Rab GTPases mature the LC3-associated midbody phagosome

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ABSTRACT

Post-mitotic midbody remnants have recently been added to the list of structures degraded via LC3-associated phagocytosis (LAP). LAP involves proteins of the autophagy pathway to degrade phagosomes and facilitate their fusion with lysosomes. Together, these data reveal that Rab GTPases play similar roles during LAPosome maturation and phagosome maturation.

Introduction

Phagocytosis and autophagy degrade cargos of different origins. Cells use phagocytosis to ingest extracellular particles, extending the plasma membrane to isolate particles in a phagosome membrane. Macroautophagy (hereafter referred to as autophagy) is used to wrap cytosolic particles in a double membrane, isolating them from the cytoplasm. Given the different origin of the degraded cargo, it was thought that phagocytosis and autophagy use different molecular mechanisms. However, both pathways converge on the lysosome and certain proteins involved in autophagy have also been shown to be required for phagosome degradation. This led to the proposal of LC3-associated phagocytosis (LAP), where autophagy-associated proteins including the ubiquitin-like Atg8/LC3 family appear on phagosomes and facilitate their fusion with lysosomes.

Recently, we discovered that post-mitotic midbody remnants are degraded by LAP. The midbody is formed in the cytosol during cell division, but released extracellularly by abscission as the midbody remnant (referred to hereafter as midbody). The post-mitotic regulation of midbodies is of particular interest because their positioning can affect cell polarity and cell fate. Previously, it was thought that cytosolic midbodies were degraded by autophagy and that released midbodies were phagocytosed and degraded by an Atg8/LC3-independent pathway. To test these hypotheses, we systematically examined 5 different models for midbody degradation in C. elegans embryos (Fig. 1) and demonstrated that only LAP was consistent with all the data in the field.

The classical phagocytosis model proposes that released midbodies are internalized by phagocytosis. The phagosome then matures using early and late endosomal GTPases Rab5 and Rab7 before fusing with a lysosome for degradation (Fig. 1A). Although this model was supported by the evidence for release and phagocytosis of the midbody, it does not explain the appearance of autophagy-related proteins on the midbody nor the role of autophagy-associated proteins in midbody degradation. The macroautophagy model suggests that abscission occurs on one side of the midbody before it is released into the cytoplasm. A phagophore membrane then isolates the midbody, whose elongation is mediated by the class III PI3K. Fusion of autophagosomes with lysosomes depend on the Atg8/LC3 family proteins LGG-1/2 and the RAB-7 GTPase (Fig. 1B). However, 3 pieces of evidence speak against the macroautophagy model. First, using a degradation tag on a midbody ring component, we revealed that the midbody is completely released after symmetric abscission and is not exposed to the cytoplasm in wild type embryos or autophagy mutants. Second, others and we have shown that the...
midbody is internalized via receptor-mediated phagocytosis. Third, we have shown that the autophagy pre-initiation factor UNC-51/ULK1 and the macroautophagy-specific PI3K complex are required for formation of the LGG-2/LC3-containing phagophore membrane that wraps the midbody. The autophagosome recruits RAB-7, which mediates tethering and fusion with a lysosome together with the Atg8/LC3 homologs LGG-1 and LGG-2. In the phagocytosis sealed by autophagy model, phagocytosis internalizes the midbody after asymmetric abscission. A phagophore seals the exposed midbody. In the "autophagy after phagocytosis" model, the midbody phagosome is wrapped by a phagophore, resulting in a quadruple membrane structure. We finally considered whether a non-canonical form of autophagy, LC3-associated phagocytosis (LAP), would be consistent with the observed data. The LAP model of midbody degradation suggests that after symmetric abscission, the released midbody is internalized by phagocytosis (Fig. 1E). The phagosome acquires LGG-1/2 by a PI3K complex that lacks the Atg14 homolog EPG-8. The LC3-associated phagosome (LAPosome) uses both RAB-7 and LGG-1/2 to fuse with a lysosome and degrade the midbody.

