), disrupting TORC1 activation by amino acids with *slc-36.1* RNAi (Fig. S2A), over-activating TORC1 in *aak-2(ok524)* deletion mutants (Fig. S2C), or disrupting TORC1 recruitment and BORC inhibition in *lmtr-2(tm2367)* mutants (Fig. S2D). We further confirmed that *TOR/let-363* RNAi did not significantly alter ARL-8::mCit colocalization with

SAM-4 (Fig. S2E-G). These data indicate that BORC regulates ARL-8 localization independent of TORC1, leaving the molecular character of TORC1 regulation on ARL-8 unclear.

As BORC deletion would lead to chronic disruption of ARL-8 localization, which could also alter lysosomes, we used a degron approach to determine the effect of acute loss of SAM-4 on ARL-8 localization and phagolysosomal degradation. We tagged endogenous SAM-4 with mScarlet-I and a ZF1 degron to induce ZIF-1-mediated degradation starting in anterior cells at the late 4-cell stage (Fig. 5D-E) (Armenti et al. 2014). Prior to the onset of degradation, ARL-8::mCit localized on discrete puncta (half together with SAM-4::mSc::ZF1) (Fig. 5D). After SAM-4::mSc::ZF1 degradation, ARL-8::mCit quickly dispersed (Fig. 5E). Next, we asked whether phagolysosomal degradation is also affected by acute loss of SAM-4 and mislocalization of ARL-8. SAM-4::mSc::ZF1 embryos showed a two-fold decrease in phagolysosome fission events and delayed degradation by a half hour (Fig. 5F-H). These data indicate that SAM-4 needs to constantly promote ARL-8 localization to lysosomal and/or phagolysosomal membranes to efficiently degrade phagolysosome protein content.

The pathway of phagolysosome vesiculation is conserved in mammals

Finally, we asked whether proteins required for phagolysosome vesiculation in *C. elegans* are also needed to resolve phagolysosomes in mammals. Given the challenges in observing single phagocytic events through resolution in mammalian cells, we instead used an overfeeding assay that caused a two-fold reduction in growth of control murine macrophage J774 cells (Fig. 6A). Overfeeding macrophages with opsonized red blood cells was not as detrimental to cell growth if phagolysosomal degradation was compromised by using CRISPR/Cas9 to mutate the lysosomal protease Cathepsin B (Fig. 6A), suggesting that phagocytes defective in phagolysosomal degradation would grow better in this assay (see discussion). Treating macrophages with sgRNAs targeting ARL8B or BORC proteins BORCS1 or Lyspersin also protected J774 cells from reduced growth after overfeeding the phagocytes with red blood cells (Fig. 6A). These data suggest that the TORC1-BORC-ARL8 pathway of phagolysosome resolution is conserved from nematodes to mammals.

Discussion

We used an *in vivo* system to observe and analyze the dynamics of single phagolysosomes containing a physiological cargo and uncovered the timing and molecular mechanisms of phagolysosome resolution. We previously showed that digestion of corpse cargo by lysosomal hydrolases starts after fusion of phagosomes to lysosomes and after breakdown of the corpse membrane inside the phagolysosome (Fazeli et al. 2018). Degradation of cargo proteins by lysosomal hydrolases generates free amino acids within the phagolysosome that are then exported by solute carriers, including SLC-36.1, to recruit and activate TORC1 on the phagolysosome surface (Fig. 6B-C). Independent of TORC1, BORC recruits the small GTPase ARL-8 to the phagolysosome (Fig. 6B), likely in its nucleotide-free form (Niwa et al. 2017). Based on the ability of SAM-4 to promote GTP exchange (Niwa et al. 2017), we predict that downstream of TORC1, BORC promotes GTP binding of ARL-8 (Fig. 6C). ARL-8 then needs to constantly cycle between a GDP-, GTP-, and unbound state to dynamically link the phagolysosomal membrane to motor proteins to extend tubules and release phagolysosomal vesicles (Fig. 6D), a process that facilitates cargo degradation and compartment resolution.

We discovered that similar TORC1-BORC-ARL8 machineries are involved in lysosome trafficking and phagolysosome tubulation (Pu et al. 2015; Farias et al. 2017; Niwa et al. 2017; De Pace et al. 2020). This raises the possibility that forces are pulling the phagolysosome in opposite directions, which could be accomplished by different microtubule motors. Indeed, phagosomes interact with dynein motors in addition to kinesins via ARL8 (Harrison et al. 2003). Alternatively, an organelle could hold the phagolysosome to prevent movement and allow tubulation driven by kinesin motors. One intriguing possibility is that the newly discovered ER-phagolysosome contacts hold the phagolysosome in place to enable tubulation (Levin-Konigsberg et al. 2019).

Although we show that BORC and ARL-8 act downstream of TORC1 in phagolysosomal tubulation, we did not observe mislocalization of ARL-8 in embryos deficient

for amino acid transporters, Ragulator, or TORC1. In the absence of nutrients, LAMTOR/Ragulator is thought to keep BORC and ARL8 away from kinesin motor proteins that transport lysosomes towards the cell surface (Filipek et al. 2017; Pu et al. 2017), but not to influence ARL8 localization to the lysosome. Our data show that the mere release of BORC from Ragulator in *lmtr-2* mutants is not sufficient to allow ARL-8-mediated phagolysosome tubulation, contradicting the idea that the sole role of mTORC1 activation in tubulation is to release BORC inhibition. As LAMTOR/Ragulator also acts as a GEF for Rag GTPases, which in turn recruit mTORC1 to lysosomes (Sancak et al. 2010; Bar-Peled et al. 2012), *lmtr-2* mutants are predicted to not only fail to inhibit BORC, but also fail to recruit TORC1 to lysosomes, supporting the idea that TORC1 activation has an as yet unidentified role in phagolysosome tubulation beyond relieving BORC inhibition or influencing ARL-8 localization.

Small GTPase localization is often guided by a GEF localized to a specific membrane, which in turn recruits and promotes membrane association of the GTPase (Blumer et al. 2013), however we revealed that a GEF is not sufficient to localize ARL-8 to lysosomes. We observed that GTP-locked ARL-8(Q75L) localizes non-specifically to membrane compartments but fails to localize to lysosomes. This is likely due to SAM-4 binding exclusively to nucleotide-free ARL-8 (Niwa et al. 2017) and being unable to recruit GTP-locked ARL-8(Q75L). Similarly, SAM-4 is unable to stably recruit ARL-8 G4 mutants to lysosomes, likely because nucleotide-binding of G4 mutants is weakened (McCray et al. 2010), and they cannot stably bind GTP or GDP. Thus, SAM-4 binding to nucleotide-free ARL-8 and the ability to remain GTP-bound may be necessary to stably and specifically localize ARL-8 to lysosomes and phagolysosomes.