Figure 1. Models for midbody internalization and degradation. (A) Under the classical phagocytosis model, released midbodies are phagocytosed, and the phagosome matures using RAB-5 (light blue) and RAB-7 (brown) sequentially before fusing with a lysosome (L) for degradation. (B) The macroautophagy model suggests that abscission occurs on one side of the midbody before it is released into the cytoplasm. The pre-initiation factor UNC-51/ULK1 and the class III PI3K complex are required for formation of the LGG-2/LC3-containing phagophore membrane (dark blue) that wraps the midbody. The autophagosome recruits RAB-7, which mediates tethering and fusion with a lysosome together with the Atg8/LC3 homologs LGG-1 and LGG-2 (red). In the phagocytosis sealed by autophagy model, phagocytosis internalizes the midbody after asymmetric abscission. A phagophore seals the exposed midbody. (D) In the autophagy after phagocytosis model, the midbody phagosome is wrapped by a phagophore, resulting in a quadruple membrane structure. (E) In the LC3-associated phagocytosis (LAP) model, the released midbody is phagocytosed; the phagosome matures by RAB-5 and RAB-7 and acquires Atg8/LC3 family proteins LGG-1/2 by a PI3K complex that lacks the Atg14 homolog EPG-8. The LC3-associated phagosome (LAPosome) uses both RAB-7 and LGG-1/2 to fuse with a lysosome and degrade the midbody.
degradation in *C. elegans*, *Drosophila*, and mammals. However, whether proteins that regulate phagosome maturation are required for midbody LAPosome maturation is still an open question.

Rab GTPases are central players of membrane identity during vesicle maturation, including endosomes and phagosomes, and there is evidence that Rab GTPases also mature LAPosomes. For example, RAB-5 was previously detected on engulfed apoptotic cells in *C. elegans*, which are later decorated by LC3. Rab-7 was also found on engulfed cell corpses, suggesting that it could also be on LAPosomes. Finally, the Rab2 homolog UNC-108 is required for the acidification of cell corpse phagosomes and their fusion with lysosomes. Therefore, we tested whether midbody LAPosomes also mature using Rab GTPases.

**Results**

Given that phagosomes mature through Rab5 to Rab7 exchange, we asked whether RAB-5 and RAB-7 are also involved in midbody LAPosome maturation. We used GFP-tagged RAB-5 and RAB-7 reporters to examine whether they colocalize with the midbody after internalization. Midbody rings and remnants were marked with mCherry-tagged NMY-2 (non-muscle myosin II). Analyzing the midbody remnant from the first division of the *C. elegans* embryo (P0), we found that GFP::RAB-5 typically appears on the P0 midbody within 20 seconds of internalization (n = 10, Fig. 2A) and disappears around 1 ± 0.5 min later, which is shorter than what is reported for cell corpses in *C. elegans*. GFP::RAB-7 typically appears on the midbody 40 seconds after internalization (n = 14, Fig. 2B) and remains on the midbody for 9 ± 2 minutes (n = 11). It takes another 20 ± 9 min for the midbody marker to disappear after GFP::RAB-7 is lost from the P0 midbody (n = 8), which is comparable to what is reported for somatic cell corpses. Similar trends were observed for midbodies formed during subsequent embryonic divisions (data not shown). Thus, RAB-5 and RAB-7 appear sequentially and transiently on the midbody LAPosome, suggesting they have a role in its maturation.

We next wanted to test whether RAB-5 and RAB-7 are required for midbody degradation. As RAB-5 depletion blocked midbody phagocytosis, we could not directly test whether RAB-5 is required for RAB-7 recruitment to the midbody LAPosome or for LAPosome degradation. We next examined RAB-7 for a role in LAPosome degradation. We found that degradation of the P0 midbody was delayed (n = 3/8) or blocked (n = 5/8, not degraded by ~60 min after internalization) in *rab-7*-depleted embryos (Fig. 2C). In comparison, control midbodies normally degraded 36 ± 7 min after internalization (n = 11). These data demonstrate that midbody LAPosome degradation requires RAB-7 and suggest that RAB-5 and RAB-7 direct maturation of midbody LAPosomes similar to classical phagosomes.