Similarly, the early endosomal localization of ARL-8 suggests the presence of an early endosomal Arl GEF that promotes endosomal membrane binding. This GEF is unlikely to be BORC because we observed ARL-8 accumulating on enlarged RAB-5-positive endosomes in BORC mutants. RAB-5 endosomes are enlarged upon interfering with ARL-8-lysosome association or the GDP-GTP cycle of ARL-8, suggesting that ARL-8 may need to be turned over on early endosomes to allow endosome maturation. These giant early endosomes resemble those formed in GTP-locked Rab5(Q79L) mutants (Stenmark et al. 1994), which also

stably localize to early endosomes and disrupt endosome maturation. Intriguingly, Sasaki *et al.* observed that cell corpse phagosomes colocalized with RAB-5 for slightly longer in strong *arl-8* loss-of-function mutants (Sasaki et al. 2013), suggesting that ARL-8 may also promote Rab turnover for the maturation of early phagosomes and early endosomes.

By studying single phagosomes over their lifetimes, we uncovered the conserved mechanisms of how large phagolysosomes tubulate to form smaller vesicles and facilitate degradation. Given the difficulty in unambiguously tracking a single phagosome in mammalian cells, let alone mammals, clearance of the *C. elegans* polar body is a powerful system to better understand proteins involved in phagolysosome resolution. Although we observed that disrupting mTORC1, BORC, and ARL8B protected phagocytes from overfeeding similar to cathepsin, we can only speculate that this is due to an effect on phagolysosome resolution. As RBCs are iron-heavy cargo and lysosomes are master regulators of iron metabolism and trafficking (Rizzollo et al. 2021), we predict that overfeeding RBCs could increase the availability of redox-active iron, which is known to damage cellular membranes via lipid oxidation and induce ferroptosis (Chen et al. 2020). However, as iron is not membrane permeant, cells deficient in phagocytic steps from engulfing RBCs to phagolysosome resolution should be protected from the damage induced by cytosolic iron. Thus, we hypothesize that phagolysosomal tubulation also promotes RBC degradation in mammals and that evolutionarily conserved mechanisms conduct phagolysosome resolution. Given the importance of effectively resolving phagolysosomes containing pathogens or cell corpses, we believe that this pathway has a critical impact on modulation of the immune response as well as metabolism.

Methods

Worm strains and maintenance

Caenorhabditis elegans strains were maintained at room temperature according to standard protocol (Brenner 1974). For a list of strains used in this study, see Table S1. ARL-8::mCitrine knock-in did not alter developmental timing, viability, or fertility. While ARL-8(T34N) mutants were also viable and fertile, the combination of the mCitrine tag with the T34N mutation led to maternal-effect embryonic lethality in ARL-8(T34N)::mCit worms, similar to the strong loss-of-function deletion allele *tm2504*, suggesting that both perturbations partially disrupt ARL-8 function. Worms were PCR genotyped using primers in Table S2. Restriction fragment length polymorphisms (RFLP) using the following restriction enzymes were performed: *arl-8(jpn1)* with MboII, *arl-8(syb3658D133A)* with NlaIV, *arl-8(wy270)* with EcoRI, *arl-8(syb2255Q75L)* with Hpy188I, *arl-8(syb2260T34N)* with BstXI, *blos-8(js1354)* with HinDIII, *blos-9(js1352)* with BclI, *kxd-1(js1356)* with Hpy188III. *glo-2* mutants were phenotyped on a DM5500 wide-field fluorescence microscope (Leica) for the absence of auto-fluorescent and birefringent gut granules.

RNAi experiments

RNAi was performed by feeding dsRNA-expressing bacteria at 25˚C from the L1 larval stage through adulthood (64-72 hours) according to established protocols (Fraser et al. 2000). RNAi constructs were published previously (*daf-15* and *rict-1* RNAi (Qi et al. 2017)) or were obtained from available libraries (Source BioScience, *let-363 (mv_B0261.2)* and *slc-36.1 (sjj_Y43F4B.7)*).

Light Microscopy

Embryos dissected on a cover slip in M9 buffer were mounted on a slide on top of an agarose pad. Sixteen 1.2 µm step Z-stacks were acquired sequentially for (GFP and) mCherry and DIC every minute at room temperature using a DM5500 wide-field fluorescence microscope with a HC PL APO 40X 1.3 NA oil objective lens supplemented with a DFC365 FX CCD camera

controlled by LAS AF software (Leica). Confocal images were obtained using a Leica SP5 confocal with a HCX PL APO CS 40.0x1.25-NA oil objective with PMT detectors. To image ARL-8::mCit localization, dissected embryos were transferred into egg salts in a 4- or 8-chamber glass bottom slide (ibidi), illuminated using a Tilt light sheet (Mizar), and imaged using an Axio Observer 7 (Zeiss) with a 40X 1.4 NA oil objective lens and ORCA-Fusion sCMOS camera (Hamamatsu) controlled by SlideBook software (3i).

Image Analysis

Time-lapse series were analyzed using Imaris (Bitplane). Internalization was defined as the first frame where the polar body moves away from the plasma membrane, which is likely to closely reflect closure of the phagocytic cup. Vesiculation events were scored when the vesicles were clearly distinct from each other, likely underestimating budding events. Each vesicle was followed individually until disappearance. Degradation was defined as the last frame where mCh reporters were observed after examining the following five frames for mCh puncta reappearance. A maximum of 170 min past engulfment was considered as the end of each time lapse if the phagolysosome was not yet degraded.

Colocalization analysis

z-stacks of 1-4-cell embryos where deconvolved and smoothened using a median filter to reduce the noise and the background was masked in Imaris. For each channel, surfaces were created to annotate the fluorescent structures using 3D View in Imaris. Thresholded colocalization of ARL-8::mCit with SAM-4::mSc or mCh::RAB-5 was measured using the Coloc tool of Imaris and percent of ARL-8 colocalization and Pearson coefficient in colocalized volume was reported.

Antibody staining

Gravid worms were dissected in water on a coverslip to release embryos and transferred to 0.1% polylysine-coated slides and frozen on dry ice. Eggshells were cracked by flicking off the

coverslip and embryos were fixed in methanol before staining. The following antibodies were used: mouse α-LMP-1 antibody (1:50, AB_2161795, Developmental Studies Hybridoma Bank), chicken α-GFP (1:500, AB_2307313, Aves). Slides were counterstained with DAPI to label DNA and mounted using DABCO.