We next tested whether proteins implicated in phagosome acidification play a role in LAPosome

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Rab GTPases decorate and acidify the midbody LAPosome. (A) GFP::RAB-5 and (B) GFP::RAB-7 (cyan) localize on phagocytosed P0 midbodies (NMY-2::mCh, yellow, purple squares). No colocalization was detected with released midbodies before internalization (red squares). The scale bar is 10 μm in the main pictures and 2 μm in insets. (C) NMY-2::mCh disappearance on P0 midbodies was significantly delayed or blocked by depleting RAB-7 compared to controls (p < 0.001). (D) NMY-2::GFP::ZF1 disappearance due to acidification was significantly delayed by depleting the Rab2 homolog UNC-108 (p < 0.05) or the V0-ATPase subunit VHA-1 (p < 0.01) compared to control embryos. The open circle denotes one frame after the end of a time-lapse series when midbody disappearance was not observed. Control data in C and D are from ref. 3.
acidiﬁcation, starting with the Rab2 homolog UNC-108. We used a GFP(S65C)-tagged midbody reporter, which loses ﬂuorescence at lower pH.3 While NMY-2::GFP(S65C)::ZF1 disappeared from P0 midbodies 22 ± 7 min after internalization in control embryos \( n = 17 \), P0 midbodies in unc-108 RNAi-treated embryos lost ﬂuorescence 34 ± 13 min after internalization \( n = 9 \), Fig. 2D). As LGG-2/LC3 rapidly appears on midbody phagosomes after internalization,3 the observed delay in acidiﬁcation demonstrates that the Rab2 homolog UNC-108 acidifies both phagosomes and LAPosomes.

It was proposed that UNC-108 could regulate acidiﬁcation of phagosomes by recruiting the main proton pump in the cell, the V-type ATPase.20 Therefore, we tested whether acidiﬁcation of midbody LAPosomes depends on the V-type ATPase. We targeted VHA-1, an essential subunit of the \( V_{5} \) proton translocation domain.21 Long-term treatment with \( vha-1 \) RNAi results in larval arrest or sterility,22 so we performed “germ line-speciﬁc” RNAi treatments using the \( rrf-1 \) mutant,23 similar to previous experiments with \( rab-5 \) RNAi.3 Disappearance of the NMY-2::GFP(S65C)::ZF1 reporter was delayed after treating worms with \( vha-1 \) RNAi \( (39 ± 13 \) min, \( n = 9 \), Fig. 2D). This delay was not due to the \( rrf-1 \) mutant background, because acidiﬁcation of the P0 midbody was normal in untreated \( rrf-1 \) mutants \( (22 ± 8 \) min, \( n = 9 \)). Thus, these data demonstrate that the V-type ATPase is required for midbody LAPosome acidiﬁcation.

Discussion

Our data reveal the roles of Rab GTPases during internalization and maturation of the midbody LAPosome (Fig. 3). We predict that RAB-5 acts at 2 separate stages during LAP. First, RAB-5 is required for the trafﬁcking of the phagocytic receptor CED-1 to the plasma membrane and thereby midbody phagocytosis.3 Second, after internalization, RAB-5 appears on the midbody LAPosome and is rapidly exchanged for RAB-7. Thus, although the process occurs more quickly, RAB-5 is likely to recruit RAB-7 to mature the midbody LAPosome similar to phagosome maturation during cell corpse clearance in \( C. \) elegans.18-20 Studying midbody degradation is therefore likely to yield insights into the mechanisms of cell corpse clearance during development and homeostasis.

RAB-7 is required for lysosome tethering and localizes on the midbody LAPosome for a considerably longer time than RAB-5 (Fig. 3). During the last steps of autophagy, autophagosomes acquire PLEKHM1, an adaptor protein with binding sites for both RAB-7 and LC3.14 PLEKHM1 subsequently recruits the HOPS complex (homotypic fusion and vacuole protein sorting), which is required to tether autophagosomes with lysosomes in worms and mammals.14,24 As RAB-7 and the Atg8/LC3 homologs are recruited to the midbody phagosome and required for midbody degradation (Fig. 2C and ref. 3), we speculate that RAB-7 and the Atg8/LC3 homologs will recruit the PLEKHM1 homolog and the HOPS complex to tether midbody LAPosomes with lysosomes for degradation.

Our data demonstrate that the Rab2 homolog UNC-108 is required for the timely acidiﬁcation of the midbody LAPosome (Fig. 3). UNC-108 is likely to act in parallel with other Rab GTPases to trafﬁc proteins to or from the LAPosome for acidiﬁcation. For example, UNC-108 and RAB-14 are both recruited to cell corpse phagosomes and are redundantly required to regulate

![Figure 3](image-url)
the acidification of cell corpse phagosomes.20 One candidate cargo of UNC-108 and RAB-14 trafficking is the endosomal V-type ATPase, a large transmembrane proton pump known as the main acidifier in cells.25 Our data showing that acidification is delayed in unc-108 and vha-1 mutants are consistent with the hypothesis that UNC-108 could mediate acidification of midbody LAPosomes through trafficking the V-type ATPase. However, LAPosome acidification is independent of Atg8/LC3, because the midbody phagosome acidified normally in atg-7 mutants.3 Thus, UNC-108 and the V-type ATPase are able to acidify LAPosomes irrespective of Atg8/LC3 on the phagosome surface.