Image processing

For clarity, images were rotated, colorized and the intensity was adjusted using Adobe Photoshop. All images show a single optical section (Z), except Fig. 1D, where the last two panels are maximum projection of two Zs with 1.2 μm steps. Several Zs with 1.2 μm steps were maximum projected in Imaris for videos.

Mammalian cell culture

J774 murine macrophages were cultured in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum. Cells were passaged by scraping when they reached 80 – 90% confluency.

Generation of mutant macrophage pools

Cas9-expressing J774 cells were transduced with lentivirus based on pMCB320 (Addgene plasmid #89359) with mCherry replaced by BFP and containing the sgRNA sequences given in the table below. Two days after transduction, sgRNA-expressing cells were selected with puromycin (2 μg/ml) for an additional two days. Pooled cells were then incubated in complete media for an additional two days to recover before use in overfeeding assays.

Gene	sgRNA sequence
Control	GAAATGGATCTAACGCAGC
BORCS1	GCCTGACCGAAGCGTTGG
Lyspersin	GACCGAAAGCTCCGGCTC
ARL8B	GTTCGCTCTTCTGGAAGG
CTSB	GGTCTCTAATCTGTCCAA

Macrophage growth assays

J774 cells were plated in 24-well plates (10K cells/well) and imaged on the next day “T0” in the brightfield channel (cell confluency) using a high-throughput Incucyte imaging system (Essen). Sheep red blood cells (SRBC) were opsonized with rabbit anti-sheep IgG (MP Biomedical) as described (Montano et al. 2018), and macrophages were either left untreated (unfed control) or challenged with IgG-coated SRBCs at a ratio of 25 SRBC per macrophage (fed samples). J774 cells were imaged every 3 h for a duration of 4 days. Cell growth was assessed based on confluency at day 4 normalized to confluency at “T0”.

Statistical evaluation

Student’s one-tailed *t*-test and one-tailed Fisher’s exact test were used to test statistical significance using the Bonferroni correction to adjust for multiple comparisons. Mean ± standard error of the mean is depicted in graphs and text.

Competing Interest Statement

The authors declare no competing commercial interests related to this work.

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Author Contributions

GF designed, performed, analyzed, and supervised most worm experiments and wrote the manuscript together with RLK and AMW. RLK designed, performed, and analyzed mammalian CRISPR experiments. MCB and CS supervised experiments. AMW designed and supervised experiments, performed light sheet microscopy, and analyzed data.

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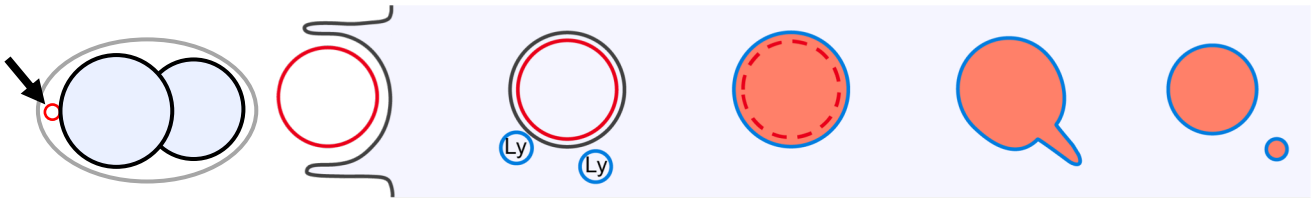
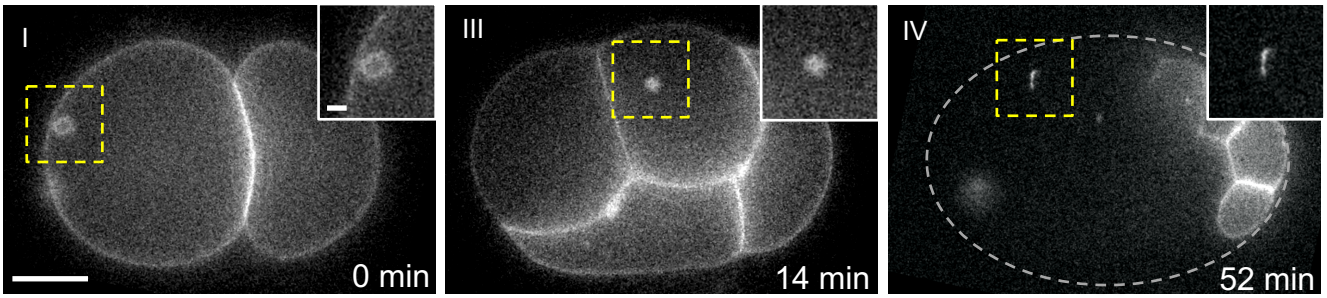
Fig. 1: Phagolysosome vesiculation requires amino acid release and TORC1 activation

A



I. Internalization | II. Lysosome fusion | III. Corpse membrane breakdown | IV. Tubulation | V. Vesiculation

B



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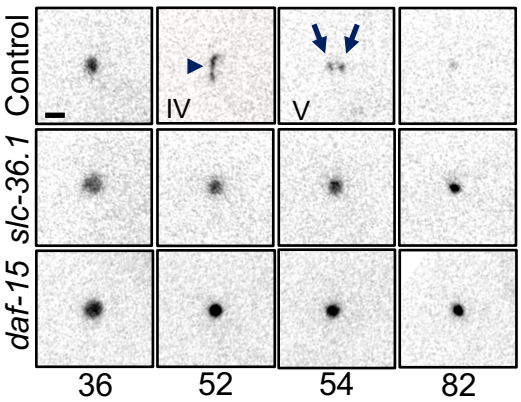

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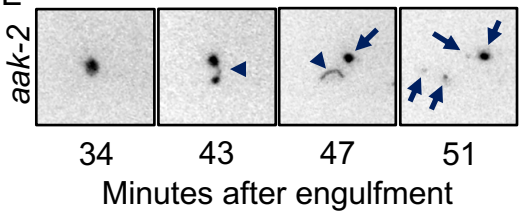

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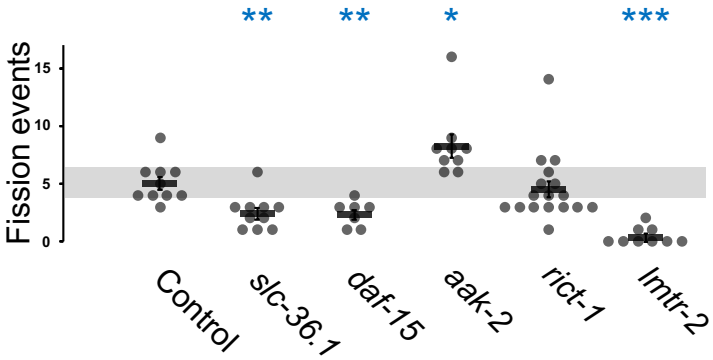

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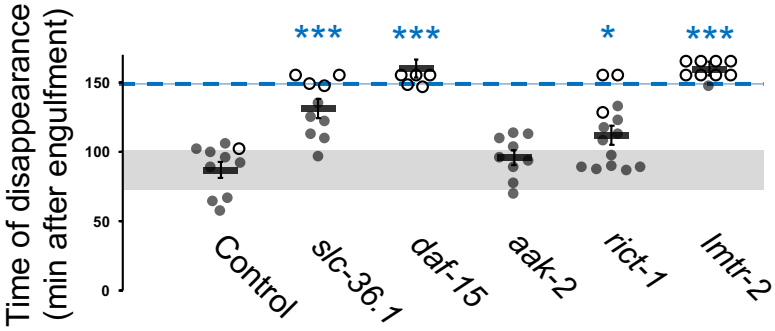


Fig. 1: Phagolysosome vesiculation requires amino acid release and TORC1 activation

A) Schematic representation of the fate of the second polar body corpse. The corpse is phagocytosed (I) by a blastomere cell and the phagosome is fused with lysosomes (II). Next the corpse membrane (red) breaks down inside the phagolysosome membrane (blue), dispersing the membrane tag inside the lumen of the phagolysosome (III). Around 40 min later tubules containing cargo are extended from phagolysosome (IV), which then are released as small vesicles (V) to facilitate degradation. B) Representative images of an embryo expressing mCh::PH::ZF1 with a corpse (yellow square) at the stages of internalization (I), corpse membrane breakdown (III) and phagolysosome tubulation (IV). Scale bars in the main panel and magnified inset show 10 and 2 μm respectively. Indicated times are minutes after engulfment. Grey dotted line in the left panel shows the outline of the embryo. See also Supplemental Movie 1. C) The polar body phagolysosome tubulates (arrowhead) to release vesicles (arrows) in a control embryo, but not after knockdown of the lysosomal amino acid transporter SLC-36.1 or a TORC1 subunit RAPTOR/DAF-15. Panels are inverted for better contrast. D) Fission events are decreased compared to control (5±1) after knockdown of SLC-36.1 (3±0) or DAF-15 (2±0), but not after TORC2 subunit RICTOR/RICT-1 knockdown (5±1). *aak-2(ok524)* mutants show increased vesiculation (8±1). Fission events were decreased in Ragulator *lmtr-2(tm2367)* mutants (0±0). E) Disrupting the negative regulator of TORC1, AMPK/AAK-2, results in increased fission events (arrowheads) to produce more phagolysosomal vesicles (arrows). F) The 2nd polar body cargo mCh::PH::ZF1 disappears from the phagolysosome 88±6 min after internalization. Timely disappearance of the corpse cargo depends on SLC-36.1 (131±7 min), DAF-15 (>150 min) and RICT-1 (113±7 min). Phagolysosome disappearance was not altered in *aak-2(ok524)* mutants (96±5 min). Disappearance of the phagolysosome cargo was delayed in *lmtr-2(tm2367)* mutants (>150 min). Each circle denotes one phagolysosome. Open circles denote the last frame of a time-lapse series in which the polar body phagolysosome did not disappear. Mean ± SEM is shown. Disappearance times and means beyond 150 min after engulfment are grouped above the dashed blue line. Grey bars show standard deviation of the mean in controls. *p<0.05,

**p<0.01, ***p<0.001 compared to control embryos using Student's t-test with Bonferroni correction.

Fig.2: BORC but not BLOC-1 is required for phagolysosome tubulation and cargo degradation

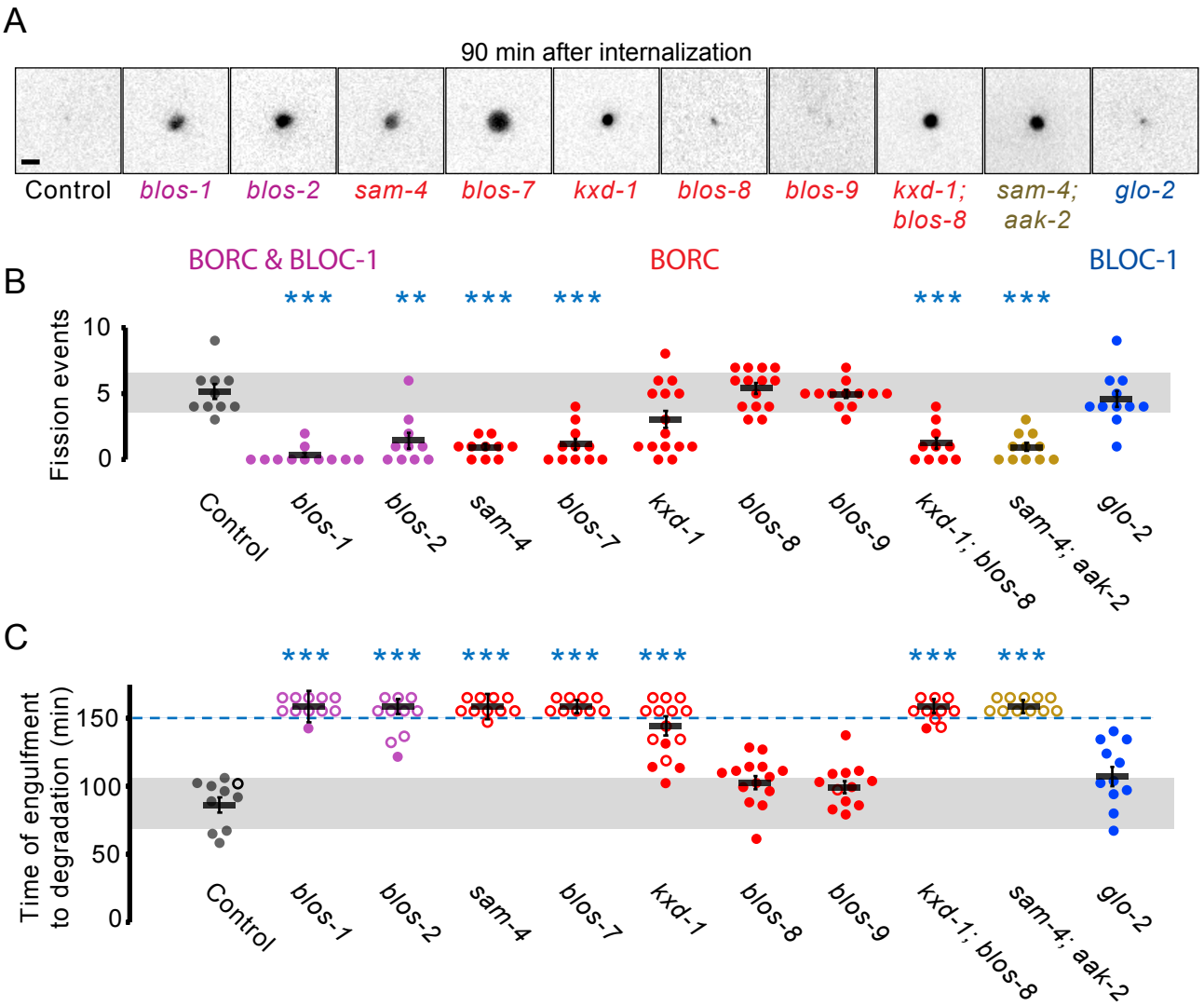


Fig. 2: BORC but not BLOC-1 is required for phagolysosome tubulation and cargo degradation