Since the first description of LAP, it is becoming clear that proteins used as hallmarks of autophagy like Atg8/LC3 can also be recruited to phagosomes to facilitate their fusion with lysosomes. Although, some phagosomes can tether with lysosomes independent of LC3,26 LC3 binding to adaptors such as PLEKHM1 may aid tethering. Further investigation is needed to understand why LAPosomes require LC3 to tether with lysosomes. As multiple membranes usually wrap LAPosomes (often 2 plasma membrane-derived vesicles), we speculate that LC3 is needed to signal to lysosomes that the LAPosome contains internal membranes that require degradation or fusion. LC3 could also recruit a subtype of lysosomes that are specialized for the degradation of multi-membrane wrapped cargo. LC3 would then play a similar role in the degradation of double-membrane wrapped autophagosomes.

Materials and methods

Worm strains and maintenance

Caenorhabditis elegans strains were maintained according to standard protocols.27 The following strains were used in this study: FT23: xnIs8 [pJN343: nmy-2p::NMY-2::mCherry; unc-119(+)] unc-119(ed3) III; RT122: unc-119(ed3) III; pwsIs20 [pie-1p::gfp::rab-5; unc-119(+)]29 RT123: unc-119(ed3) III; pwsIs21 [pie-1p::gfp::rab-7; unc-119(+)],30 WEH02: ltlIs38 [pie-1p::GFP::PH(PLC1); unc-119(+)] xnIs8 [pJN343: nmy-2p::NMY-2::mCherry; unc-119(+)] unc-119(ed3) III,3 WEH51: unc-119(ed3) III; xnIs65 [nmy-2p::nmy-2::gfp::zf1; unc-119(+)] IV; WEH212: xnIs8 [pJN343: nmy-2p::NMY-2::mCherry; unc-119(+)] unc-119(ed3) III; pwsIs21 [pie-1p::gfp::rab-7, unc-119(+)]. WEH212 and WEH214 were generated by crossing FT23 with RT122 and RT123, respectively.

RNAi experiments

unc-108 and vha-1 RNAi were performed by feeding worms dsRNA-expressing bacteria from the L1 larval stage through adulthood at 25°C (60–70 h) according to established protocols.31 For rab-7 RNAi, L3/L4 worms were treated at 25°C for 18–25 h as longer treatment caused sterility. Control animals were fed OP50. RNAi bacteria were obtained from available libraries (Source BioScience). The following clones were used: rab-7 (mv_W03C9.3), unc-108 (mv_F53F10.4), and vha-1 (mv_R10E11.8).

Time-lapse imaging

Embryos were dissected from gravid adults and mounted in M9 buffer on an agarose pad on a slide. For the colocalization analysis in Fig. 2A–B, live embryos were imaged simultaneously using a Leica TCS SP5 confocal microscope with a HCX PL APO 63x 1.4 NA oil objective lens supplemented with a Leica HyD hybrid detector. Z-stacks were collected every 0.5 μm every min. For RAB-5 and RAB-7 timing and the acidification/degradation analysis in Fig. 2C–D, Z-stacks were acquired sequentially for GFP and then mCherry every 1.2 μm every 20 seconds using a Leica DM5500 wide-field fluorescence microscope with a HC PL APO 40x 1.3 NA oil objective lens supplemented with a Leica DFC365 FX CCD camera controlled by LAS AF software.

Image analysis and manipulation

Time-lapse series were analyzed using Imaris (Bitplane). Internalization is defined as the first frame where the midbody moves away from the plasma membrane. For clarity, images were rotated, colorized to cyan and yellow, and the intensity was adjusted using Adobe Photoshop. In Fig. 2A–B, 4 Z-slices were projected (Z interval of 0.5 μm), except only one Z-plane is shown in inset images.

Statistical evaluation

Student’s one-tailed t-test was used to test statistical significance. Mean ± standard error of the mean is depicted in Fig. 2C–D. Mean ± standard deviation is reported in the text.
Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Author contributions

GF performed all experiments. GF and AMW designed and analyzed all experiments and wrote the manuscript.

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