A) Inverted images of corpse phagolysosomes 90 minutes after engulfment in control and indicated mutants. Scale bar is 2 μm. B) Phagolysosomal fission events were reduced compared to control embryos (5±1) in BORC subunit *blos-1(ok3707)* (0±0), *blos-2(js1351)* (1±1), *sam-4(tm3828)* (1±0), or *blos-7(wy1159)* (1±0) mutants. Single *kxd-1(js1356)* (3±1), *blos-8(js1354)* (5±0) or *blos-9(js1352)* (5±0) mutants did not significantly decrease fission events, but *kxd-1(js1356); blos-8(js1354)* double mutants decreased fission events (1±0), implying redundancy of some BORC subunits. Vesiculation was disrupted in *sam-4(tm3828); aak-2(ok524)* double mutants (1±0), which was significantly different from *aak-2(ok524)* single mutants ($p<0.001$). Disrupting the related complex BLOC-1 with *glo-2(zu455)* mutants did not affect vesiculation (5±1). C) The 2nd polar body cargo mCh::PH::ZF1 disappears from the phagolysosome 88±6 min after internalization. BORC mutants *blos-1(ok3707)*, *blos-2(js1351)*, *sam-4(tm3828)*, *blos-7(wy1159)* (all >150 min), or *kxd-1(js1356)* (146±7 min), delayed cargo disappearance but not *blos-8(js1354)* (104±5 min) or *blos-9(js1352)* (101±5 min) mutants. Cargo degradation was delayed >150 min in *kxd-1(js1356); blos-8(js1354)* or *sam-4(tm3828); aak-2(ok524)* double mutants. Disappearance of the phagolysosome cargo was not affected in *glo-2(zu455)* mutants (108±7 min). Open circles denote the last frame of a time-lapse series in which the polar body phagolysosome did not disappear. Mean ± SEM is shown. Disappearance times and means beyond 150 min after engulfment are grouped above the dashed blue line. Grey bars show standard deviation of the mean in controls. **$p<0.01$, ***$p<0.001$ compared to control embryos using Student's t-test with Bonferroni correction. See also Supplemental Movie 2.

Fig. 3: Dysregulating the ARL-8 GTPase cycle disrupts phagolysosome tubulation

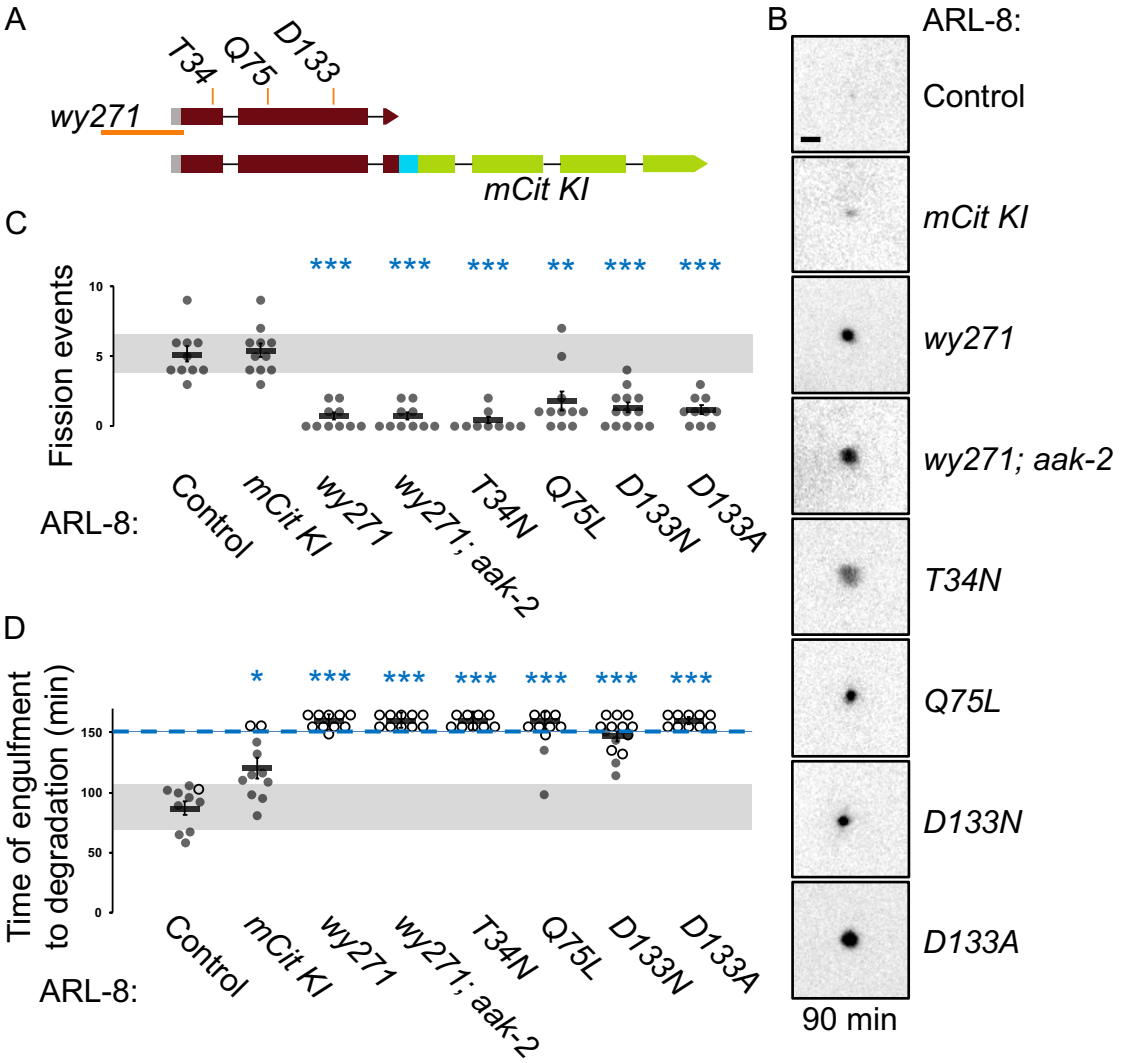


Fig. 3: Dysregulating the ARL-8 GTPase cycle disrupts phagolysosome tubulation

A) Genomic *arl-8* locus with *wy271* deletion (orange line) and the point mutations T34N (GDP-bound), Q75L (GTP-bound), D133N and D133A mutations (G4 motif) indicated. mCitrine was inserted in the C-terminus of *arl-8* with a flexible linker (cyan) with or without the above point mutations. B) Inverted images of corpse phagolysosomes 90 minutes after engulfment in control and indicated mutants. Scale bar is 2 μm. C) Knocking mCit into *arl-8* did not affect phagolysosomal fission events (5±1 vs. 5±1 in controls), while fission events were dramatically reduced in *arl*-8(*wy271)* (1±0), *T34N* (0±0), *Q75L* (2±1), *D133N* (1±0) or *D133A* (1±0) mutants. Vesiculation was also disrupted in *arl-8(wy271); aak-2(ok524)* double mutants (1±0), which was significantly different from *aak-2(ok524)* mutants ($p<0.001$). D) The ARL-8::mCit knock-in slightly delayed the disappearance of the 2nd polar body phagolysosome cargo marker mCh::PH::ZF1 (120±8 min), compared to control (88±6 min), whereas cargo degradation was severely delayed in *arl*-8(*wy271)*, *T34N*, *Q75L*, *D133A* (all >150 min) or *D133N* (146±4) mutants, as well as *arl-8*(*wy271); aak-2(ok524)* double mutants (>150 min). Open circles denote the last frame of a time-lapse series in which the polar body phagolysosome did not disappear. Mean ± SEM is shown. Disappearance times and means beyond 150 min after engulfment are grouped above the dashed blue line. Grey bars show standard deviation of the mean in controls. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to control embryos using Student's t-test with Bonferroni correction.

Fig. 4 ARL-8 localizes to lysosomes, phagolysosomes, and early endosomes

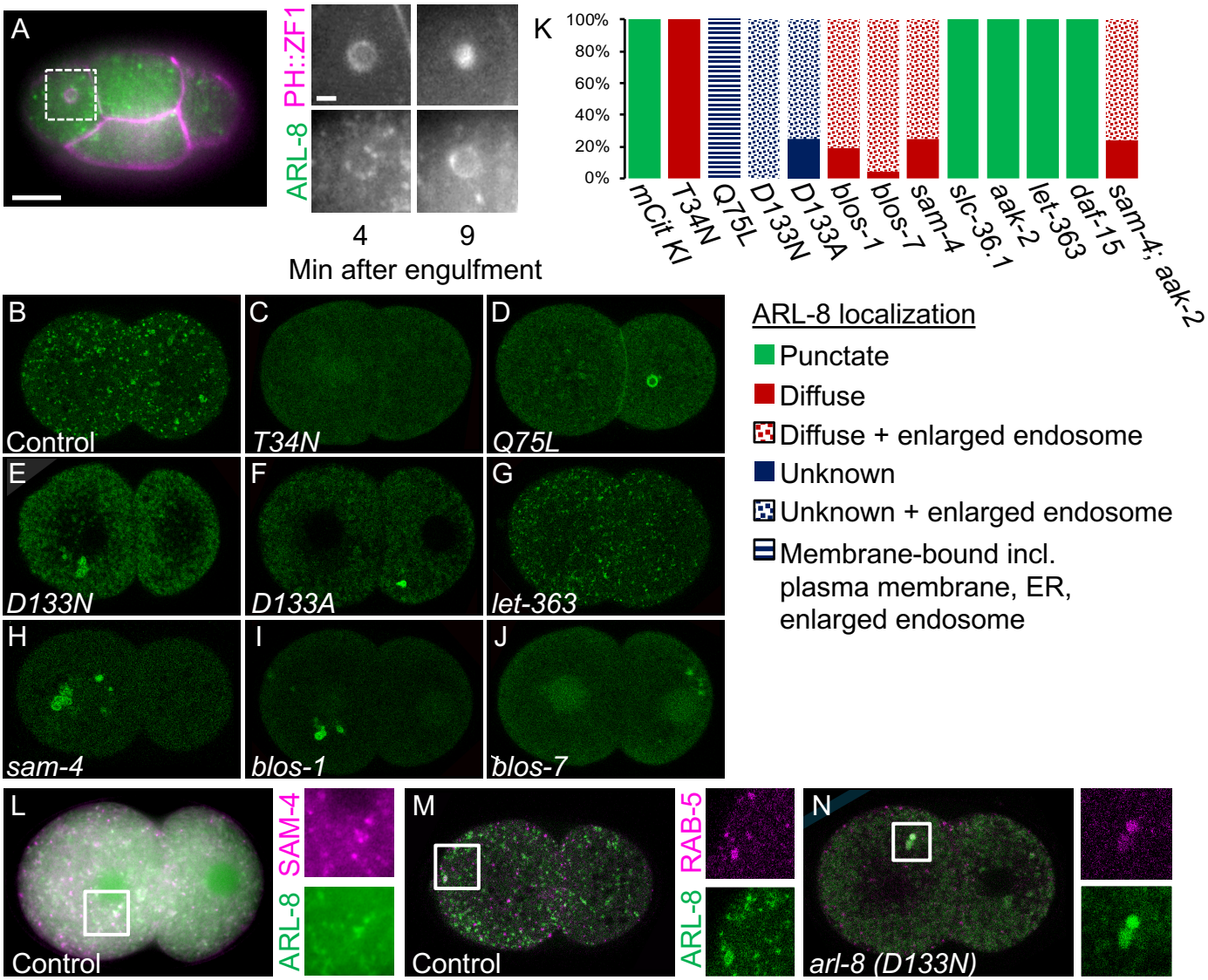
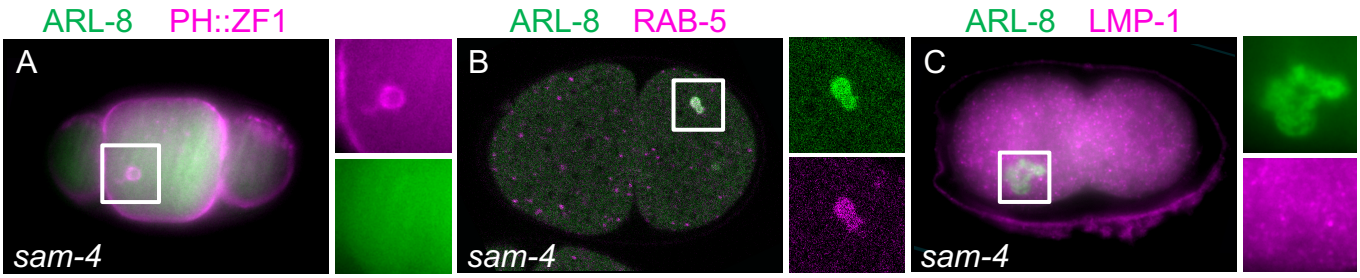
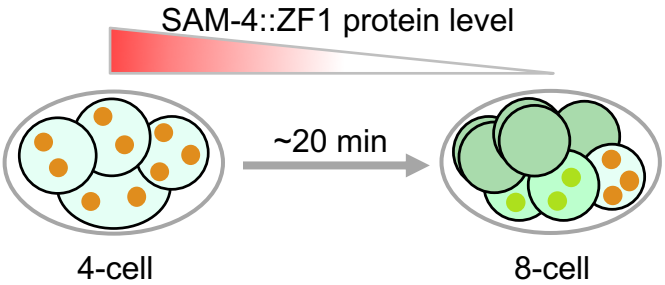


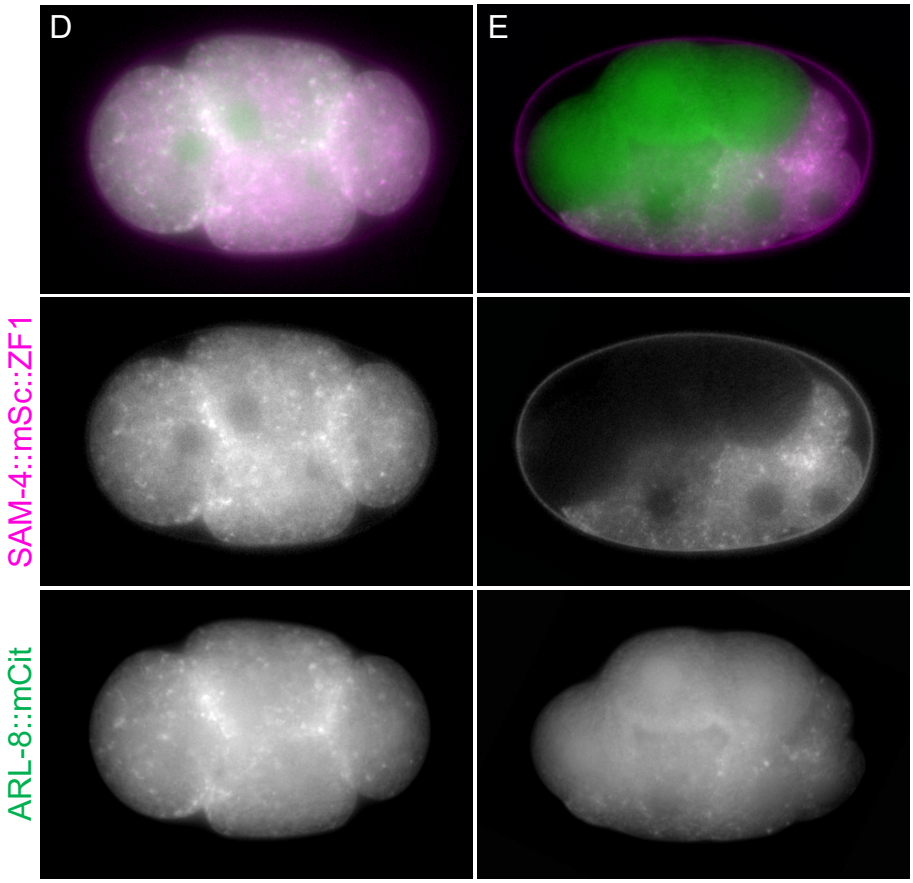
Fig. 4: ARL-8 localizes to lysosomes, phagolysosomes, and early endosomes

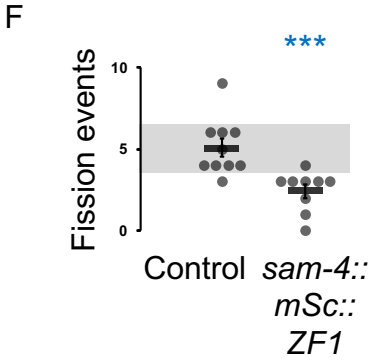
A) ARL-8::mCit puncta (green) appear around the polar body membrane (mCh::PH::ZF1, magenta) shortly after internalization. By the time of the corpse membrane breakdown inside the phagolysosome, ARL-8::mCit appears as a ring around the phagosome. Scale bar is 10 μm. Insets show the magnified dashed white square at the indicated time after engulfment. Scale bar is 2 μm. B) In control embryos, endogenously tagged ARL-8::mCit localizes to puncta (lysosomes and endosomes). C) ARL-8(T34N)::mCit is mostly diffuse. D) ARL-8(Q75L)::mCit localizes to the plasma membrane, ER, and large vesicles. E-F) ARL-8(D133N)::mCit and ARL-8(D133A)::mCit associate with unknown structures in cytoplasm and large interconnected endosomes. G) ARL-8::mCit localizes to puncta after RNAi-mediated *TOR/let*-363 knockdown, similar to control. H-J) In BORC subunit *sam-4 (tm3828)*, *blos-1(ok3707)*, or *blos-7(wy1159)* mutants, ARL-8::mCit appears diffuse but also associates with large interconnected endosomes. K) Quantification of ARL-8::mCit localization in ARL-8 or BORC mutants or embryos deficient in TOR-related proteins. See also Supplemental Fig. S1 and S2. L) ARL-8::mCit colocalizes with the lysosomal resident BORC subunit SAM-4. M-N) ARL-8::mCit infrequently colocalizes with the early endosomal marker mCh::RAB-5 (M), while the large interconnected endosomes in *arl-8(D133N)* mutants are RAB-5-positive (N).

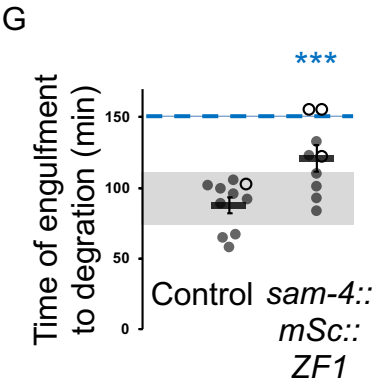
Fig. 5: ARL-8 localization to lysosomes requires SAM-4











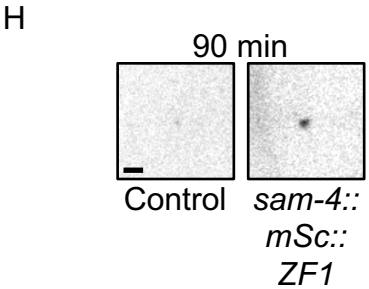


Fig. 5: ARL-8 localization to lysosomes requires SAM-4

A) ARL-8::mCit (green) does not appear around the polar body membrane (mCh::PH::ZF1, magenta) after engulfment in *sam-4(tm3828)* mutants (see also Fig 4.A). B-C) ARL-8::mCit colocalizes with mCh::RAB-5 on large interconnected vesicles in *sam-4 (tm3828)* mutants (B), but not with lysosomal marker LMP-1 (C). D) SAM-4::mSc::ZF1 (magenta) and ARL-8::mCit (green) colocalize in puncta in a 4-cell embryo prior to onset of ZIF-1-mediated degradation. E) SAM-4::mSc::ZF1 degrades in the anterior cells of an 8-cell embryo and ARL-8::mCit disperses in the cytosol. In posterior germline cells, ZF1-tagged proteins are not degraded and the punctate pattern of SAM-4 and ARL-8::mCit and their colocalization is conserved. F) Degrading SAM-4::mSc::ZF1 significantly decreased phagolysosome fission events (2±0 vs. 5±1 in control). G) Disappearance of the 2nd polar body phagolysosome cargo marker mCh::PH::ZF1 was delayed (121±9), compared to control (88±6 min). Open circles denote the last frame of a time-lapse series in which the polar body phagolysosome did not disappear. Mean ± SEM is shown. Disappearance times beyond 150 min after engulfment are grouped above the dashed blue line. Grey bars show standard deviation of the mean in controls. ***$p<0.001$ compared to control embryos using Student's t-test. H) Inverted images of corpse phagolysosomes 90 minutes after engulfment in control and a SAM-4::mSc::ZF1 knock-in embryo. Scale bar is 2 μm.

Fig.6: The pathway of phagolysosome vesiculation is conserved in mammals

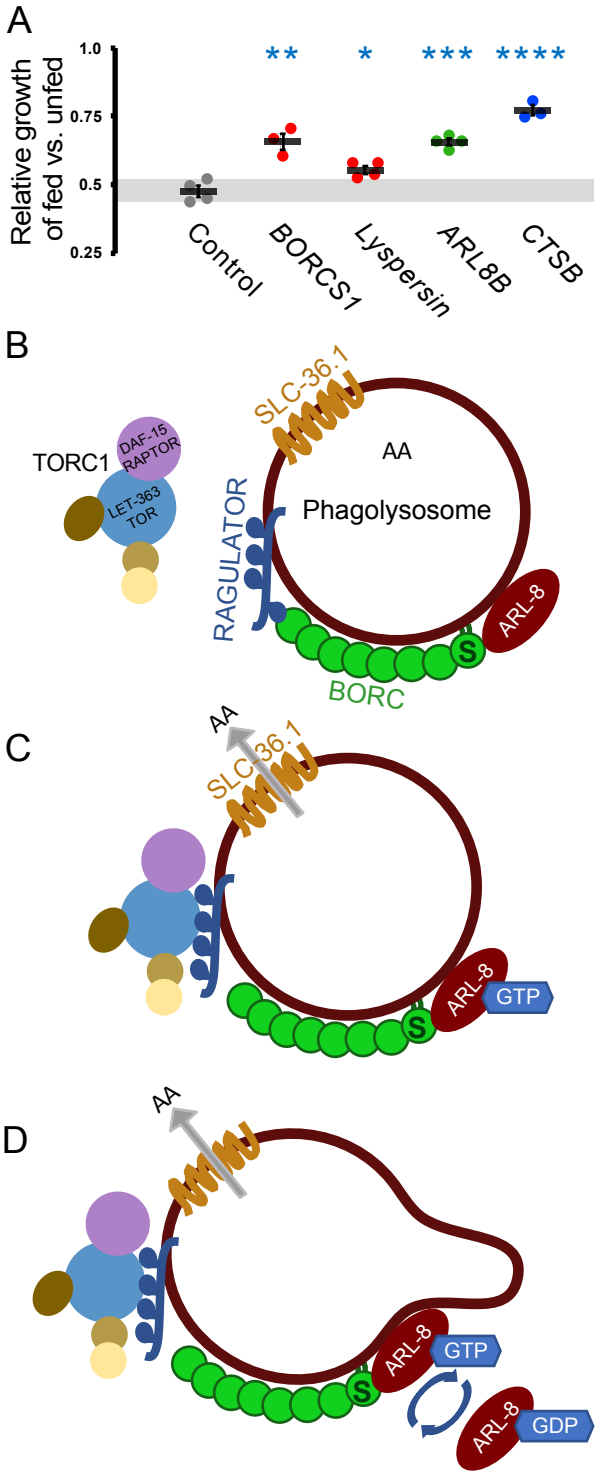


Fig. 6: The pathway of phagolysosome vesiculation is conserved in mammals

A) Overfeeding murine macrophage J774 cells with 25:1 excess sheep red blood cells reduced their growth compared to unfed cells (0.48±0.02). Partially mutating the lysosomal protease Cathepsin B (CTSB) attenuated the growth reduction (0.77±0.02). Partially mutating ARL8B (0.66±0.01) or BORC proteins BORCS1 (0.66±0.03) or Lyspersin (0.56±0.01) also protected macrophages from growth inhibition after overfeeding with red blood cells. Grey bar shows standard deviation of the mean in controls. *$p<0.05$, **$p<0.02$, ***$p<0.002$, ****$p<0.0003$ compared to control fed cells using Student's t-test with Bonferroni correction. B-D) Model of phagolysosome vesiculation. B) After phagosome-lysosome fusion, phagolysosomes are decorated with lysosomal membrane proteins, including solute carriers, Ragulator, and BORC and the BORC subunit SAM-4 (S) recruits ARL-8 to the phagolysosome membrane. Digestion of corpse cargo by lysosomal hydrolases leads to amino acids in the phagolysosome lumen. C) Amino acids are released by solute carriers, including SLC-36.1, and activate TORC1 to release BORC from Ragulator. The BORC subunit SAM-4 (S) promotes GTP-binding by ARL-8. D) ARL-8 needs to cycle between a GDP- and GTP-bound state to maintain its localization and promote tubulation.